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Combined effects of antifouling biocides on the growth of three marine microalgal species

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HIGHLIGHT

- The toxicity of antifouling binary mixtures was tested on three microalgae species.
- Both methods used to predict interactive effects of mixtures gave similar results.
- Mixtures of similarly acting chemicals were close to the CA model predictions.
- Mixture of ZnPT and Cu induced strong synergism on *Tetraselmis suecica*.
- Transchelation of ZnPT into CuPT in presence of Cu^{2+} was demonstrated.

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ABSTRACT

The toxicity of the antifouling compounds diuron, irgarol, zinc pyrithione (ZnPT), copper pyrithione (CuPT) and copper was tested on the three marine microalgae *Tisochrysis lutea*, *Skeletonema marinoi* and *Tetraselmis suecica*. Toxicity tests based on the inhibition of growth rate after 96-h exposure were run using microplates. Chemical analyses were performed to validate the exposure concentrations and the stability of the compounds under test conditions.

Single chemicals exhibited varying toxicity depending on the species, irgarol being the most toxic chemical and Cu the least toxic. Selected binary mixtures were tested and the resulting interactions were analyzed using two distinct concentration-response surface models: one using the concentration addition (CA) model as reference and two deviating isobole models implemented in R software; the other implementing concentration-response surface models in Excel®, using both CA and independent action (IA) models as reference and three deviating models. Most mixtures of chemicals sharing the same mode of action (MoA) were correctly predicted by the CA model. For mixtures of dissimilarly acting chemicals, neither of the reference models provided better predictions than the other. Mixture of ZnPT together with Cu induced a strong synergistic effect on T. suecica while strong antagonism was observed on the two other species. The synergy was due to the transchelation of ZnPT into CuPT in the presence of Cu, CuPT being 14-fold more toxic than ZnPT for this species. The two modelling approaches are compared and the differences observed among the interaction patterns resulting from the mixtures are discussed.

1. Introduction

Diuron (1-(3,4 dichlorophenyl)-3,3 dimethyl urea), irgarol (2-methylthio-4-tertbutylamino-6-cyclopropylamino-*s*-triazine), Zinc Pyrithione and Copper Pyrithione (ZnPT/CuPT, bis(2-

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pyridylthio)zinc/copper 1,1′-dioxide) are among the proposed chemicals to be used as “booster” biocides in Cu-based antifouling paints (Konstantinou and Albanis, 2004). These biocides are usually used alone, in combination with Cu, although two can co-occur in some paint formulations (Environment Agency, 1998). Leaching of these substances to the environment occurs directly from both the ship hull (Readman et al., 1993; Takahashi, 2009) and the discarded antifouling paint particles (Turner et al., 2008; Turner, 2010; Hasan et al., 2014), especially during maintenance and cleaning (Links et al., 2006).

Recently, the use of diuron (Regulation (EU) No 528/2012) and irgarol (Regulation (EU) No 2016/107) as biocides has been prohibited in Europe because of their high toxicity towards aquatic life and both have been included in the list of “48 priority pollutants to be monitored in European waters” in the Water Framework Directive (2000/60/EC and 2013/39/EU). Nonetheless, diuron and irgarol are still found in European fresh and coastal waters. Concentrations up to 0.27 $\mu g\,L^{-1}$ diuron and 0.19 $\mu g\,L^{-1}$ irgarol were reported by Caquet et al. (2013) in Vilaine Bay (Brittany, France) and even higher concentrations up to 2.60 $\mu g\,L^{-1}$ diuron and 0.82 $\mu g\,L^{-1}$ irgarol were reported in careening areas of several French ports (Cozic and Durand, 2013). Diuron (phenylurea) and irgarol (*S*-triazine) both act as photosystem II (PSII) inhibitors by competing with the quinone Q_B on its binding site located in the D1 protein, thus preventing electron transfer between Q_A and Q_B and inhibiting Hill's reaction (Nimbal et al., 1996; Jones and Kerswell, 2003). Several studies reported the high toxicity of these compounds towards microalgae: Koutsaftis and Aoyama (2006) determined 72-h 50% inhibitory concentrations (IC50) of 36.0 and 1.10 $\mu g\,L^{-1}$ on the growth of the diatom *Chaetoceros gracilis* for diuron and irgarol, respectively. Bao et al. (2011) reported 96-h EC50 values for diuron and irgarol of 5.90 and 0.57 $\mu g\,L^{-1}$, and 4.30 and 0.39 $\mu g\,L^{-1}$ on the growth of the marine microalgae *Skeletonema costatum* and *Thalassiosira pseudonana*, respectively.

