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Original scientific paper

Investigation of spermiotoxic, embryotoxic and cytotoxic effects of copper pyrithione on *Paracentrotus lividus* (Lamarck, 1816)

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SUMMARY

Spermiotoxic, embryotoxic and cytotoxic effects of the widely used biofouling biocide copper pyrithione (CuPt) were evaluated in bioassays to examine the inhibition of fertilization rate, offspring quality and effects on early development of the sea urchin *Paracentrotus lividus*. CuPt was non-spermiotoxic for fertilization rates but the frequency of embryonic malformations increased in a concentration-dependent manner when eggs were fertilized with CuPt-exposed sperm. CuPt EC_{50} was calculated to be 13.58 µg/l for embryotoxicity. While the frequency of normally developed plutei decreased, the number of larvae with skeletal deformations increased. The IC_{25} and IC_{50} values in cytotoxicity assays were calculated to be 12.79 and 47.85 µg/l, respectively. The study revealed statistically significant decrease in the number of mitotically dividing cells, increase in the percentage of interphase cells and increased chromosomal abnormalities in the exposed cells. According to these results, CuPt can be said to have a highly toxic effect on sea urchin embryos at the applied concentrations. This situation suggests that there may be a potential risk of marine contamination with CuPt for this species.

Keywords: biocides, copper pyrithione, developmental biology, sea urchin

INTRODUCTION

Biofouling is the growth of unwanted organisms on submerged/semi-submerged structures, adding friction to ships leading to increased fuel consumption and economic loss (Egardt et al., 2017). Anti-fouling biocides are used to impede the growth of aquatic organisms, such as mussels, crustacea, barnacles and algae, on the submerged parts of marine vessels. They interact with organisms and have variable toxic impacts, and high level of toxicity in some cases. Sublethal effects on aquatic organisms include decreased growth rates and lower reproducibility (Fernández-Alba et al. 2002). Common anti-fouling agents, such as tributyltin (TBT), were therefore banned in 2008. Alternative biocides which cause less toxicity to non-target organisms, broad

antimicrobial activity, low water solubility and high degradability have been used in marine antifouling paints as replacements for TBT. Pyrithione salts, such as zinc pyrithione (ZnPt) and copper pyrithione (CuPt), were introduced on the market in the 1990s as alternatives to TBT. They are both lipophilic metal complexes, which can interact with free metal ions in seawater by exchanging their metal ions (Maraldo & Dahllof, 2004; Martins et al. 2018).

The widespread use of CuPt has become a focal concern of recent studies and more research has been conducted on its ecotoxicity to non-target aquatic organisms, such as fish and invertebrates (Koutsaftis & Aoyama, 2007; Onduka et al., 2010; Bao et al., 2011; Wang et al., 2011). High CuPt toxicity to non-target species of different trophic levels, such as the algae *Skeletonemacostatum* (72-h EC_{50} = 1.5 μg/l), crustacean *Tigriopus japonicus* (24-h EC_{50} = 23 μg/L), and fish *Pagrus major* (96-h LC_{50} = 9.3 μg/L), was reported (Onduka et al., 2010). In a study where toxic effects of CuPt, zinc pyrithione (ZnPt), Sea Nine-211, diuron, Irgarol 1051 (also termed co-biocide) and KH101 to *Oncorhynchus tshawytscha* were studied, the highest toxicity was found to result from CuPt (Okamura et al., 2002). Embryotoxicity of CuPt to the sea urchin *Strongylocentrotus intermedius* was reported as EC_{50} = 32.93 nM (Wang et al., 2011). In another study using the same species, the EC_{50} value in the first 6 h after fertilization was 4000 nM (Xue et al., 2011).

In different studies with aquatic organisms, such as *Perinereis nuntia* (Mochida et al., 2011), *Artemia salina* (Koutsaftis & Aoyama, 2007) and sea urchins (Kobayashi & Okamura, 2002), the toxicity of various biocides was investigated, and the CuPt biocide was shown to be highly toxic to these organisms.

Toxicity bioassays are biological tools complementing analytical chemistry techniques for assessing biological effects of pollution in complex matrices, such as sediment, and they usually involve the use of test organisms in the laboratory to predict ecosystem-level effects. Their additional advantage is that they detect new/emerging contaminants for which no analytical techniques have yet been developed or validated, providing an insight into the bioavailability of pollutants or integrating the toxic effects of different substances in the environment. The sea urchin and bivalve embryo development test is a standardized, economic, rapid chronic toxicity tool successfully used in screening the toxicity of known, emerging contaminants and other pollutants and their mixtures in natural matrices, such as water and sediments (Carballeira et al., 2012; Soares & Junior, 2016; Pagano et al., 2017; Rial et al., 2017). *Paracentrotus lividus* is an indicator marine species, widely found in the Mediterranean and Aegean Seas, and its occurrence is not restricted and therefore is representative for a variety of ecosystems (Martins et al. 2018).

The ecotoxicology studies cited above have drawn attention to CuPt as an emerging marine pollutant with a potential to adversely affect marine life. A literature survey showed that no research had been conducted on the effects of CuPt on *P. lividus*. Therefore, the present study aims to reveal the spermiotoxic, embryotoxic and cytotoxic effects of CuPt on sea urchins as an important model organism.

MATERIAL AND METHODS

Adult samples *of Paracentrotus lividu*s, a test organism used in embryotoxicity and spermiotoxicity bioassays, were hand collected in unpolluted areas of the Dardanelles Strait, Güzelyalı, Çanakkale province (Turkey) and transferred to the laboratory in portable boxes. Salinity, temperature, and pH of the seawater were measured to be ‰ 31, 20.3°C, and 8.14, respectively. CuPt (purity: 95-100%; CAS: 14915-37-8; molecular weight: 315.86 g/mol) was obtained from Arch UK Biocides. Stock solution of CuPt was prepared in dimethyl sulfoxide (DMSO; purity: >99%, Amresco), at 400 μg/ml. Exposure solutions were prepared from stock solutions at concentrations of 1, 10, 20, 40, 60, 80 and 100 μg/l. According to the relevant literature, concentration range was made between concentrations with the highest mortality range and the lowest inhibitory effect. A positive control group was exposed to $3x10^{-4}$ M cadmium chloride ($CdCl_2$, CAS no:10108-64-2, purity: 99.9%; Fischer Scientific, Waltham, MA).

