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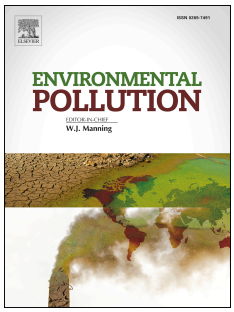

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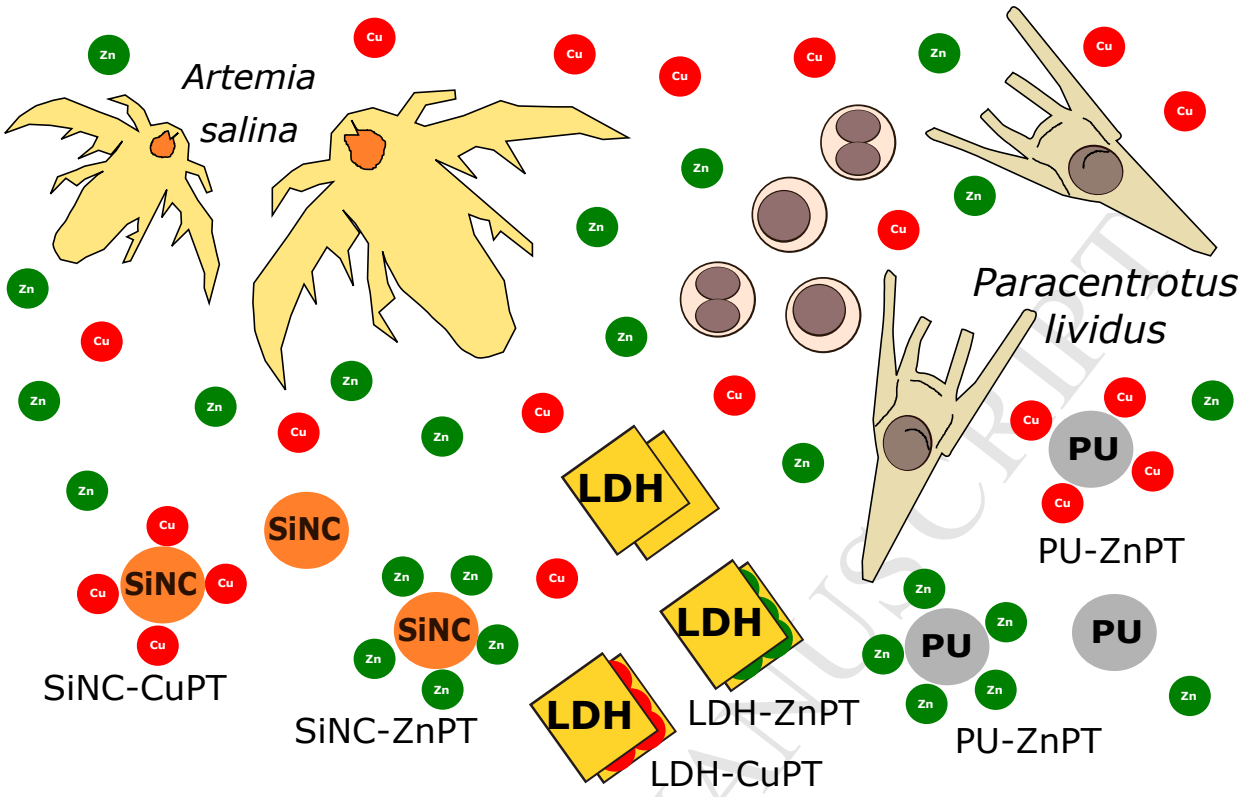

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Toxicity of engineered micro- and nanomaterials with antifouling properties to the brine shrimp *Artemia salina* and embryonic stages of the sea urchin *Paracentrotus lividus*

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Abstract

Antifouling booster biocides are chemicals used in protective paints to tackle the adhesion of fouling organisms to maritime artificial structures. However, they are also known to exert toxic effects on non-target organisms. Recent research developments have highlighted the potential use of engineered micro/nanomaterials (EMNMs) as carriers of antifouling booster biocides in order to control their release and to reduce the harmful effects on living biota. In the present study, we sought to assess the toxicity of two commercially-available booster biocides: (zinc pyrithione (ZnPT) and copper pyrithione (CuPT)); three unloaded engineered micro/nanomaterials (EMNMs); layered double hydroxides (LDH), silica nanocapsules (SiNC), polyurea microcapsules (PU);, and six novel EMNMs (loaded with each of the two biocides).

The exposure tests were conducted on the larval stage (nauplii) of the brine shrimp *Artemia salina* and on two embryonic developmental stages of the European purple sea urchin *Paracentrotus lividus*. The findings indicate that the unloaded LDH and PU (i.e. both biocide-free EMNMs) have non/low toxic effects on both species. The unloaded SiNC, in contrast, exerted a mild toxic effect on the *A. salina* nauplii and *P. lividus* embryos. The free biocides presented different toxicity values, with ZnPT being more toxic than CuPT in the *P. lividus* assays. LDH-based pyrithiones demonstrated lower toxicity compared to the free forms of the state-of-the-art compounds, and constitute good candidates in terms of their antifouling efficacy.

Highlights:

- CuPT and ZnPT have different modes of activity on the larval stages of *P. lividus*.
- The unloaded LDH nanocarrier is an environmentally safe compound.
- Lower toxicity of LDH-CuPT and LDH-ZnPT compared to state-of-the-art free compounds.
- PU nanocarrier exhibits low toxicity, being environmentally-friendly nanocarrier.

Keywords: fouling; engineered micro/nanomaterials (EMNMs); layered double hydroxides (LDH); silica mesoporous nanocapsules (SiNC); polyurea microcapsules (PU); pyrithione

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1. Introduction

Innovative compounds intended for maritime application need to be tested in terms of ecotoxicity, effects on human health, and safety before being introduced into the market. Among such compounds, the introduction of engineered micro-/nanomaterials (EMNMs) has been rapidly increasing in recent years and these have since became regarded as potential environmental pollutants (e.g. Kango et al., 2013; Garillo et al., 2015). The use of EMNMs has been recently proposed to minimize two of the major problems that affect human-made maritime structures: corrosion (Tedim et al., 2010; Maia et al., 2016; Martins et al., 2017) and biofouling (Geiger et al., 2004; Hart et al., 2011; Zheng et al., 2013; Avelelas et al., 2017; Figueiredo et al., *in press*), with the latter being the subject of the present study.