On contrary to diuron and irgarol, very little is known about the occurrence of the two organometals ZnPT and CuPT in the environment. Indeed, literature about pyrithione concentrations in water is very scarce: to our knowledge, only one study reported the occurrence of pyrithione (PT, Hydroxy-2(1H)-pyridinethione) in the marine environment, at a concentration of $13.4 \pm 0.60\,\mu g\,L^{-1}$ measured by cathodic stripping voltammetry in a marina from Mersey estuary (United Kingdom) (Mackie et al., 2004). ZnPT and CuPT usually co-occur in the marine environment as they are both present in antifouling paints, and because ZnPT easily transchelates into CuPT in presence of Cu (Thomas, 1999; Maraldo and Dahllöf, 2004; Grunnet and Dahllöf, 2005). ZnPT has long been used for its bactericidal and fungicidal activity, especially in antidandruff shampoos (Yebra et al., 2004), and has been proposed as one of the most relevant compounds to replace TBT in antifouling paints during the past decade (Doose et al., 2004). It is assumed to act by disrupting cell membrane integrity and inhibiting ATP synthesis and membrane transport (Chandler and Segel, 1978; Dinning et al., 1998b, 1998a). No study specifically evaluated the mode of action (MoA) of CuPT, though it is reasonable to think that it shares the same mechanism as ZnPT. Regarding their toxicity on microalgae, Yamada (2006) reported 72-h EC50 of 2.10 and 28.4 $\mu g\,L^{-1}$ on the growth of *S. costatum*, and 28.0 and 35.0 $\mu g\,L^{-1}$ on the growth of *Selenastrum capricornutum*, for ZnPT and CuPT, respectively. In another study on *S. costatum*, the 72-h EC50 were 1.60 and 1.50 $\mu g\,L^{-1}$ for ZnPT and CuPT (Onduka et al., 2010), while Devilla et al. (2005) determined a 72-h EC50 of 0.54 $\mu g\,L^{-1}$ for ZnPT on the growth of the microalga *Emiliania huxleyi*.

Concerning copper, since its bioavailable form is the dissolved ionic form Cu^{2+}, the abbreviation Cu will refer to Cu^{2+} ions throughout this article. Most antifouling paints contain copper in the form of copper(I) oxide (or cuprous oxide, Cu_2O), or more rarely copper(I)thiocyanate (or cuprous thiocyanate, CuSCN). Once in seawater, Cu_2O and CuSCN are oxidized in Cu^{2+} (Vetere et al., 1997). As a result, marinas, coastal and estuarine waters are often contaminated by elevated concentrations of Cu in sediments and surface waters. Average dissolved Cu concentrations of 8.50 and 11.2 $\mu g\,L^{-1}$ have been reported in marinas of the San Diego region (USA) (Schiff et al., 2007) and beach waters in Acapulco (Mexico) (Jonathan et al., 2011), respectively. Cu is an essential component in many metabolic processes in microalgae, however, concentrations above the optimum level can become toxic (Baron et al., 1995). Toxic MoA of Cu is thought to inhibit electron transport by damaging acceptor and donor sides of the PSII (Patsikka et al., 1998), hence decreasing the photosynthetic efficiency (El Berdey et al., 2000). Regarding its toxicity towards microalgae, a 96-h EC50 of 970 $\mu g\,L^{-1}$ was reported by Bao et al. (2008) on the growth of the microalgae *Thalassiosira pseudonana*, while Koutsaftis and Aoyama (2006) determined a 72-h IC50 of 1200 $\mu g\,L^{-1}$ on the growth of *Chaetoceros gracilis*.

Numerous studies have shown the importance of studying mixtures of chemicals, as it is more environmentally relevant and because chemicals in mixtures can exhibit higher toxicity than they would alone (Fernandez-Alba et al., 2002; Franklin et al., 2002; Cedergreen et al., 2006; Koutsaftis and Aoyama, 2006). Cedergreen (2014) reported that approximately 5% of the tested pesticide mixtures exhibit larger effects than predicted, while for antifouling mixtures it was approximately 26% of the tested mixtures. Two main reference models are used to predict the toxicity of mixtures. The most frequently used is the concentration addition (CA) model, also referred as Loewe additivity (Loewe and Muischnek, 1926), which is based on the assumption that chemicals sharing the same molecular target can thus be considered as dilutions of each other. On the contrary, the independent action (IA) model considers that chemicals acting on independent targets can result in a binary response: either affected or non-affected. Hence, the probability of surviving a mixture following IA is equal to the product of the probabilities of surviving each of the chemicals individually. Several other models (Hewlett, 1969; Vølund, 1992; Jonker et al., 2005) describe types of deviations from these two reference models, being either synergistic (greater effect than predicted), antagonistic (smaller effect than predicted) or a mixture of the two.

Phytoplankton is responsible for over half of the global annual primary production on earth (Beardall and Raven, 2016) and occupies a key role in the oceanic food web. As many phytoplankton species are living in marinas and harbor areas, they are exposed to cocktails of chemicals, especially antifouling biocides. In this study, binary mixtures of antifouling biocides (including Cu) were tested on three marine microalgal species: the haptophyte *Tisochrysis lutea*, the diatom *Skeletonema marinoi* and the chlorophyte *Tetraselmis suecica*.

To evaluate the extent to which the combined toxicity of antifouling biocides together with Cu can harm marine microalgae, the goals of this study were: i) to determine the toxicity of diuron, irgarol, ZnPT, CuPT and Cu on the three species of microalgae; and ii) to evaluate and compare the interaction patterns of six chosen binary mixtures through two different modelling approaches testing deviations from the CA and IA models.

2. Materials and methods

2.1. Chemical/toxicant preparation

Diuron, irgarol®, (PESTANAL®, analytical standard), Zinc Pyrithione (ZnPT) and copper(II) sulfate pentahydrate ($CuSO_4$, ≥ 98%) were purchased from Sigma-Aldrich. Copper Pyrithione (CuPT) was