Gametes were obtained and embryos were allowed to develop according to Pagano's bioassay protocol (1986; 2001). The gametes were harvested by injecting 1-3 ml of 0.5 M KCl into the ceolomic cavity along the perioral membrane. The eggs were placed in sterilized beakers containing 25 ml of filtered seawater (FSW) and sperm was retrieved in dry media (without gametes being put into FSW). Bioassay protocols for fertilization, embryo development and quality control at all stages of the procedures were conducted as reported by Pagano et al. (1986) and Chapman et al. (1995). The spermiotoxicity experiment was carried out in quadruplicate, while the embryotoxicity experiment was conducted in triplicate to use less chemical and save time during counting. In the control group, the fertilization rate was expected to

be at least 70-90% and normal plutei to be at least 80% (Chapman et al. 1995).

Spermiotoxicity experiment

The spermiotoxicity experiment was carried out in two stages. In the first stage, the egg fertilization rate of CuPt-exposed sperms was evaluated: 20 µl aliquot of sperm harvested from 3 males was exposed to each consentration of CuPt diluted with 10 ml of FSW. The experiments were conducted in amber colored glass containers to avoid sunlight-induced degradation of copper pyrithione and to prevent its adhesion to plastic culture plates (Bao et al., 2011). Sperm was exposed to different concentrations at room temperature for 30 min, then 40 µl aliquot of sperm suspension was added to FSW containing 4 ml of egg suspension (about 50 eggs/ml) (Pagano et al., 1986). The eggs were left to incubate at 18 ± 1°C for 30 min. At the end of exposure time, fertilization was terminated with 1% formaldehyde (Novelli et al. 2003). Randomly selected 100 embryos were taken from each sample and evaluated blind under a light microscope and the percentage of fertilization was determined. Only embryos with fertilization membranes which were observed clearly were considered as fertilized.

In the second stage of the spermiotoxicity bioassay, embryos were allowed to develop from CuPt exposed sperms, and morphologically assessed. Eggs were fertilized with the sperm exposed to CuPt for 30 min and left to incubate at 18 ± 1°C for 48-72 h. After that developmental abnormalities were determined under the light microscope (Pagano et al. 2001).

Embryotoxicity experiment

The zygote solution was prepared by adding sperm suspension onto the egg solution prepared for the embryotoxicity experiment and left to undergo fertilization for 20 min. One ml of the fertilized egg solution was sampled and added into the control group and concentration series of test solutions (1, 10, 20, 40, 60, 80 and 100 µg/l CuPt concentrations) and 10 ml of aliquot was obtained. The embryos were left for incubation at 18 ± 1°C for 48-72 h until they reached the pluteus larva stage. At the end of the experiment, embryo development was terminated with 1% formaldehyde and 100 embryos were randomly enumerated under the light microscope for the frequency of malformations detected in embryos. Then the percentage of developmental anomalies were evaluated in the plutei: (1) normal pluteus size and symmetry, (2) malformed larvae P1+P2 (P1: malformed skeletal system; P2: missing skeletal system); (3) development retardation (R) (sizes ≤ 1/2 normal larvae and embryos having failed to reach pluteus stage (blastula and gastrula) (as modified from Pagano et al., 1986, 2001; Cairns, 1986).

Cytogenetic analysis

Fertilization and domestication of cells in cytotoxicity experiments were carried out as in the embryotoxicity bioassay. However, after 5-6 h fertilization exposure, the embryos were filtered and put into 10 ml of Carnoy's fluid (absolute ethanol:chloroform:acetic acid, 6:3:1) and fixed in this fixative for 30 min. Thirty min after fixation, the fixative was discharged and 10 ml of absolute ethanol was added. The samples were kept in absolute ethanol for 24 h and the solvent was replaced at the end of 24 h, and then stained with 2% of acetocarmine dye and examined under the light microscope (1000x). Enumeration and morphological measurements of mitotic degradation and chromosome anomalies, used to assess cytogenetic effects, were carried out in line with Pagano et al (1986) and Ferreira et al. (2009).

Statistical analysis

Dose-response relationships for CuPt were established to calculate the EC_{50} defined as the toxicant concentration causing 50% reduction in embryogenesis success. Data showing the percentages of abnormal larvae (identified for each exposure concentration and each control group: negative, solvent and positive controls) were analysed and are given as mean ± standard error or with 95% confidence interval (CI). The median effect concentration (EC_{50}) is the calculated value for 50% of the estimated toxic effect associated with CuPt exposure concentrations. The toxic endpoint in the embryotoxicity assay was observed as the percentage of abnormal larvae (25%, EC_{25}, and 50%, EC_{50}). In the spermiotoxicity and cytotoxicity experiments, IC_{25} and IC_{50} values were calculated using: i) a linear interpolation method (ICPIN Version 2.0, USEPA), and ii) a Toxicity Relationship Analysis Program (TRAP, USEPA, Duluth, MN, USA). The spermiotoxicity data (fertilization rate) were transformed into arc-sin square root and exposure concentrations log-transformed for analysis. Differences between mean deformities in embryotoxicity and cytotoxicity bioassays were analyzed using the non-parametric Kruskal-Wallis H test, since the data did not meet normal distribution assumptions. The significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

The sea urchin spermiotoxicity, embryo development and cytotoxicity bioassays were used in the present study to evaluate the toxicity of CuPt using the indicator species *Paracentrotus lividus*.

In the first stage of the spermiotoxicity test, control and DMSO control fertilization rates were very similar and the average was 90.87±4.84, therefore the tests were considered valid. However, spermiotoxicity of CuPt calculated as fertilization rate was minimum 75%, therefore the confidence intervals for IC_{25} and IC_{50} (95%) could not be calculated, since at least one dose (concentration) has to be under 50%. It is possible to conclude that CuPt did not significantly affect the fertilization rate of *P. lividus* sperms.