Following the global ban on the use of organotin-based antifouling paints, such as tributyltin (TBT) and tributyltin oxide (TBTO), alternative booster biocides have been tested and adopted by the maritime antifouling paint industry (Abbott et al., 2000). These include certain organic tin-free biocides, such as Irgarol® 1051, Sea-Nine 211™, zinc pyrithione (ZnPT), and copper pyrithione (CuPT), all of which have been introduced into the marine environment as biocides in antifouling paints (Voulvoulis, 2006). However, several studies have demonstrated that these compounds also produce undesired toxic effects on non-target species, thus posing a risk to the marine ecosystem through the massive amounts of biocides that are released into the seawater (Dafforn et al., 2011; Price and Readman, 2013; Tornero and Hanke, 2016; Chen and Lam, 2017; Figueiredo et al., *in press*). In this context, the encapsulation/immobilization of biocides was proposed as a way to reduce their impact on the marine environment, in addition to the benefits in terms of stability and durability of such antifouling coatings (Maia et al., 2015; Avelelas et al., 2017; Figueiredo et al., *in press*). The delivery of a given booster biocide can be

mediated by a wide-range of low/non-toxic EMNMs that function as micro- or nano-sized carriers/reservoirs/containers (Avelelas et al., 2017; Martins et al., 2017; Gutner-Hoch et al., 2018; Figueiredo et al., *in press*). These include: (a) layered double hydroxides (LDH), a class of anionic nanoclays with a lateral size ranging 20 to 40 nm, high specific surface area, and improved chemical stability (e.g. Choy et al., 2004; Tedim et al., 2010); silica mesoporous nanocapsules (SiNC), comprising hollow spheres with high loading capacity and a sustained release profile (He and Shi 2011); and (c) polyurea microcapsules (PUMC), featuring high biocompatibility and used as drug carriers (Morral-Ruiz et al., 2012), but which have never previously been assessed in terms of ecotoxicity.

The UN Agenda 2030 includes a set of goals closely related to those pertaining to worldwide sustainable development. The introduction of a toxicity assessment in the early stages of a material's development, before its entrance into the market and the environment, offers one of the possible ways by which to achieve some of these goals, particularly the promotion of ocean conservation and sustainable use. In order to assess the potential effects of novel chemicals on marine organisms, acute and rapid-screening chronic toxicity assays are highly used and recommended. The brine shrimp *Artemia salina* is a widely-used model species in marine ecotoxicology, due to its reliability, ease of performance, and cost-effectiveness (e.g., Nunes et al., 2006, Kokkali et al., 2011, Rajabi et al., 2015). The sea urchin *Paracentrotus lividus* is an additional model-system that has been frequently used for acute toxicity tests. The short-term chronic toxicity tests are based on monitoring the sea urchins' early developmental stages following egg fertilization, the cleavage stages of the embryos (the 2-cell stage occurs at 90 minutes post fertilization), and the normal or abnormal development of its pluteus-larvae, which occurs at 24-48 hours post-fertilization (e.g., His et al., 1999; Kobayashi and Okamura 2002,

Bellas 2007, Fabbrocini and D'Adamo 2011). *P. lividus* is also one of the most common organisms used in biomonitoring studies of various pollutants, including the antifoulant tributyltin (e.g. Marin et al., 2000), organic biocides (e.g. Bellas et al., 2005), and nanoparticles (NPs) (Fairbairn et al., 2011; Siller et al., 2013). Fairbairn et al. (2011) found that ZnO NPs are more toxic to sea-urchin embryos compared to CeO_2 and TiO_2 NPs. Siller et al. (2013) described the dose-dependent developmental defects as well as behavioral changes following exposure of sea urchin embryos to polymer-coated silver NPs. Both *A. salina* and *P. lividus* are model-systems adopted by the US Environmental Protection Agency (EPA 2002) and by the European OECD (OECD 2013).

The current study engaged with testing the toxicity of (1) the booster biocides, zinc pyrithione (ZnPT) and copper pyrithione (CuPT); (2) three unloaded EMNMs, comprising layered double hydroxides (LDH), silica mesoporous nanocapsules (SiNC), and polyurea microcapsules (PU); and (3) six novel antifouling micro/nanomaterials that correspond to EMNMs loaded with each of the two above-mentioned biocides. The tests were conducted on nauplii of *A. salina* and on the 2-cell stage embryos and pluteus-larvae of *P. lividus*. The results provide comparative data on the toxicity of the different compounds and enable us to score them as potential antifoulants for incorporation into antifouling paints.

2. Material and Methods

2.1. *Chemical compounds*

The compounds tested in the present study comprised two commercial biocides: zinc (ZnPT) and copper pyrithione (CuPT), provided by LONZA (www.lonza.com, Basel, Switzerland) and three unloaded EMNMs: layered double hydroxides (LDH), spherical mesoporous silica nanocapsules

(SiNC), and polyurea microcapsules (PU), all produced by Smallmatek (www.smallmatek.pt, Aveiro, Portugal) (Table 1). In addition, six loaded EMNMs corresponding to both commercial biocides were individually immobilized/encapsulated in the nanomaterials: LDH-ZnPT, LDH-CuPT, SiNC-ZnPT, SiNC-CuPT, PU-ZnPT, and PU-CuPT, all similarly produced by Smallmatek.

All details regarding the synthesis and characterization of LDH- and SiNC-related materials (loaded and unloaded), tested in the present study, can be found in Avelelas et al. (2017). Polyurea microcapsules were produced by interfacial polycondensation using two grams of diethylenetriamine (DETA) and three grams of 2,4-toluene diisocyanate (TDI) as monomers for polyurea polymerization. The encapsulation of pyrithiones was performed by dissolving them in 10 mL of dichloromethane, which was the dispersed phase in the formed microemulsion. The procedure was adapted from Maia et al. (2016), using pyrithiones in place of 2-mercaptobenzothiazole.

