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Prediction and assessment of mixture toxicity of compounds in antifouling paints using the sea-urchin embryo-larval bioassay

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ABSTRACT

The ecotoxicological assessment of alternative "booster" biocides is urgently needed in order to develop environmentally acceptable antifouling paints. However, research has focused mainly on single compounds, and there is still a lack of data on their mixture toxicity. The present study investigated the single and mixture toxicity of three of the most widely used antifouling biocides: zinc pyrithione, chlorothalonil and Sea-Nine, using the sea-urchin (*Paracentrotus lividus*) embryo-larval bioassay. Also, the predictive ability of the concentration addition (CA) and independent action (IA) concepts for antifouling mixtures was evaluated. Both concepts failed to accurately predict the toxicity of the antifouling mixtures, with the exception of the zinc pyrithione and Sea-Nine mixture, which was accurately predicted by the IA concept, suggesting a dissimilar mode of action of those substances. In general, CA predicted consistently higher toxicity than IA; however, CA overestimated the toxicity of the studied mixtures by a factor of only 1.6, representing a reasonable worst-case approach to be used in the predictive hazard assessment of antifouling mixtures. Finally, the present study demonstrates that the risk of antifouling mixtures for the early developmental stages of sea urchin is higher than the risk of each single substance, and therefore, the inclusion of mixture considerations in the development of water quality criteria for antifouling compounds is strongly recommended.

1. Introduction

Antifouling coatings containing biocides are used to prevent or minimize the impact of biofouling and their economic importance is widely recognized. Among the several methods that have been used to prevent biofouling, TBT-based coatings, introduced in the early 1970s, have been the most efficient due to their acute toxicity to target fouling organisms. However, the environmental stability and the extreme toxicity of TBT to non-target organisms (Bryan and Gibbs, 1991; Alzieu, 2000) have prompted the global banning of organotin-based coatings (IMO Resolution A. 895 21, 25 November 1999).

The development of environmentally acceptable antifouling compounds represents, at the moment, a major challenge for the producers of coatings. New alternative formulations based on copper oxide in combination with a range of organic compounds termed "booster" biocides have been recently marketed. Zinc pyrithione (Zpt), chlorothalonil and Sea-Nine are three of the most widely used alternative "booster" biocides and their use is expected to increase after the phase out of organotin-based coatings in January 2008 (Voulvoulis et al., 1999; Konstantinou and Albanis, 2004). Zpt has been used as a bactericide and fungicide in agriculture and in antidandruff shampoos, and during recent years in antifouling paints (Konstantinou and Albanis, 2004; Yebra et al., 2004). Chlorothalonil is the second most widely used agricultural fungicide in the U.S., also employed as a preservative for paints and adhesives and as a "booster" biocide (Cox, 1997; Cima et al., 2008). Sea-Nine 211 is a highly effective biocide against a wide range of fouling organisms which gained the "Green Chemistry Challenge Award" from the U.S. EPA in the category "Designing Safer Chemical Products" for its environmental safety (USEPA, 1996). As a result of their use in antifouling applications high concentrations of chlorothalonil and Sea-Nine have been reported in coastal areas (Voulvoulis et al., 2000; Martínez et al., 2001; Sakkas et al., 2002).

Therefore, it is crucial to perform adequate hazard assessment of the alternative biocides in the marine environment in order to develop environmentally acceptable coatings. Although much effort has been devoted during recent years to the study of the toxicity and the characteristics associated with alternative antifouling biocides (e.g. Callow and Willingham, 1996; Maraldo and Dahllöf, 2004; Turley et al., 2005; Bellas, 2006), research has focused mainly on single compounds, and there is still a lack of data on their mixture toxicity. The assessment of the effects of mixtures of antifouling agents should be considered an issue of high prior-

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ity, since aquatic organisms are usually exposed simultaneously to numerous biocides that leach from the coatings rather than to single compounds. The experimental evaluation of all possible combinations of antifouling compounds is not feasible but several models have been developed for the prediction of combination effects on the basis of the concentration–response relationships of individual mixture components (Berenbaum, 1989). Those models are based on two reference concepts, concentration addition (CA) (Loewe and Muischnek, 1926) and independent action (IA) (Bliss, 1939). CA assumes that the mixture constituents share a common target site and present a similar mechanism of action, whereas IA assumes different target sites and dissimilar mechanisms of action of all mixture components (Faust et al., 2003; Backhaus et al., 2004).

The objective of the present study was to investigate the toxicity of selected antifouling compounds. The effects of Zpt, chlorothalonil and Sea-Nine on early developmental stages of sea urchin were studied individually and in combination, using the CA and IA concepts to describe their mixture toxicity, and the potential risk for the marine environment was discussed. Since the tested biocides present rather heterogeneous chemical structures we expect them not to be strictly similarly acting compounds, which suggests that IA would be more suitable for the prediction of mixture toxicities. However, although several studies have undertaken the investigation of the toxicity modes of action of Zpt, chlorothalonil and Sea-Nine, their mechanisms of action are still not clearly established (Sujkowski et al., 1995; Bragadin et al., 2003, 2005), and the application of the CA concept should not be discarded.