In the second stage of the spermiotoxicity test, malformations of embryos developed from the CuPt-exposed sperms increased depending on concentration. A decrease in the frequencies of normal (N) ($P < 0.05$, Figure 1) developed plutei from the CuPt-exposed sperm was significantly different from controls. Malformations of embryos (P1+P2 combinations) developed from CuPt-exposed sperm increased in a concentration-dependent way, and mean differences were significant between normal and malformed embryos (P1+P2) ($P<0.05$, Figure 2). There were no significant differences between the development retarded (R) embryos exposed to the given concentrations (1, 10, 20, 40, 60, 80 and 100 µg/l CuPt concentrations) ($P>0.05$, Fig. 3).

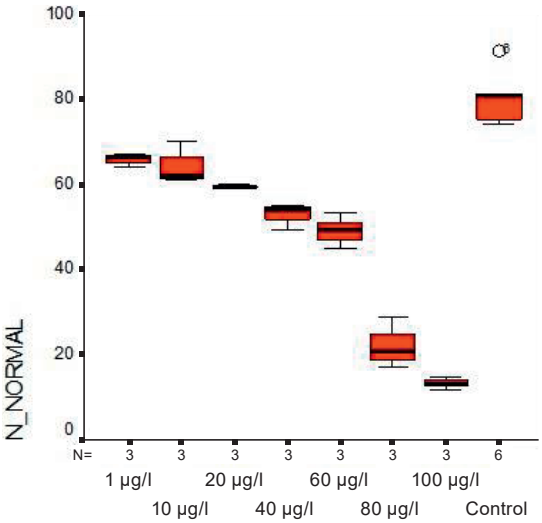

Figure 1. Alteration (expressed as percentage) in normal plutei (N) developed from eggs fertilized with sperm exposed to different CuPt concentrations

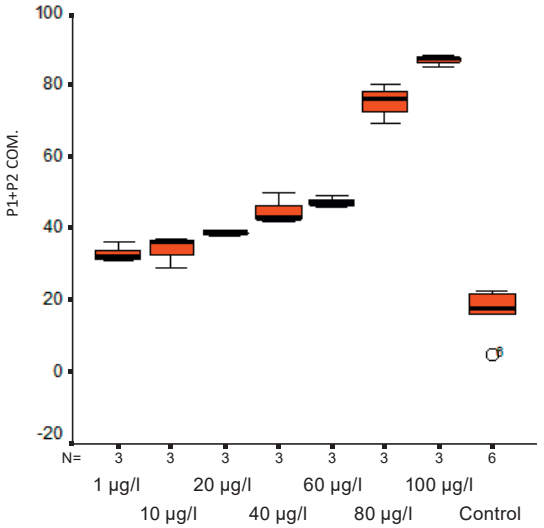

Figure 2. Alteration (expressed as percentage) in P1+P2 combination developed from eggs fertilized with sperm exposed to different CuPt concentrations

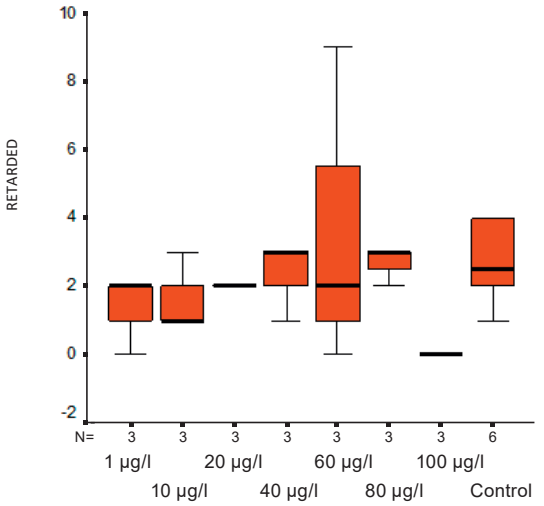

Figure 3. Alteration (expressed as percentage) in retarded embryos (R) developed from eggs fertilized with sperm exposed to different CuPt concentrations.

Embryotoxicity test

In embryotoxicity experiments, solvent control was considered safe. The percentage of abnormal embryos increased significantly after applying CuPt at all of its seven exposure concentrations (i.e., 1, 10, 20, 40, 60, 80, 100 µg/l). Decrease in the frequencies of normal (N) developed plutei in exposure groups was significantly different from controls ($P < 0.05$, Figure 4). A positive control group was exposed to $3x10^{-4}$ M cadmium chloride and 100% of the embryos showed development

retardation (R) malformation. The EC_{50} value of CuPt was calculated to be 13.58 µg/l for embryotoxicity to *P. lividus*. While the average value of normal pluteus was 61.33 ± 1.75 in the group exposed to 1 µg/l CuPt, it was 3.83 ± 7.52 in the group exposed to 100 µg/l ($P < 0.05$). As CuPt concentration increased, the mean percent of normally developing specimens decreased, whereas skeletal deformities increased with P_1+P_2 combinations (Figures 5-6).

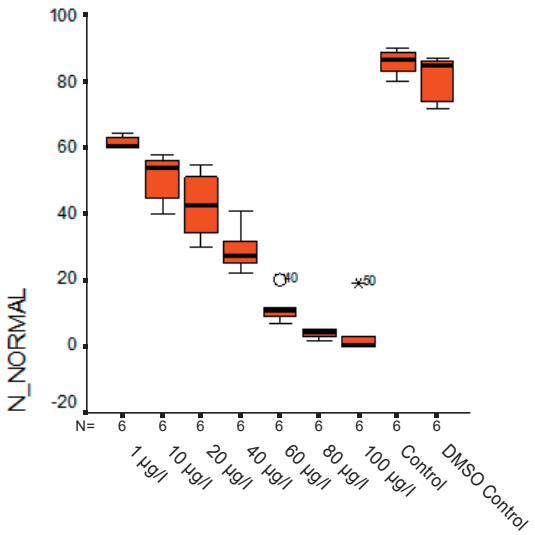

Figure 4. Embryotoxicity results for normal plutei (N, expressed as percentage) developed from normal eggs and sperm showing larval development effects due to different CuPt concentrations