2.2. Model-systems and layout of toxicity assays

Both *A. salina* and *P. lividus* assays were conducted during November 2015-April 2016 at the Interuniversity Institute for Marine Sciences (IUI) in Eilat, northern Gulf of Aqaba, Israel. In order to conduct the toxicity assays stock solutions of the free biocides (ZnPT, CuPT) and dispersions of unloaded ENMs (LDH, SiNC, PU), and novel anti-fouling ENMs (LDH-ZnPT, LDH-CuPT, SiNC-ZnPT, SiNC-CuPT, PU-ZnPT, and PU-CuPT) were prepared in filtered natural Eilat seawater 0.45 μm (FSW, 40.8±0.1‰ salinity) by vigorous shaking in an ultrasonic bath for 1 hour in order to maximize dispersion and homogenous dissolution. These novel

products are not yet in the market and, therefore in order to cover different possible environmental contamination scenarios, a wide range of exposure concentrations was used to assess the effects of the nanomaterials on early development stages of both crustaceans and echinoderms. Exposure concentrations ranged from 0.001 mg/L to 100 mg/L (biocide loading dry weight content), on a logarithmic scale; a negative control containing only FSW was also included, and a total of three treatments per compound was performed. Cysts of the brine shrimp *A. salina* were hatched in aerated 0.45 µm FSW at 30°C for 24-30 h. Nauplii were then transferred into 6-well tissue culture plates (three wells, each containing 10 nauplii per treatment) filled with the test solutions, and then incubated at 24±2°C for 24 h under a 12:12 h light regime. Mortality of animals (as indicated by lack of mobility) was recorded after 24 h under a dissecting microscope, following Rajabi et al. (2015).

Adult sea urchin *P. lividus* were kept in a flow-through seawater system at the IUI and fed with *Ulva* spp. macroalgae. The temperature, pH, level of nutrients, and water salinity were monitored and corresponded to the ambient values at the time of the experiment (24±1°C, 8.18±0.01, 0.06±0.01 µmol/l, 150±5 nmol/l, and 40.8±0.1‰ for Temperature, pH, PO_4, NH_4, and water salinity, respectively). Spawning of eggs and sperm was induced by injecting 1 mL 0.5 M KCl into the coelomic cavity of the individual animals (two females and two males for each fertilization trial). Subsequently, the animals spawned for several minutes and the gametes were mixed in 0.45 µm FSW. The fertilized eggs were immediately placed in 24-well tissue culture plates with four replicates per test concentration, each replicate containing 200 fertilized eggs. The total volume in each well was 2 mL and the plates were gently shaken at 22±2 °C in an incubator under a 12L/12D regime. In order to determine the effect of a given compound on the 2-cell embryo stage (see Vaschenko et al., 1999), 100 embryos were removed from each well 90

min. after gamete mixing and fixed in 5 µL 5% glutaraldehyde in 0.45 µm FSW. The number of embryos that reached this stage out of the total number of introduced eggs in each batch was determined under a dissecting microscope. At 48 h post-fertilization the number of larvae that had reached the pluteus-larvae stage was determined under a dissecting microscope (Fernández and Beiras 2001; Bellas et al., 2005).

Assessment of each EMNM was evaluated as a score from the averages of toxicity ranking of *A. salina* and *P. lividus* assays, with toxicity ranking of high (+++) < 10 mg/mL < medium (++) < 100 mg/mL < low (+) for the *A. salina* assays, and for the *P. lividus* both 2-cell and pluteus are ranking as high (+++) < 1 mg/mL < medium (++) < 100 mg/mL < low (+).

2.3. Statistical analysis

Lethal concentration value (LC_{50}) of *A. salina* assays and effective concentration value (EC_{50}) of *P. lividus* assays for each tested compound were determined using Graphpad Prism V.5, by plotting a dose-response sigmoidal curve through a non-regression analysis. For each compound and species, the non-linear regression equation that best fit the data was selected, considering the R^2 value, absolute sum of squares, and the 95% confidence intervals.

3. Results and Discussion

3.1. Material characterization

Synthesized PU presents a spherical morphology with the typical core-shell structure (Fig. 1A). It features a broad size distribution, ranging from 200 nm to 10 micrometers, and a tendency to

shrink due to the evaporation of entrapped solvent inside the microcapsules. Chemically, the prepared PU displays the typical urea band as a result of the polymerization of TDI with DETA, illustrated in Fig. 1B. The LDH particles present a hexagonal morphology with size distribution between 300 and 600 nm in width and length, while SiNC presented an uniform and spherical morphology, with size generally ranging between 100 and 500 nm.

3.2. Assessment of the exposure effects on early developmental stages

3.2.1 Unloaded micro-/nanocarriers (EMNMs)

The results indicate that unloaded LDH and PU caused no acute toxicity to *A. salina* nauplii, even under the highest exposure concentration (Fig. 2A). Unloaded (i.e. biocide-free) PU and LDH demonstrated no short-term chronic toxicity in any of the early developmental stages assessed in the sea-urchin *P. lividus* (Figs 2B and 2C); however, unloaded LDH demonstrated a EC_{50} of 2 mg/L specifically on the development of the pluteus-larvae. Empty SiNC demonstrated EC_{50} of 31.87 mg/L and EC_{50} of 2.93 mg/L for the 2-cell embryo stage and pluteus-larvae, respectively.

The unloaded LDH revealed no toxicity towards the nauplii of *A. salina* and the 2-cell stage of *P. lividus*, similarly to previous tests conducted on other invertebrate species (Avelelas et al., 2017; Martins et al., 2017; Gutner-Hoch et al., 2018). However, the current study also demonstrates that the unloaded LDH did exert toxic effects on the pluteus-larvae of *P. lividus*. The toxicity of unloaded LDH to the pluteus-larvae corresponds to the findings on the efficacy of LDH on *Bugula neritina* larvae, which demonstrated EC_{50} values of 9.4 mg/L and 4.3 mg/L for the Mediterranean Sea and Red Sea animals, respectively (Gutner-Hoch et al., 2018). In the current

study, the unloaded LDH inhibited the development of the pluteus-larvae, thus demonstrating its embryotoxicity and suggesting a mild toxic effect of the nanocarrier itself.