It is generally accepted that embryos and larvae are the most sensitive developmental stages in the life cycle of marine invertebrates. Because of their high sensitivity, rapid response and ecological relevance, the embryo-larval bioassays with marine invertebrates, in particular with bivalves and sea urchins, have been used for decades in the toxicity evaluation of marine pollutants (Kobayashi, 1995; His et al., 1999). We report here toxicity tests with the edible sea-urchin *Paracentrotus lividus* (Lamarck, 1816), a large regular sea-urchin widely distributed throughout the Mediterranean Sea and European Atlantic coast with important ecological roles in the functioning, dynamics and structure of benthic assemblages (Hayward and Ryland, 1990; Boudouresque and Verlaque, 2007). Also, several studies have shown the importance of sea-urchin pluteus larvae in the composition and biomass of zooplankton communities, playing a significant role in the pelagic food web (Luis et al., 2005). In some European countries *P. lividus* is exploited for its highly valued gonads (Boudouresque and Verlaque, 2007).

2. Materials and methods

2.1. Biological material

Mature *P. lividus* were collected in a pristine site located at the Ría de Vigo (Galicia, NW Spain). Animals were transported to the laboratory in a portable icebox and maintained in aquaria with running natural seawater for at least 1 week until the experiments. Sea-urchins were fed with the green algae *Ulva lactuca*.

2.2. Experimental procedure

P. lividus gametes were obtained by dissection from a single pair of adults according to methods described elsewhere (Beiras and Saco-Álvarez, 2006). Mature oocytes were transferred to a 100-ml measuring cylinder and their quality was checked under microscope. Only batches of mature eggs that were spherical and undamaged were used for the experiments. Sperm mobility was checked under microscope and the sperm solution was stored at 4 °C until use. A few microliter of motile sperm were added to the egg suspension and carefully stirred to allow fertilization. Fertilized eggs from one female and one male were used to minimize genetic variability (Stebbing et al., 1980; Klöckner et al., 1985). Approximately 350 fertilized eggs (i.e. those with a fertilization membrane) were delivered into glass vials with airtight Teflon-lined screw caps containing 20 ml of the experimental solutions. The vials were incubated in darkness at 20 °C for 48 h (until larvae reached the four-arm pluteus stage).

After the incubation period sea-urchin larvae were preserved by adding a few drops of 40% buffered formalin, and the percentage of four-arm pluteus larvae ($n = 100$) and the mean larval growth ($n = 35$) were recorded. Larval growth was defined as the maximum dimension in the first 35 individuals per vial (including embryos), subtracting the average of the diameter of the fertilized eggs (90.49 ± 4.53). Length of individuals was recorded in the inverted microscope using the Leica QWin image analysis software. Four replicates per treatment, 10 artificial seawater (ASW) controls, and 10 dimethylsulfoxide (DMSO) controls were assayed for each experiment. Control embryogenesis success was always above 90%. Physico-chemical conditions of the experiments were 35.10 ± 0.64 ppt salinity, 7.58 ± 0.01 mg/l O_2 and 8.23 ± 0.01 pH (mean ± S.D.).

2.3. Experimental solutions

The selected biocides were analytical grade zinc pyrithione (1-hydroxypyridine-2-thione (pyrithione) zinc salt) (Sigma–Aldrich), chlorothalonil (2,4,5,6,-tetra-chloro-isophthalonitrile) (Sigma–Aldrich) and Sea-Nine 211 (4,5-dichloro-2-*n*-octyl-4-isothiazolin-3-one) (Rohm and Haas). Stock solutions were freshly prepared in a non-toxic organic dissolvent, dimethylsulfoxide (Bellas et al., 2005a), approximately 1 h before the beginning of the experiments. The experimental concentrations were obtained by serial dilution of the stock solution in artificial seawater (ASW) prepared as in Zaroogian et al. (1969). The range of experimental concentrations, chosen on the basis of range-finding trials, was below the water-saturation levels of the tested compounds, covering concentrations measured in the environment for chlorothalonil and Sea-Nine (Voulvoulis et al., 2000; Martínez et al., 2001; Sakkas et al., 2002), whilst predicted environmental concentrations were used for Zpt (Madsen et al., 1999). All glassware was acid-washed (HNO_3, 10% vol.) and rinsed with acetone and distilled water before the experiments.

2.4. Mixture experiments

In designing binary and ternary mixture experiments we have employed a fixed ratio design, i.e. the mixture ratio of the individual components was kept constant whilst the total concentration of the equitoxic mixture was varied. The individual compounds were mixed at the ratio of their EC_{50}s. The following ratios were used: 0.1, 0.3, 0.44, 0.67, 1, 1.5, 2.25 and 3.38. Therefore, the total concentration in the mixture c_{mix} is the sum of the concentration of the n components i (c_i) which are present as fractions p_i as shown in the following equation:

$$c_{mix} = \sum_{i=1}^{n} c_i = \sum_{i=1}^{n} (p_i c_{mix}) \quad (1)$$

Using this design, the concentration–response relationship of the mixture can be described on the basis of the single substance experiments.