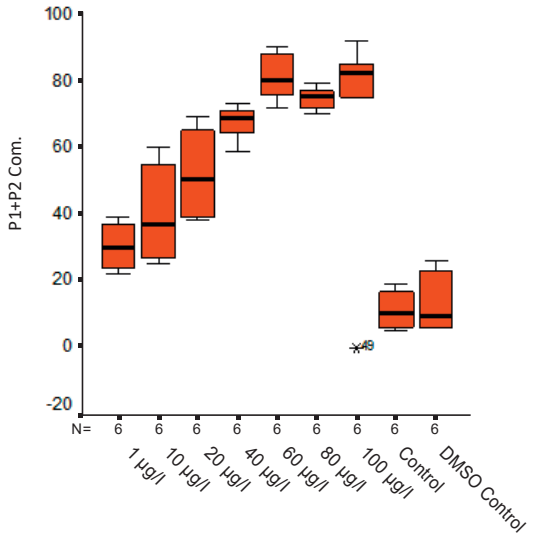

Figure 5. Variations in embryotoxicity expressed as P1 + P2 combination of developmental defects (%) depending on different CuPt concentrations

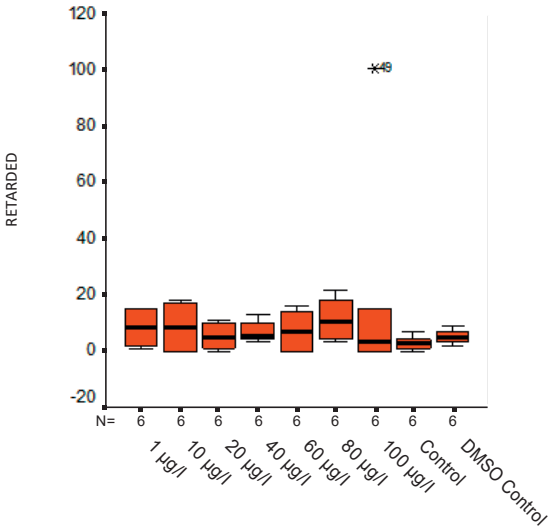

Figure 6. Variations in embryotoxicity expressed as retarded (R) developmental defect (%) depending on different CuPt concentrations

Cytotoxicity test

In cytotoxicity experiments, the IC_{25} and IC_{50} values for percentage mitotic index were calculated to be 12.79 µg/l and 47.85 µg/l, respectively. Our results showed the percentage mitotic index of the groups as: 28% ± 0.007 (control); 28% ± 0.005 (1 µg/l); 24%± 0.008 (10 µg/l); the percentages decreased from 16 to 7% under doses increasing from 20 to 100 µg/l (Figure 7). DMSO solvent control was not cytotoxic, based on the mitotic index. The positive control cadmium chloride was 3% ± 0.001. Mitotic index decreased in a concentration dependent manner. Interphase percentage per embryo decreased inversely with decreased mitotic index (Figure 8).

The metaphase and anaphase ratio (M/A) was 4.4 in the 60 µg/l exposure group and close to 2 in all other concentration groups, suggesting that cell transition from metaphase to anaphase was inhibited by the concentration of 60 µg/l (Figure 9). The number of lagged chromosomes, a chromosome abnormality, exhibited no significant difference between the 10 and 20 µg/l concentrations but tended to increase with concentration. The number of lagged chromosomes was 0.47 ± 0.06 and 2.9 ± 0.24 under 1 and 60 µg/l concentrations, respectively. The control group was 9.6 ± 0.23. Under 100 µg/l, the number was found to be 6 ± 0.19 due to much fewer cells undergoing mitosis. The number

of free and broken chromosomes also tended to increase depending on concentration. The numbers of free chromosomes were 0.35 ± 0.07, 0.68 ± 0.01 and 1.1 ± 1.1 in the control group and under 1 µg/l and 60 µg/l concentrations, respectively. In addition, the number of broken chromosomes was 1.6 ± 0.16, 3.2 ± 0.17 and 6.8 ± 0.21 in the control group and under 1 µg/l and in 100 µg/l, respectively. No increase in the number of sticky chromosomes was observed under concentrations up to 60 µg/l but they increased under 80 and 100 µg/l concentrations. No prominent differences in the formation of acentric fragment and anaphase bridge were observed between concentrations.

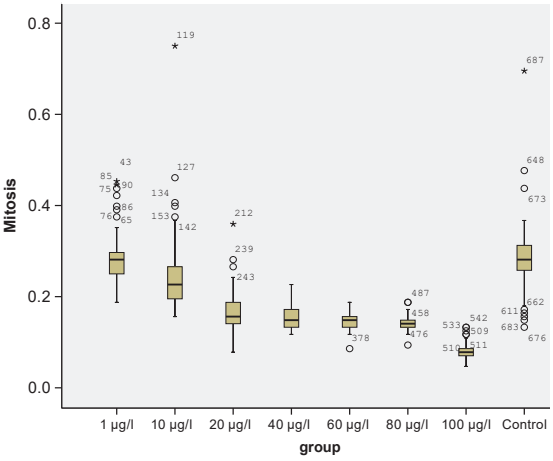

Figure 7. Variation in mitotic index counts per embryo due to CuPt exposure concentrations

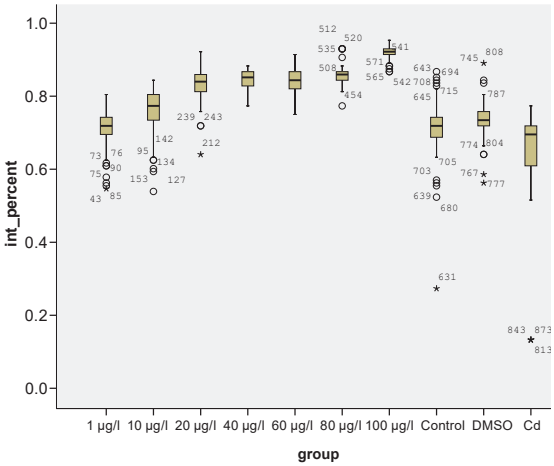

Figure 8. Variation in interphase percentages per embryo due to CuPt exposure concentrations