The unloaded PU microcapsules appear to present the potential to function as safe reservoirs, as they did not cause mortality to the *A. salina* nauplii or to the *P. lividus* early 2-cell stage or their larvae. This study is the first to examine the toxicology of PU microcapsules to marine organisms. It is anticipated that future studies will further test their effects on the marine environment across the food web.

The silica nanocapsules (SiNC) demonstrated a moderate toxicity effect on the larval stage of *A. salina* nauplii (12.29 mg/L) and embryos of *P. lividus* (2.93 mg/L for pluteus stage assays) (Fig. 3), which may be explained by the presence of residuals of quaternary ammonia, a harmful surfactant used in capsules synthesis, as recently discovered by Figueiredo et al. (*in press*).

3.2.2. Free anti-fouling biocides

ZnPT demonstrated the highest acute and short-term chronic toxicity among all tested compounds for both species, with LC_{50}=1.37 mg/L in the *A. salina* test, EC_{50}=0.063 mg/L in the 2-cell embryo stage assay, and EC_{50}=0.002 mg/L in the pluteus-larvae assay. CuPT also yielded high toxicity, with LC_{50} =4.58 mg/L in the *A. salina* assay, EC_{50}=0.011 mg/L in the pluteus-larvae assay, and no inhibition in the first cleavage of the fertilized eggs into the 2-cell embryos (Table 2).

The biocide CuPT did not inhibit the development in *P. lividus* towards the 2-cell embryo stage (in contrast to ZnPT), but did inhibit development toward the pluteus-larvae. It is suggested that these results may have been due to a gradual accumulation of the biocide in the embryos, leading

to a delayed effect that was expressed only in the larvae. The mode of action of CuPT has been attributed to an oxidation process targeting the mitochondria (Almond and Trombetta 2016). Additionally, Rhee et al. (2013) suggested that copper-related toxicity might mediate the apoptotic process by means of oxidative stress. Apoptotic phenotypes have been noted among sea urchin embryos treated with nanocontainers loaded with CuPT (cf. Fig. 4). CuPT has been reported to cause embryotoxicity in fish by promoting distortion of the larval notochord and disorganizing skeletal muscles in zebrafish embryos (*Danio rerio*) (Almond and Trombetta 2016). The current findings (for both model species) indicate that ZnPT is more toxic than CuPT in agreement with the efficacy results obtained for the mussel *B. pharaonis* (EC_{50}=4.2 mg/L for the ZnPT *vs*. no effect up to 100 mg/L for CuPT, see Gutner-Hoch et al., 2018) and with the effects on the dinoflagellate *Pyrocystis lunula* (Bao et al., 2011). Similarly, Kobayashi and Okamura (2002) found that embryo development of the sea urchin *Anthocidaris crassispina* was more inhibited when exposed to ZnPT than to CuPT ($EC_{50}=10^{-14}$ mg/L and $EC_{50}=10^{-9}$ mg/L, respectively for pluteus-larvae development). However, both biocides significantly inhibited the growth of photosynthetic species at very low and similar concentrations (Avelelas et al., 2017; Bao et al., 2011), with CuPT being more toxic than ZnPT to fish, corals, polychaetesm and crustaceans (such as *Artemia salina*, in contrast to the present findings) (Bao et al., 2014; Koutsaftis and Aoyama 2007; Mochida et al., 2006). It therefore seems that the toxicity of these two biocides varies according to the model-system used. In the current study CuPT did not reveal embrytoxicity, in contrast to ZnPT, which inhibited cell cleavage towards 2-cell embryos (Fig. 2B). ZnPT has been reported to have a high potential for accumulation in the tissues of marine mollusks (Marceselli et al. 2011). In addition, although shown to be photodegradable (Sakkas et

249 al. 2007), concentrations of zinc pyrithione can build up in deep waters or in muddy coastal
250 areas.

251

252 3.2.3 Novel anti-fouling micro/nanomaterials

253 The compounds LDH-ZnPT and SiNC-ZnPT demonstrated low EC_{50} values in the *P. lividus* 2-
254 cell assay (cf. Table 2), although higher than ZnPT; while LDH-CuPT and SiNC-CuPT
255 demonstrated a lower toxicity than CuPT. In the pluteus-larvae stage assay, LDH-ZnPT and
256 LDH-CuPT were more toxic compared to SiNC-ZnPT and SiNC-CuPT. In *A. salina* the
257 compounds LDH-ZnPT, SiNC-CuPT, ZnPT, and CuPT demonstrated similar toxicity and were
258 slightly more toxic than LDH-CuPT and SiNC-ZnPT. PU-ZnPT and PU-CuPT did not cause *A.*
259 *salina* nauplii mortality (Fig. 3A); and, while PU-CuPT did not inhibit the development to 2-cell
260 embryos, it did however inhibit the pluteus development (EC_{50}=20.96 mg/L). PU-ZnPT was
261 more toxic than PU-CuPT (Figs 3B and 3C).

262 Differences were noted in the *P. lividus* developmental stages that arrested under the free booster
263 biocides and the loaded ENMs (Fig. 4). ZnPT was more toxic toward the early embryonic stages
264 than CuPT (Figs 4A-D). Under the compound LDH-ZnPT, the fertilized eggs did not undergo
265 cleavage and remained at a single cell stage, similar to the inhibition demonstrated under ZnPT
266 (Figs 4E and 4F). LDH-CuPT demonstrated a lower toxicity compared to ZnPT and LDH-ZnPT,
267 with *P. lividus* embryos reaching a 4-cell stage under 10 mg/L and a morula stage under 1 mg/L
268 (Figs 4H and 4G). Under both SiNC-ZnPT and SiNC-CuPT the *P. lividus* embryos reached the
269 pluteus-larvae stage 48 h post-fertilization under the 1 mg/L (Figs 4J and 4L), while under
270 exposure of 10 mg/L, embryogenesis was inhibited and remained as 4-cell embryos at the most

(Figs 4I and 4K). Among the free CuPT, LDH-CuPT, and SiNC-CuPT apoptotic embryos were noted, featuring shrinkage of cells and membrane budding (Elmore 2007). This phenotype mostly appeared under the free CuPT exposure treatments (Figs 4C and 4D).

