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Toxicity of four antifouling biocides and their mixtures on the brine shrimp *Artemia salina*

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Abstract

Zinc pyrithione (ZPT), Copper pyrihione (CPT), Chlorothalonil and Diuron are four of the most widely used as alternative to tributlytin (TBT) antifouling biocides in boat paints. As most previous laboratory bioassays for these biocides have been conducted solely based on acute tests with a single compound, information on the possible combined toxicity of these common biocides to marine organisms are limited. In this study, the toxicity of binary (in several proportions), ternary and quaternary mixtures were evaluated using the brine shrimp *Artemia salina* as test organism. Mixture toxicities were studied using the concentration addition model (isobolograms and toxic unit summation), and the mixture toxicity index (MTI). The ZPT-CPT combination had a strictly synergistic effect which requires attention because the coexistence of ZPT and CPT in the marine environment, due to transchelation of ZPT, may occur. The binary mixtures of Diuron with the metal pyrithiones exhibited various interactive effects (synergistic, antagonistic or additive) depending on concentration ratios, whereas all binary mixtures that contained Chlorothalonil exhibited antagonistic effects. The different types of combined effects subsequent to proportion variation of binary mixtures underline the importance of the combined toxicity characterization for various ratios of concentrations. The four ternary mixtures tested, also exhibited various interactive effects, and the quaternary mixture exhibited synergism. The models applied were in agreement in most cases. The observed synergistic interactions underline the requirement to review water quality guidelines, which are likely underestimating the adverse combined effects of these chemicals.

Keywords: Antifouling biocides; Combined toxicity; *Artemia salina*; Concentration addition model; Mixture toxicity index

1. Introduction

Antifouling paints are used to prevent the attachment of fouling organisms such as barnacles, mussels and algae on submerged structures, such as the hulls of watercrafts (Fernandez-Alba et al., 2002; Karlsson and Eklund, 2004). Since the ban on using tributyltin (TBT) as an antifouling agent on vessels under 25 m, paint manufacturers now often utilize alternative "TBT-free" chemically active paint systems that rely on the use of sea water soluble pigments as Cu_2O, CuSCN or ZnO in combination with organic boosting co-biocides for fouling control (Konstantinou and Albanis, 2004; Yebra et al., 2004). These alternative to TBT organic booster biocides are also toxic to other non-target organisms, and their contamination in the aquatic environment has recently been a topic of increasing importance (Konstantinou and Albanis, 2004; Karlsson and Eklund, 2004).

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is a substituted urea-based herbicide employed principally for

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the control of vegetation in non-crop areas since the 1950s, although it is now used as booster biocide in antifouling paints (Yebra et al., 2004). The occurrence of Diuron as an antifouling agent has been reported in a number of European countries and Japan, and is no longer approved for use in the UK as an active ingredient in antifouling paints, on any size of vessel (Konstantinou and Albanis, 2004). Zinc pyrithione (ZPT), the zinc complex of 2-mercaptopyridine-1-oxide, is marketed as an environmentally neutral, non-persistent antifouling biocide (Maraldo and Dahllöf, 2004) and although its high toxicity to a broad spectrum of aquatic organisms is well known (Goka, 1999; Okamura et al., 2002), a few studies report on zinc pyrithione effects on marine organisms (Madsen et al., 2000; Kobayashi and Okamura, 2002; Okamura et al., 2002, 2006; Mochida et al., 2006). Copper pyrithione (CPT), the copper complex of 2-mercaptopyridine-1-oxide, offers several advantages over zinc pyrithione for many applications, notably a lower solubility and short half-lives. The former, as compared to zinc pyrithione, increases its effective availability as a biocidal agent over a longer period of time, when exposed to marine environments (Yebra et al., 2004). Chlorothalonil, (2,4,5,6-tetrachloroisophthalonitrile), is a fungicide that is used currently as a booster biocide in antifouling paints (Yebra et al., 2004). Chlorothalonil is very highly toxic to fish, and concentrations as low as 2 parts per billion can cause gill damage and anemia. It is also toxic to shrimp, frogs, beneficial microorganisms, earthworms and in plants it causes a variety of effects (Cox, 1997).

The combined toxic effects of multiple chemicals have been recognized as an important consideration in ecotoxicology because mixtures of chemicals can have a greater negative impact than the individual constituents of the mixture (Fernandez-Alba et al., 2002). The possible coexistence of the above described antifouling chemicals in the marine environment and the possibility of synergistic effects, emphasize the need for tests also on mixture and complex products and not to rely only on effects from single chemicals (Karlsson and Eklund, 2004). In the present study, the combined toxic effects of the above described antifouling chemicals are examined and the possibility of marine environmental risk due to their coexistence is investigated.

Mixture toxicity studies can be divided roughly into two types: Those investigating the effect of multiple chemicals at predefined mixture ratios (proportions of the individual chemicals) and those investigating binary mixtures at various mixture ratios (Cedergreen et al., 2007). In previous studies (Fernandez-Alba et al., 2002; Koutsaftis and Aoyama, 2006; Zhou et al., 2006), mixtures of antifouling paint biocides at predefined ratios were studied and synergistic effects for a majority of them were found. However, chemicals in mixture can produce different types of combined action depending on their relative concentrations; thus, a global characterization for even a single pair of chemicals—in varying ratios and concentrations of the chemicals—requires experimental confirmation (Borgert et al., 2004) and in the present study this is attempted. The effects of binary mixtures of antifouling chemicals in varying proportions on the brine shrimp *Artemia salina* are examined; multiple mixtures at predefined ratios are tested as well.

Artemia spp. (brine shrimp), are found in salt lakes, salt pans and marine environments. It has gained popularity as a test organism because of its ease of culture, short generation time, cosmopolitan distribution and the commercial availability of its dormant eggs (cysts). Since test animals hatching from cysts are of similar age, genotype and physiological condition, test variability is greatly reduced (Barahona and Sanchez-Fortun, 1999).

The aim of the present work is to assess the adverse effect of four organic antifouling biocides ZPT, CPT, Chlorothalonil and Diuron alone on *A. salina* and to investigate the interactive effect of the binary, tertiary mixtures and all four compounds applied together. Mixture toxicity is determined using the concentration addition model visualized by isobolograms (for the binary mixtures) or by using toxic unit summations, a mixture toxicity index (MTI) and statistical comparisons. The outcomes of the above methods are compared and their applicability and consistency are evaluated.

2. Materials and methods

2.1. Toxicity test

The Artoxkit M with the brine shrimp *A. salina*, which was used in the study as a standardized test, was obtained from Microbiotest Inc. A 24-h LC50 bioassay was performed in a multiwell test plate using instar II–III larvae of the brine shrimp *A. salina*. The test was conducted according to the standard operating procedure (25 ± 1 °C, 35‰ salinity) with three replicates for each treatment and ten animals per replicate under dark conditions (Artoxkit, 1990).

2.2. Preparation of solutions

Dimethyl sulfoxide, (DMSO, Wako Pure Chemicals) was used to dissolve Zinc pyrithione and Copper pyrithione (Hayashi Pure Chemical Ind., Co., Ltd), as well as Diuron and Chlorothalonil (Riedel-de Haen). All