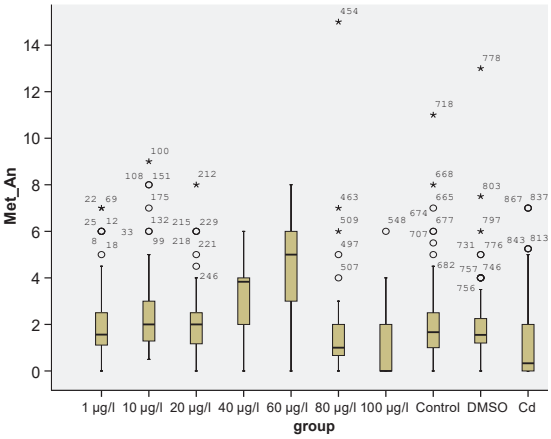

Figure 9. Changes in M/A ratio per embryo due to CuPt exposure concentrations

In toxicology, hormetic dose response occurs associated with exposures of biological organisms to environmental stressors. Some adaptive pathways extend the region of cellular homeostasis and are protective against toxicity. Hormesis denotes that cells can positively maintain their metabolic activities and adapt to lower concentrations of toxic agents (Calabrese, 2008; Tang et al., 2019). DMSO used as a solvent is known to protect cells from various lesions, to modify membrane permeability and to act as an antioxidant in biological systems (Grigoryan & Shiladzhyan, 2009). In the spermiotoxicity test of the present study, an increase of 9% in the number of fertilized embryos in 1 µg/l, compared with the control group, is considered to have stemmed from a probable hormetic effect of lower doses of CuPt. It is also thought that DMSO might have suppressed the toxic effect of CuPt on sperm motility most probably due to antioxidant effects. Onduka et al. (2010) estimated CuPt 24 h toxicity to the crustacean *Tigriopus japonicus* as EC_{50} = 23 µg/l, and our results agree with them. Xu et al. (2011) reported individual and joint-action toxicity of binary or multiple mixtures of heavy metal compounds for embryonic toxicity and spermiotoxicity in the sea urchin *Strongylocentyotus intermedius*. Among four metals, Cu was the most toxic with EC_{50} = 1.32 µM, while toxicities to larval development in sea urchin embryos decreased in the order: Cu > Pb > Zn > Cd. Cu was still very toxic (EC_{50} = 6.40 µM) regarding sea urchin fertilization success, spermiotoxicity, and the ranking was: Zn > Cu > Pb > Cd based on the EC_{50} values. Fernandez & Beiras (2001) found very comparable data for Cu embryotoxicity to *P. lividus*.

Exposure of sea urchin *Evechinus chloroticus* adults to moderately high levels of waterborne copper led to a significant increase in copper burden in gonads of both sexes; however spawning success was not impaired. Larval size was also significantly affected by the copper burden of mothers, and many larvae were severely abnormal at this early stage. The study concluded that in a polluted environment there is likely to be an overall reduced reproductive output across the population (Phillips & Rouchon, 2018).

The toxicity of CuPt to *Artemia salina*, the brine shrimp, was significantly influenced by the organic matter content, salinity and proportions of constituent salts in water. Upon combining with cupric ions in the medium, the non-hazardous degradation product 2-mercaptopyridine-N-oxide (HPT) exhibited increased toxicity due to its rapid transformation to the parent biocide. The 48 h CuPt acute survival effect to *A. salina* was estimated as EC_{50} = 250 μg/l (Lavtizar et al. 2018). The ranges agree with data in the present study.

Basallote et al. (2018) demonstrated effects of pH and pCO_2 on sediment metal toxicity to *Paracentrotus lividus* embryotoxicity and spermiotoxicity, and metal toxicity was significant at pH 7.0 and 6.5. The antifouling biocide CuPt easily photolyses (Lavtizar et al. 2018), similar to zinc pyrithione (ZnPt), and can interact with free metal ions by releasing the metal ion (zinc-zinc pyrithione or copper) and take up new free metal ions, e.g., manganese, iron or copper from seawater. The degradation half-life of ZnPt and CuPt was estimated to be between 7 and 9 min. when exposed to light under experimental conditions. Marinas and harbours have a high-risk potential for ZnPt and CuPt accumulation due to high concentration of boats in them that are sources of these biocides as paint particles continue to release them and cause persistent contamination of the sediment (Thomas et al., 2000; Maraldo & Dahllof, 2004).

Egardt et al. (2017) studied antifouling paints in sediments in a national park on the Swedish west coast and determined several banned antifouling compounds in surface sediments. Furthermore, the reported Cd and Cu levels were significantly higher than background values, and copper was significantly correlated with Cd, Cr and Pb, suggesting similar sources and similar correlation between Cd and Zn. Although Cu is an essential metal, it may pose a potential risk of being toxic in high concentrations, and may have sub-lethal effects on the benthic environment, raising concern for all sources of Cu whether organic, organometallic or metallic. Metal contamination of marine waters is not limited to known abundant pollutants, heavy rare earth elements (HREEs) are of concern too due to growing applications in several advanced technologies, necessitating in-depth, ad hoc investigations for potential environmental toxicology. Oral et al. (2017) studied HREE-associated toxicities to *Arbacia lixula* and *Paracentrotus lividus* and found different toxicities of the tested HREEs in terms of effects on embryogenesis, fertilization, cytogenetic and redox endpoints at 10^{-5} M. In agreement with our results, sperm exposure to HREEs (10^{-5}-10^{-4} M) resulted in a concentration-related decrease in fertilization success along with increase in offspring damage. The same research group extended their research to other sea urchin species for comparing species sensitivities, and found similar impacts. Gravina et al. (2018) reported effects of broadly unexplored HREEs effects to early life stages of the sea urchin *Sphaerechinus granularis* and observed significant developmental defect (DD) increases, i.e. significant decrease in mitotic activity with increased mitotic aberrations in embryos even at 10^{-5} M. *S. granularis* lives further offshore and deeper than the other species, *P. lividus* and *A. lixula,* and it is historically more sensitive to pollutants.

Toxic effects (spermiotoxicity) of three forms of selenium to *P. lividus* were also reported for the larval stage, and hypothesized to be associated with sperm since selenium was prominent in the early reproductive stages (Oral 1997). As in that study, it is considered in the present study that CuPt toxicity might have been conveyed to the embryo through the genetic material of the sperm.