During the development of the embryos towards the pluteus-larvae stage, LDH-ZnPT and LDH-CuPT demonstrated a higher toxicity than SiNC-ZnPT and SiNC-CuPT. However, in the 2-cell assay LDH-ZnPT and SiNC-ZnPT demonstrated a higher toxicity than LDH-CuPT and SiNC-CuPT. Such differences may imply different modes of activity of the compounds, as similarly revealed in the free biocide results. It seems, therefore, that the early developmental stages in the sea urchin are more sensitive to ZnPT than to CuPT, regardless of the type of EMNMs used. These results might be due to the behavior of the nanomaterials or to the biocides' release-rate within the organisms, which can also be related to the type of nanocarrier and internal bio-physical and chemical conditions, which may vary among different developmental stages and organisms (Barnes et al., 2001). In addition interactions with biomolecules, ions, organelles, or organs might also affect the results (Figueiredo et al. *in press*). Following Onduka et al. (2010) and Avelelas et al. (2017), the persistence of high intracellular levels of metallic ions and unstable ionized pyrithiones (derived from the chemical dissociation of ZnPT or CuPT), which can in turn react with other metals and form other even more toxic metal-based pyrithiones, may cause damaging and irreversible biochemical and physiological changes and lead to death of the organism.

3.3. Toxicity and corresponding antifouling efficacy of EMNMs

The current study presents a comparison between the toxicity of the different EMNMs and their respective antifouling efficacy assessments, along with their EC_{50} score (Table 3). Unpublished antifouling efficacy data on PU, PU-CuPT, and PU-ZnPT are also included in the comparison.

While the unloaded LDH exhibited a low toxicity and efficacy score, the EMNM loaded with the booster biocides revealed both increased toxicity and efficacy. Furthermore, LDH loaded with the CuPT booster biocide revealed increased efficacy and toxicity compared to the CuPT booster biocide without the LDH nanocarrier. In contrast, the toxicity of SiNC did not change when loaded with the tested booster biocides, which could be a result of the presence of residuals of quaternary of ammonia (Figueiredo et al., *in press*). The toxicity of the PU was found to be low, and when loaded with ZnPT it achieved a similar score to that of the free booster biocides. The present findings regarding the tested unloaded/empty nanomaterials agree with recent studies demonstrating their environmentally-friendly properties in regard to non-target marine organisms representing different trophic levels (e.g. Avelelas et al., 2017; Martins et al., 2017; Gutner-Hoch et al., 2018; Figueiredo et al., *in press*). The low-toxicity of these raw materials, particularly LDH and PU, along with the controlled-released technology highlights them as a class of innovative “green" materials for the upcoming sustainable industrialization. This has particular application for the coating industry, as the incorporation of these materials in the production process is expected to contribute to mitigating the harmful impact of antifouling biocides on the marine ecosystem. Indeed, the use of biocide-loaded nanocarriers as antifoulant additives possesses advantages, as they function as controlled delivery and release systems that maintain their antifouling efficacy against target species (Gutner-Hoch et al., 2018; Figueiredo et al., *in press*) while reducing the toxicity towards non-fouling organisms (Avelelas et al., 2017; Figueiredo et al., *in press*).

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5. Conclusions

The current findings demonstrate the added-value of the sea urchin embryotoxicity test for determining the toxicity of free biocides, unloaded EMNMs, and novel antifouling engineered materials:

(i) Both the 2-cell stage and pluteus-larvae assays reveal what appear to be different modes of activity by both CuPT and ZnPT on the sea urchin early developmental stages. The mode of action of both free biocides, as well as of the three types of nanocarrier, requires further studies.

(ii) LDH-based biocides have lower toxicity than the free forms of the state-of-the-art compounds, and may have the potential to act as antifoulants.

(iii) Unloaded LDH are environmentally-safe nanocarriers.

(iv) PU microcapsules exhibited very low toxicity on the *A. salina* nauplii and *P. lividus* 2-cell and pluteus-larvae stages, suggesting that PU is an environmentally-friendly nanocarrier.

Future studies (e.g. acute, chronic, mesocosm, and field tests) are highly recommended to complement and support these conclusions.

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Legends

Fig. 1. Optical microscopy images: (A) PU microcapsules and (B) FTIR spectrum of PU showing typical polyurea bands.

Fig. 2. Toxicity assays conducted under different concentrations of ENPs: (A) mortality of *Artemia salina* 24 hours post-fertilization, (B) first cleavage of fertilized eggs of *Paracentrotus lividus* 90 min post-fertilization; and (C) percentage of *P. lividus* pluteus-larvae following 48 hours. Unloaded polyurea microcapsules (PU) data are not plotted due to the lack of effects even at the highest exposure concentration.

Fig. 3. Schematic presentation of ENP toxicity in relation to their concentration in (A) *Artemia salina* assays, (B) *Paracentrotus lividus* 2-cell embryo stage bioassay, and (C) *Paracentrotus lividus* pluteus-larvae development assays (green: lowest toxicity, red: highest toxicity).

Fig. 4. Light microscopy images of *Paracentrotus lividus* developmental stages following 48 hours exposure to 10 and 1 mg/L of (A, B) ZnPT - both with single fertilized eggs; (C, D) CuPT - 4-cell stage with apoptotic bodies and morula stage with apoptotic bodies, respectively; (E, F) ZnPT in LDH - both with single fertilized eggs; (G, H morula stage) CuPT in LDH - 4-cell stage and morula stage, respectively;, (I, J) ZnPT in LDH - single eggs and partially developed pluteus larvae; (K, L) CuPT in SiNC nanocarriers - 4-cell stage with apoptotic bodies and partially developed pluteus larvae, respectively. White scale bars represent 100 micrometers.

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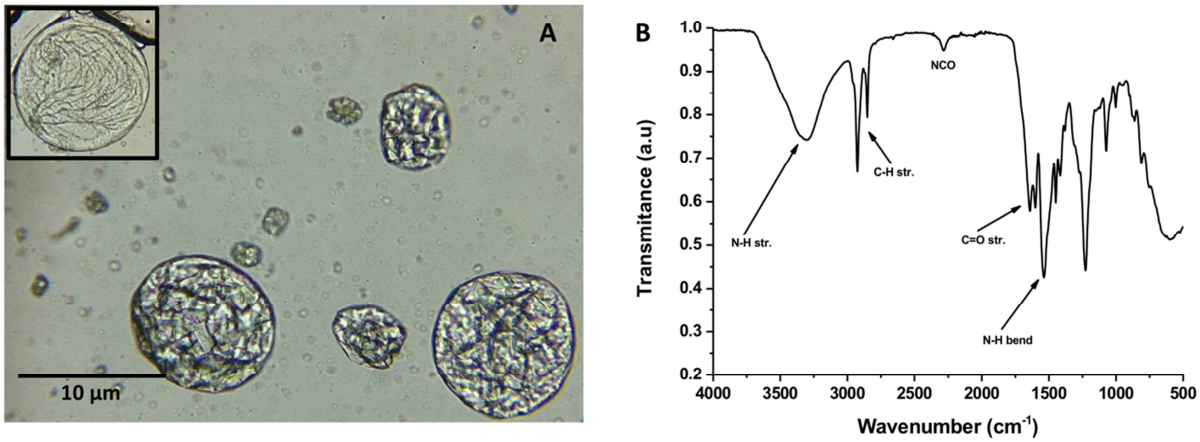
498 Fig. 1



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501 Fig. 2