Incresed skeletal deformities were induced by CuPt in the fish species *Fundulus heteroclitus*, where metabolites originating from CuPt degradation in sea water inhibited the AChE esterase enzyme (Mochida et al., 2009). Moreover, CuPt is likely to degrade cell membrane and pH gradient, and to bind such complex agents as metals and proteins (Ermolayeva & Sanders, 1995; Wang et al., 2011). It can be derived from these results that skeletal malformations observed in the embryotoxicity test on *P. lividus* may also be due to mechanisms similar to those cited in earlier studies. Bellas et al. (2005) tested four pesticides (chlorpyrifos, diuron, lindane and tributyltin) for potential threat to non-target marine species. These substances are also introduced to the marine environment and coastal areas by spray drift, surface runoff or accidental spills. Although they are not in the same chemical group with CuPt, their toxicities to early development of embryos and larvae of four marine invertebrates, chosen for their abundance, ecological importance and commercial relevance, namely: an echinoid (*Paracentrotus lividus*), an ascidian (*Ciona intestinalis*), and two crustacean species (*Maja squinado* and *Palaemon serratus*), were studied and

the presented results are in agreement with our work. Tributyltin embryotoxicity EC_{50} was 0.309 μg/l,, i.e. it was highly toxic to *P. lividus* embryogenesis, as our results also show. Another form of Cu is copper oxide nanoparticles (CuO NPs), extensively used in industrial and commercial applications. The effects of CuO NPs on the spermatozoa of the sea urchin *Paracentrotus lividus* were assessed using physiological and biochemical markers, and oxidative stress was found as the main driver of CuO NP spermiotoxic effects (Gallo et al., 2018). Male reproductive success depends on several abiotic and biotic factors. The authors emphasized sperm quality to be crucial for predicting male reproductive biology and since CuO NP exposure was associated with a reduction in sperm quality and fertilization failure, attention was drawn to such effects having potential implications for species fitness and survival.

In cytotoxicity studies on the effects of three forms of selenium on *P. lividus*, as the concentration increases the toxic agents are found to cause decrease in mitotic index by inhibiting mitotic activity, and increase in the percentage of interphase cells. Moreover, they are also thought to break chromosomes and inhibit chromosomes from binding to spindle fibers and to enable the formation of free chromosomes by hindering cell division shuttles in their varying concentrations. Inferring from all these findings on chromosome abnormalities, it is thought that these toxic agents are lethal to some embryos in early stages (blastula or gastrula) before differentiation, while causing skeletal malformations in the developed embryos (Oral 1997). Our results are in agreement with those of other studies regarding the cytogenetic toxicity analysis of CuPt (Oral, 1997; Oral et al., 2017; Gravina et al., 2018). CuPt also inhibits mitotic activity more as concentrations increase, and causes increased population of interphase cells and also affects chromosomal activities. Previous research had evidenced that CuPt used in anti-fouling paints was highly toxic to marine organisms (Mochida et al., 2009). When CuPt is rapidly disintegrated in seawater, its environmental concentrations change and therefore further research on CuPt should be carried out (Harino et al., 2007).

The present research revealed that the non-persistent antifouling biocide CuPt in the administered concentrations was highly toxic to *Paracentrotus lividus* embryos. CuPt was not toxic to sperm but to embryos developing from eggs fertilized by that sperm. Moreover, this study is significant in reporting cytogenetic effects of CuPt. Bioassays provide invaluable information on the bioavailability of pollutants, reveal complex interactions between emerging contaminants not previously considered as risks, and are rapid, reliable and simple. Limited work has been done on comparing the sensitivity of different species to pollutants, relying for hazard assessment of pollutants on single-species tests that cannot detect the full range of pollutants entering the marine environment, and the present work is a contribution to the field in that sense. There is a need to standardize these bioassays, especially for climate change impact and mixtures of pollutants, and more significantly for emerging contaminants (Pagano et al., 2017). Comparative toxicity data on pollutants across taxonomically distant echinoid species characterized by different habitats and by different sensitivities to xenobiotics will be most beneficial for standardizing and validating bioassays, especially for variable sensitivities of test species used. *Patella* spp. (Mollusca, Gastropoda) has also been evaluated for use as a standardized protocol for embryo-larval bioassay (Perez et al., 2016). Recently Morroni and co-workers (Morroni et al., 2016, 2018) developed a new Integrative Toxicity Index (ITI) for overall comparison of evaluation procedures and of results obtained from different experimental treatments. The ITI is expected to provide new insights into the capability of each metal to induce anomalies in the embryogenesis of echinoid embryos and their recovery to normal development after metal exposure, thus adding further ecological value to the sea urchin bioassay. The possibility to understand and weigh the reversibility of toxic effects improves the ecological relevance of embryo toxicity bioassays. These studies provide comparative toxicity data on single and mixture of emerging contaminants and should be considered a priority for monitoring and regulatory programs. Programs aimed at minimizing adverse health outcomes from single or mixed pollutant exposures will be used for regional and international level mitigation measures. As CuPt is already approved in the European Union, while ZnPt is pending approval (EU Commission, 2015), and Japan, Hong Kong, China, Australia and New Zealand have authorised the use of both in antifouling paint formulations (NZEPA, 2013; APVMA, 2017 as sited by Martins et al., 2018), comprehensive risk assessment studies should be launched soon.

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REFERENCES

Bao, V.W.W., Leung, K.M.Y., Qiu, J.W., & Lam, M.H.W. (2011). Acute toxicities of five commonly used antifouling booster biocides to selected subtropical and cosmopolitan marine species. *Marine Pollution Bulletin 62*, 1147-1151. doi:10.1016/j.marpolbul.2011.02.041

Basallote, M.D., Rodriguez-Romero, A., De Orte, M.R., DelValls, T.A., & Riba, I. (2018). CO_2 leakage simulation: effects of the pH decrease on fertilisation and larval development of *Paracentrotus lividus* and sediment metals toxicity. *Chemistry and Ecology, 34*, 1-21.

Bellas, J., R. Beiras, J.C. Marino-Balsa, N. Fernandez. (2005). Toxicity of organic compounds to marine invertebrate embryos and larvae: A comparison between the sea urchin embryogenesis bioassay and alternative test species. *Ecotoxicology, 14*, 337-353.

Cairns, J. (1986). *Community toxicity testing*: *ASTM* STP 920. West Conshohocken, PA: American Society for Testing and Materials.

Calabrese, E.J. (2008). Hormesis: Why it is important to toxicology and toxicologists. *Environmental Toxicology and Chemistry, 27*(7), 1451-1474.

Carballeira, C., Ramos-Gomez, J., Martin-Diaz, L., & DelValls, T.A. (2012). Identification of specific malformations of sea urchin larvae for toxicity assessment: Application to marine pisciculture effluents. *Marine Environmental Research, 77*, 12-22.

Chapman, G.A., Denton, D.L., & Lazorchak, J.M. (eds.) (1995). Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. *EPA-600-R95-136*. Cincinnati, OH: U.S. Environmental Protection Agency.

Egardt, J., Nilsson, P., & Dahllof, I. (2017). Sediments indicate the continued use of banned antifouling compounds. *Marine Pollution Bulletin, 125*, 282-288.

Ermolayeva, E., & Sanders, D. (1995). Mechanism of pyrithione induced membrane depolarization in *Neurospora crassa*. *Applied and Environmental Microbiology, 61*, 3385-3390.

EU Commission (2015). Commission implementing regulation (EU) 2015/984 of 24 June 2015 approving copper pyrithione as an existing active substance for use in biocidal products for product-type 21. *Official Journal of the European Union, L159*, 43-45.

Fernández-Alba, A.R., Hernando, M.D., Piedra, L., & Chisti, Y. (2002). Toxicity evaluation of single and mixed antifouling biocides measured with acute toxicity bioassays. *Analytica Chimica Acta, 456*, 303-312. doi:10.1016/S0003-2670(02)00037-5

Fernandez N., & Beiras, R. (2001). Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the *Paracentrotus lividus* sea-urchin. *Ecotoxicology*, *10*, 263-271.

Ferreira, K., Torres, G.A., Carvalho, I.V., & Davide, L.C. (2009). Abnormal meiotic behavior in three species of *Crotalaria*. *Pesquisa Agropecuaria Brasileira, 44*(12). doi: 10.1590/S0100-204X2009001200012

Gallo, A., Manfra, L., Boni, R., Rotini, A., Migliore, L., & Tosti, E. (2018). Cytotoxicity and genotoxicity of CuO nanoparticles in sea urchin spermatozoa through oxidative stress. *Environment International, 118*, 325-333.

Gravina, M., Pagano, G., Oral, R., Guida, M., Toscanesi, M., Siciliano, A. ...Trifuoggi, M. (2018). Heavy rare earth elements affect *Sphaerechinus granularis* sea urchin early life stages by multiple toxicity endpoints. *Bulletin of Environmental Contamination and Toxicology*, *100*, 641-646.

Grigoryan, K.R., & Shiladzhyan, A.A. (2009). The effect of solvated ions on the termal denaturation of human serum albumin in water-dimethylsulfoxide solutions. *Russian Journal of Bioorganic Chemistry, 35*(5), 581-584. doi: 10.1134/S1068162009050070

Harino, H., Yamamoto, Y., Eguchi, S., Kawai, S., Kurokawa, Y., Arai, T. ... Miyazaki, N. (2007). Concentrations of antifouling biocides in sediment and mussel samples collected from Otsuchi bay, Japan. *Archives of Environmental Contamination and Toxicology, 52*, 179-188. doi: 10.1007/s00244-006-0087-2

Kobayashi, N., & Okamura, H. (2002). Effects of new antifouling compounds on the development of sea urchin. *Marine Pollution Bulletin, 44*, 748-751. doi: 10.1016/S0025-326X (02)00052-8

Koutsaftis, A, & Aoyama, I. (2007). Toxicity of four antifouling biocides and their mixtures on the brine shrimp *Artemia salina*. *Science of the Total Environment, 387*, 166-174. doi: 10.1016/j.scitotenv.2007.07.023

Lavtizar, V., Kimura, D., Asaoka, S., & Okamura, H. (2018). The influence of seawater properties on toxicity of copper pyrithione and its degradation product to brine shrimp *Artemia salina*. *Ecotoxicology and Environmental Safety, 147*, 132-138.

Maraldo, K., & Dahllof, I. (2004). Indirect estimation of degradation time for zinc pyrithione and copper pyrithione in seawater. *Marine Pollution Bulletin, 48*, 894-901.

Martins, S.E., Fillmann, G., Lillicrap, A., & Thomas, K.V. (2018). Review: ecotoxicity of organic and organo-metallic antifouling co-biocides and implications for environmental hazard and risk assessments in aquatic ecosystems. *Biofouling, 34*, 34-52.

Mochida, K., Amano, H., Onduka, T., Kakuno, A.,& Fujii, K. (2011). Toxicity and metabolism of copper pyrithione and its degradation product, 2,20-dipyridyl disulfide

in a marine polychaete. *Chemosphere, 82*, 390-397. doi: 10.1016/j.chemosphere.2010.09.074

Mochida, K., Ito, K., Harino, H., Tanaka, H., Onduka, T., Kakuno, A., & Fujii, K. (2009). Inhibition of acetylcholinesterase by metabolites of copper pyrithione (CuP) and its possible involvement in vertebral deformity of a CuP-exposed marine teleostean fish. *Comparative Biochemistry and Physiology*, Part C: *Toxicology and Pharmagology*, *149*, 624–630. doi: 10.1016/j.cbpc.2009.01.003

Morroni, L., Pinsino, A., Pellegrini, D., & Regoli, F. (2018). Reversibility of trace metals effects on sea urchin embryonic development. *Ecotoxicology and Environmental Safety, 148*, 923-929.

Morroni, L., Pinsino, A., Pellegrini, D., Regoli, F., & Matranga, V. (2016). Development of a new integrative toxicity index based on an improvement of the sea urchin embryotoxicity test. *Ecotoxicology and Environmental Safety, 123*, 2-7.