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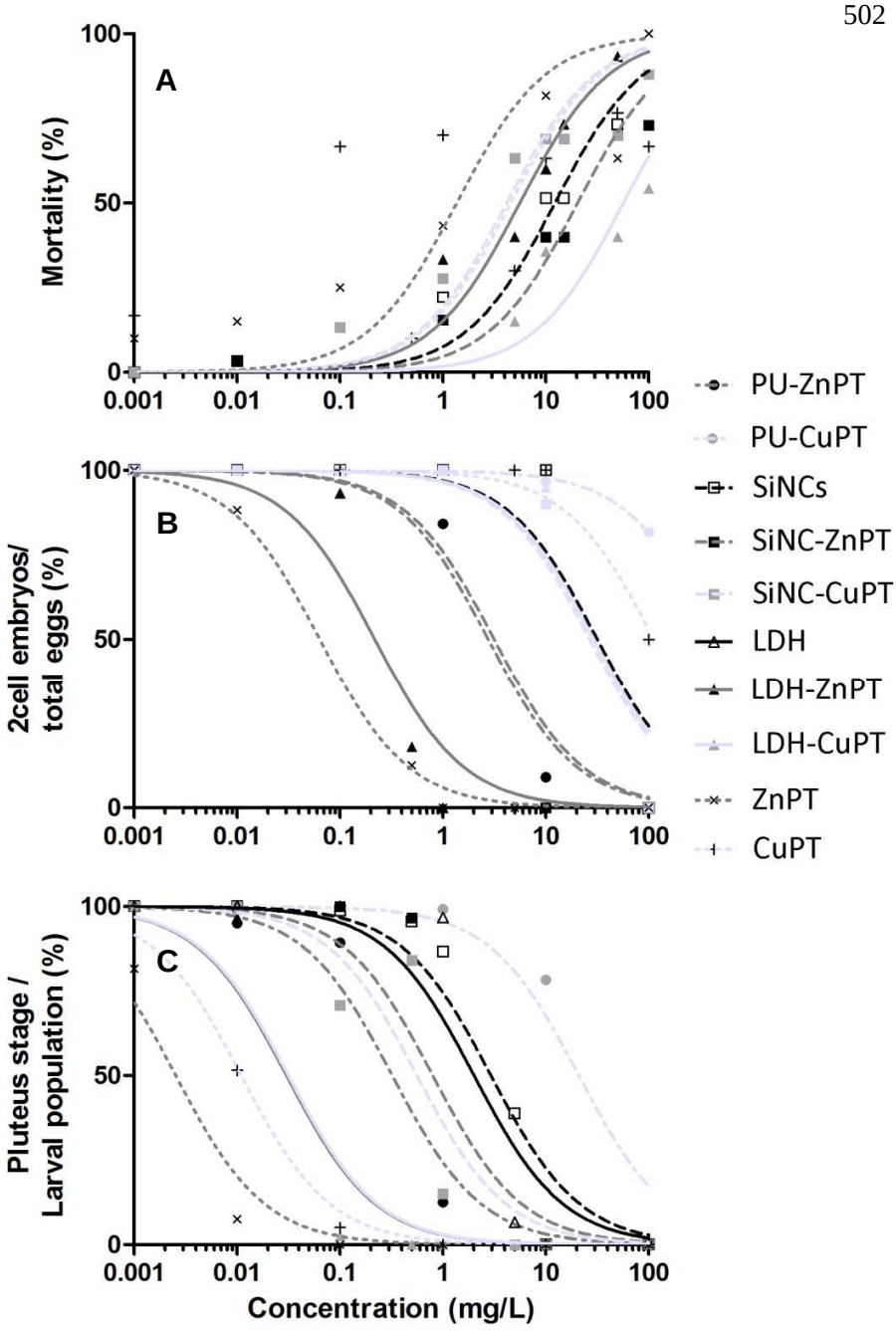
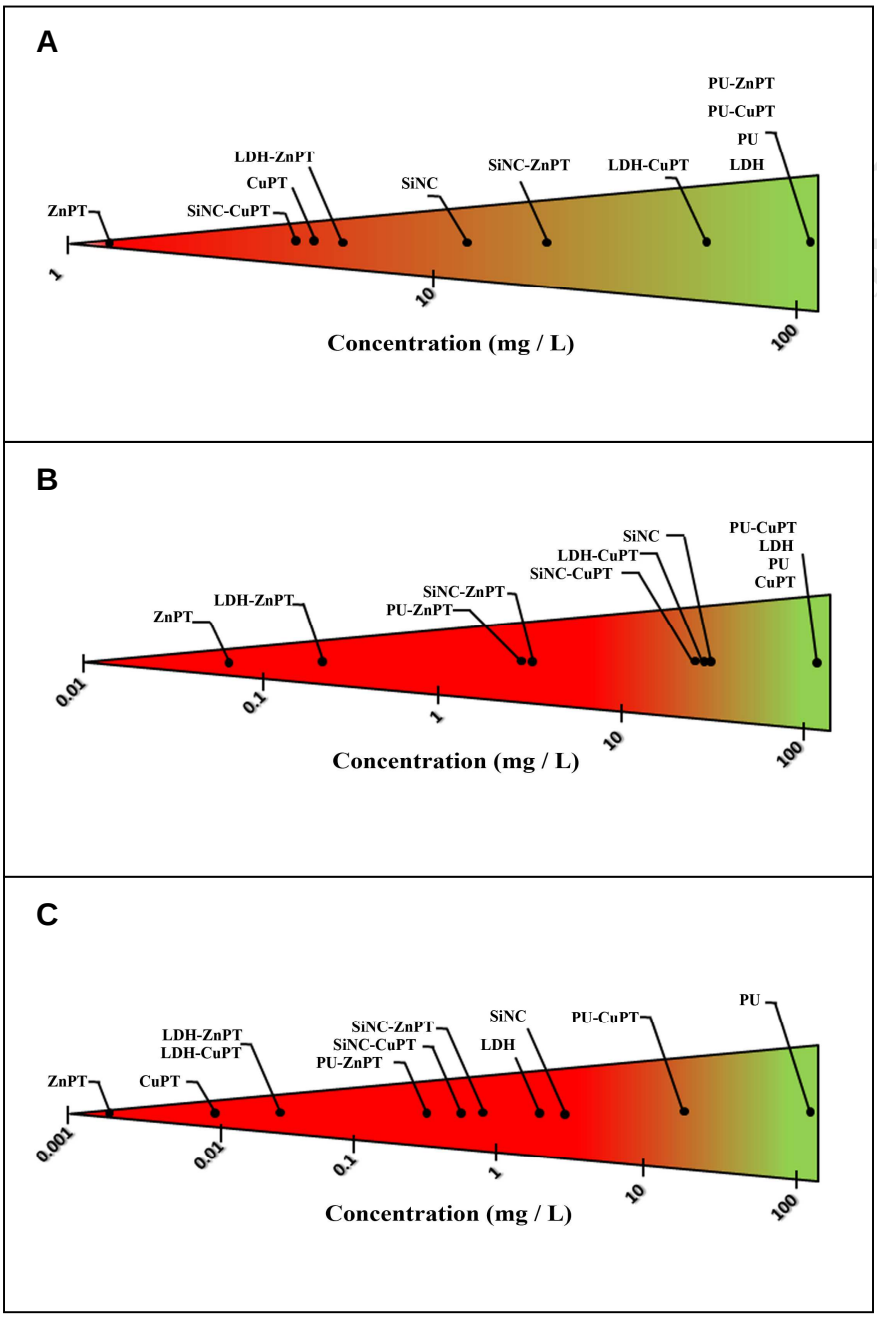