Novelli, A.A., Losso, C., Ghetti, F.P., & Ghirardini, V.A. (2003). Toxicity of heavy metals using sperm cell and embryo toxicity bioassays with *Paracentrotus lividus* (Echinodermata: Echinoidea): Comparisons with exposure concentrations in the Lagoon of Venice, Italy. *Environmental Toxicology and Chemistry, 22*(6), 1295-1301. doi: 10.1002/etc.5620220616

Okamura, H., Watanabe, T., Aoyama, I., & Hasobe, M. (2002). Toxicity evaluation of new antifouling compounds using suspension-cultured fish cells. *Chemosphere, 46*, 945-951. doi: 10.1016/S0045-6535(01)00204-1

Onduka, T., Mochida, K., Harino, H., Ito, K., Kakuno, A., & Fujii, K. (2010). Toxicity of metal pyrithione photodegradation products to marine organisms with indirect evidence for their presence in seawater. *Archives of Environmental Contamination and Toxicology, 58*, 991-997.

Oral, R. (1997). Selenat, Selenit ve Seleno-Dl- Metionin'in *Paracentrotus lividus* (Lamarck, 1816) üzerine embriyotoksik ve genotoksik etkilerinin araştırılması. *Doctoral Dissertation*. Ege University, Graduate School of Natural and Applied Science, Izmir, Turkey.

Oral, R., Pagano, G., Siciliano, A., Gravina, M., Palumbo, A., Castellano, I. ... Trifuoggi, M. (2017). Heavy rare earth elements affect early life stages in *Paracentrotus lividus* and *Arbacia lixula* sea urchins. *Environmental Research, 154*, 240-246.

Pagano, G., Cipollaro, M., Corsale, G., Esposito, A., Ragucci, E., Giordano, G.G., & Trieff, N.M. (1986). The sea urchin: Bioassay for the assessment of damage from environmental contaminants (pp 67-92). In: Cairns, J., Jr., (ed.), *Community toxicity testing*. Philadelphia, PA: *American Society for Testing and Materials*. doi: 10.1520/STP23050S.

Pagano, G., Guida, M., Trifuoggi, M., Thomas, P. Palumbo, A., Romano, G., & Oral. R. (2017). Sea urchin bioassays in toxicity testing: I. Inorganics, organics, complex mixtures and natural products. *Expert Opinion on Experimental Biology, 6,* 1-10. doi: 10.4172/2325-9655.1000142

Pagano, G., Laccarino, M., De Biase, A., Meriç, S., Warnau, M., Oral, R., & Trieff, N.M. (2001). Factors affecting R6 fungicide toxicity on sea urchin fertilization and early development: roles of exposure routes and mixture components. *Human and Experimental Toxicology, 20*, 404-411. doi: 10.1191/096032701682692982

Perez, S., Fernandez, N., & Ribeiro, P.A. (2016). Standardization of a *Patella spp*. (Mollusca, Gastropoda) embryo-larval bioassay and advantages of its use in marine ecotoxicology. *Ecotoxicology and Environmental Safety, 127*, 175-186.

Phillips, N.E., & Rouchon, A.M. (2018). A dose-dependent relationship between copper burden in female urchin gonads and developmental impairment of their offspring. *Marine Environmental Research, 136*, 120-125.

Rial, D., Leon, V.M., & Bellas, J. (2017). Integrative assessment of coastal marine pollution in the Bay of Santander and the Upper Galician Rias. *Journal of Sea Research, 130*, 239-247.

Soares, J.B., & Junior, C.R. (2016). Echinodermata in ecotoxicological tests: maintenance and sensitivity. *Brazilian Journal of Oceanography, 64*, 29-36.

Tang S., Liang, J., Xiang, C., Xiao, Y., Wang, X., Wu, J. ... Cheke, R.A. (2019). A general model of hormesis in biological systems and its application to pest management. *Journal of Royal Society Interface, 16*(157), 20190468.

Thomas, K.V., Blake, S.J., & Waldock, M.J. (2000). Antifouling paint booster biocide contamination in UK marine sediments. *Marine Pollution Bulletin, 40*(9),739-745.

Wang, H., Li, Y., Huang, H., Xu, X., & Wang, Y. (2011). Toxicity evaluation of single and mixed antifouling biocides using the *Strongylocentrotus intermedius* sea urchin embryo test. *Environmental Toxicology and Chem*istry, *30*, 692-703. https://doi.org/10.1002/etc.440

Xu, X., Li, Y., Wang, Y., & Wang, Y. (2011). Assessment of toxic interactions of heavy metals in multi-component mixtures using sea urchin embryo-larval bioassay. *Toxicology in Vitro, 25*, 294-300. doi.org/10.1016/j.tiv.2010.09.007

Xue, X., Fu, J., Wang, H., Zhang, B., Wang, X., & Wang, Y.. (2011). Influence of P-glycoprotein on embryotoxicity of the antifouling biocides to sea urchin (*Strongylocentrotus intermedius*). *Ecotoxicology, 20*(2), 419-428. doi: 10.1007/s10646-011-0593-5.

Ispitivanje spermotoksičnog, embriotoksičnog i citotoksičnog delovanja bakar piritiona na *Paracentrotus lividus* (Lamarck, 1816)

REZIME

Ispitivano je spermotoksično, embriotoksično i citotoksično delovanje biocida bakar piritiona (CuPt), koji se koristi protiv biotaloženja, kako bi se u biotestu procenila inhibicija oplodnje, kvalitet potomstva i delovanje na rani razvoj morskog ježa *Paracentrotus lividus*. U pogledu nivoa oplodnje, CuPt je pokazao odsustvo spermotoksičnosti, ali se učestalost deformacija embriona povećala kada su jaja oplođena spermom izloženom delovanju CuPt, i to u zavisnosti od koncentracije. Dobijena je CuPt EC_{50} od 13.58 µg/l za embriotoksičnost. Dok je učestalost normalno razvijenih pluteusa opadala, broj larvi sa skeletnim deformacijama se povećavao. Odgovarajuće vrednosti IC_{25} i IC_{50} u biotestovima citotoksičnosti su bile 12.79 i 47.85 µg/l. Istraživanje je otkrilo statistički značajno smanjenje učestalosti deobe ćelija mitozom, povećanje procenta interfaznih ćelija i povećanje hromozomskih abnormalnosti kod izloženih ćelija. Prema ovim rezultatima, može se reći da primenjene koncentracije CuPt imaju visoko toksično delovanje na embrione morskog ježa. Ovakva situacija ukazuje na mogući rizik od kontaminacije ove vrste bakar piritionom.

Ključne reči: biocidi, bakar pirition, razvojna biologija, morski jež