A
B
C
Mortality (%)
2cell embryos/ total eggs (%)
Pluteus stage / Larval population (%)
100
50
0
0.001
0.01
0.1
1
10
100
Concentration (mg/L)
PU-ZnPT
PU-CuPT
SiNCs
SiNC-ZnPT
SiNC-CuPT
LDH
LDH-ZnPT
LDH-CuPT
ZnPT
CuPT

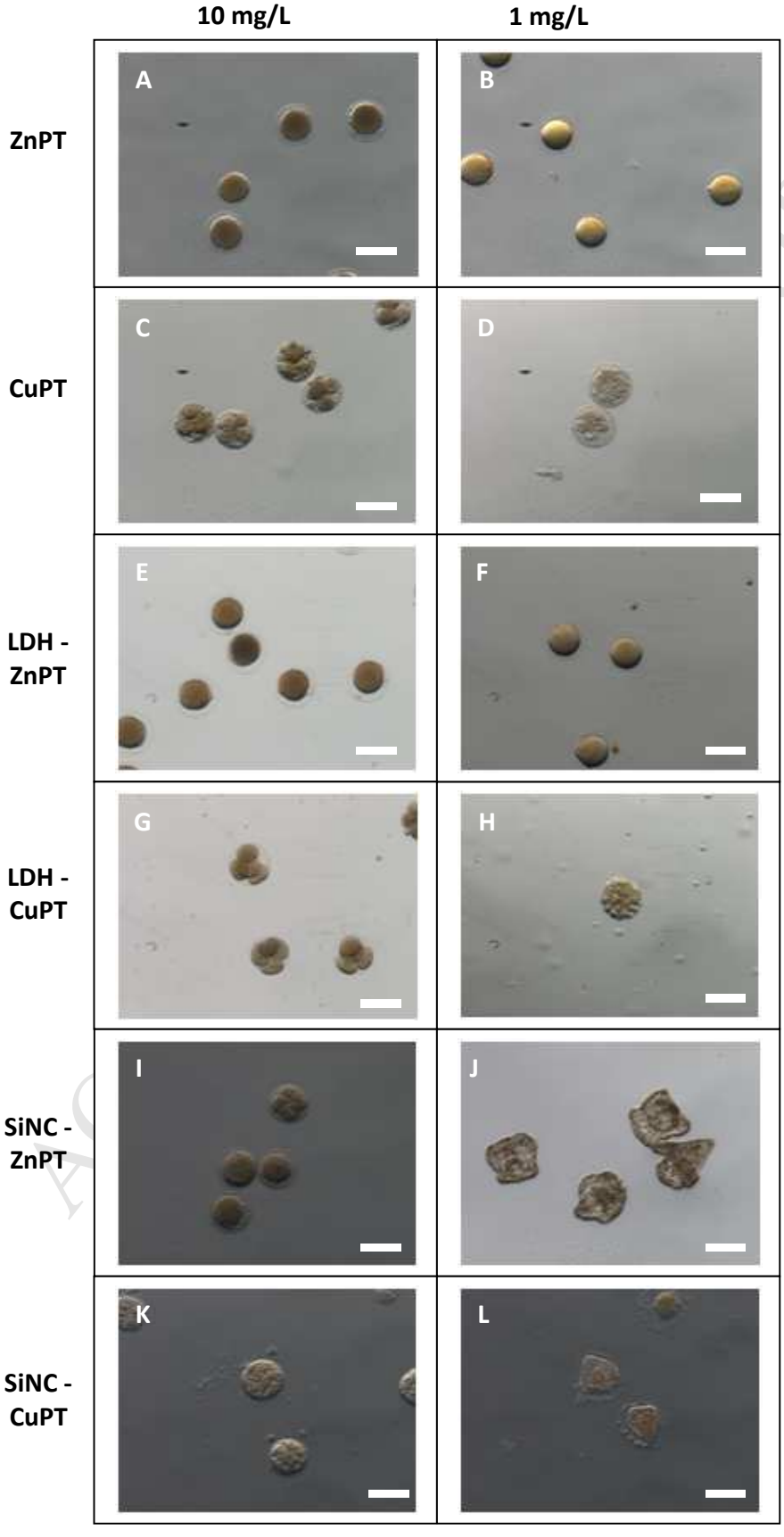
503 Fig. 3

504


A
PU-ZnPT
PU-CuPT
PU
LDH
LDH-CuPT
SiNC-ZnPT
SiNC
LDH-ZnPT
CuPT
SiNC-CuPT
ZnPT
1
10
100
Concentration (mg / L)
B
SiNC
PU-CuPT
LDH-CuPT
LDH
SiNC-CuPT
PU
CuPT
SiNC-ZnPT
LDH-ZnPT
PU-ZnPT
ZnPT
0.01
0.1
1
10
100
Concentration (mg / L)
C
PU
SiNC-ZnPT
SiNC
PU-CuPT
LDH-ZnPT
SiNC-CuPT
LDH
LDH-CuPT
PU-ZnPT
ZnPT
CuPT
0.001
0.01
0.1
1
10
100
Concentration (mg / L)

505 Fig. 4

506 507



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509 Table 1. Chemical specification of the tested compounds.

Compound abbreviation	Chemical specification of compounds
ZnPT	Zinc pyrithione (Zinc Omadine ™)
CuPT	Copper pyrithione (Copper Omadine ™)
LDH	Zn-Al layered double hydroxide (without biocide)
SiNC	Hollow silica nanocapsules (without biocide)
PU	Polyurea microcapsules
SiNC-ZnPT	Zinc pyrithione encapsulated into silica nano-capsules
SiNC-CuPT	Copper pyrithione encapsulated into silica nano-capsules
LDH-ZnPT	Zinc pyrithione immobilized in layered double hydroxide
LDH-CuPT	Copper pyrithione immobilized in layered double hydroxide
PU-ZnPT	Zinc pyrithione encapsulated into polyurea microcapsules
PU-CuPT	Copper pyrithione encapsulated into polyurea microcapsules

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Table 2. EC_{50} values of ENMs obtained in *Paracentrotus lividus* 2-cell, pluteus-larvae, and LC_{50} values of ENMs obtained in *Artemia salina* assays.

Compound	2-cell (EC_{50} mg/L)	2-cell range (mg/L)	Pluteus (EC_{50} mg/L)	Pluteus Range (mg/L)	*Artemia salina* (LC_{50} mg/L)	Range (mg/L)
ZnPT	0.063	0.042-0.095	0.002	0.001-0.004	1.37	0.38 – 4.87
CuPT	>100	-	0.011	0.007-0.015	4.58	0.82 – 25.64
PU	>100	-	>100	-	>100	
LDH	>100	-	2.00	-	>100	
SiNC	31.87	7.04-144.2	2.93	1.59-5.4	12.29	6.66 – 22.67
PU-ZnPT	2.76	1.3-5.87	0.32	0.16-0.64	>100	
PU-CuPT	>100	-	20.96	9.62-45.67	>100	
LDH-ZnPT	0.21	0.07-0.67	0.03	0.01-0.07	5.59	3.78 – 8.26
LDH-CuPT	29.33	5.7-150.8	0.03	0.01-0.08	56.68	34.14 – 94.11
SiNC-ZnPT	3.16	0.7-14.26	0.79	0.27-2.3	20.46	13.76 – 30.41
SiNC-CuPT	26.8	7.99-89.87	0.56	0.22-1.38	4.18	2.38 – 7.34

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524 Table 3. LC_{50} and EC_{50} values of tested EMNMs and their relative score obtained from toxicity
525 tests on *Artemia salina* and embryos of *Paracentrotus lividus* and from efficacy tests on
526 *Brachidontes pharaonis* and *Bugula neritina*. The scores for LC_{50} and EC_{50} presented as + to
527 +++ reflecting lowest to highest values, respectively. Score is based on data compilation
528 presented in Figure 3 (this study) and from Gutner-Hoch et al. 2018: the latter indicated by *,
529 and unpublished data indicated by **.

Compound	Toxicity (mg/L)				Efficacy EC_{50} (mg/L)		
	A. salina LC_{50}	2-cell EC_{50}	Pluteus-larvae EC_{50}	Toxicity score	*B. pharaonis*	*B. neritina*	EC_{50} score
LDH	>100	>100	2.0	+	>100 *	4.3 *	+
LDH-CuPT	56.6	29.3	0.03	++	9.6 *	0.1 *	+++
LDH-ZnPT	20.4	0.2	0.03	+++	1.3 *	0.04 *	+++
SiNC	12.2	31.8	2.9	++	20.9 *	0.1 *	++
SiNC-CuPT	4.1	26.8	0.5	++	17.3 *	2.9 *	++
SiNC-ZnPT	20.4	3.1	0.7	++	9.3 *	0.1 *	+++
PU	>100	>100	>100	+	>100	14.5 **	+
PU-CuPT	>100	>100	20.9	+	>100 **	90.0 **	+
PU-ZnPT	>100	2.7	0.3	++	40.0 **	0.2 **	++
CuPT	4.5	>100	0.01	++	>100 *	0.1 *	++
ZnPT	1.37	0.06	0.002	++	4.2 *	0.05 *	+++

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