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The influence of seawater properties on toxicity of copper pyrithione and its degradation product to brine shrimp *Artemia salina*

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ABSTRACT

Copper pyrithione (CuPT) is a biocide, used worldwide to prevent biofouling on submerged surfaces. In aquatic environments it rapidly degrades, however, one of the degradation products (HPT) is known to react with cupric ion back to its parent compound. Not much is known about the behavior and toxicity of CuPT and its degradation product HPT in different water systems. Hence, our aim was to investigate the ecotoxicity of CuPT, HPT as well as Cu^{2+} to the brine shrimp *Artemia salina* in natural seawater and organic matter-free artificial seawater. Moreover, in order to elucidate the influence of ionic strength of water on CuPT toxicity, tests were performed in water media with modified salinity. The results showed that CuPT was the most toxic to the exposed crustaceans in a seawater media with the highest salinity and with no organic matter content. HPT in a presence of cupric ion converted to CuPT, but the measured CuPT concentrations and the mortality of *A. salina* in natural water were lower than in artificial water. The toxicity of CuPT to *A. salina* was significantly influenced by the organic matter content, salinity, and proportions of constituent salts in water. In a combination with cupric ion, non-hazardous degradation product HPT exhibits increased toxicity due to its rapid transformation to its parent compound.

Keywords: antifouling biocide, copper pyrithione, mixture toxicity, brine shrimp, organic matter, salinity

1. Introduction

Biofouling, i.e. the adhesion of organisms on submerged surfaces represents a great economic burden for shipping industry. It significantly increases the fuel consumption (even up to 40%) and elevates the costs for hull maintenance (Champ, 2000). Moreover, it can represent a serious environmental threat due to the transfer of invasive species during the ship voyages. In order to prevent the accumulation of organisms, the protection of the immersed surfaces is crucial. The principal and effective strategy to restrain the fouling is by application of antifouling paints. These in most cases consist of copper compounds and one or more booster biocides (Takahashi, 2009). For the effective prevention of accumulation of fouling organisms, such as algae, barnacles, mussels and tubeworms, antifouling coatings are designed to achieve a constant leaching of a sufficient concentration of copper and biocides to the outer surface layer. Concerns occur when antifouling biocides are not selective only to target organisms, therefore the ecotoxicity studies are vital to perform.

After the ban of tributyltin, the most notorious antifoulant for its high toxicity, new antifouling biocides were developed and applied in paints (Konstaninou, 2006). Among them is copper pyrithione (CuPT) which is acknowledged for its exceptional and broad antimicrobial activity (Mochida et al., 2006). In Japan, CuPT remains one of the most commonly used antifouling booster biocide, with 240 tons of manufactured or imported quantities per year (data for year 2014, J-Check, 2017). In antifouling coatings it is most frequently combined with cuprous oxide (Okamura and Mieno, 2006). CuPT is not stable in the aquatic environment, however its presence has been reported in sediments collected from the bay in Japan in concentrations up to 22 µg/kg dry sediment weight (Harino et al., 2007). A research study of Onduka et al., 2010 has shown high CuPT toxicity to non-target species of different trophic levels, such as algae *Skeletonema costatum* (72-h EC_{50} = 1.5 µg/L), crustacean *Tigriopus japonicus* (24-h EC_{50} = 23 µg/L), and a fish *Pagrus major* (96-h LC_{50} = 9.3 µg/L).

The advantage of CuPT among several other antifoulants is in its rapid degradation under the light, which impedes the biocide to excessively accumulate in the aquatic compartments and in aquatic organisms. The estimated half life in a sterile seawater is 7.1 min (Maraldo and Dahllöf, 2004). Yet, one of the photodegradation products reported, 2-mercaptopyridine-N-oxide (HPT), was shown to transform back to its parent compound in the presence of Cu^{2+} (Onduka et al., 2010). The toxicity of the

degradation product HPT was found to be much lower than of the CuPT when tested on a crustacean *T. japonicus*, with an 24-h EC_{50} > 12 500 µg/L (EC_{50} = 23 µg/L for CuPT), and a fish *P. major* (96-h LC_{50} = 4,500 µg/L for HPT and 9.3 µg/L for CuPT). In contrast to crustaceans and fish, the toxicity of HPT was similar to the toxicity of CuPT when tested on alga *S. costatum* (72-h EC_{50} = 1.1 µg/L for HPT and 1.5 µg/L for CuPT), however researchers suggest the toxicities were similar due to the conversion of HPT to CuPT upon the reaction with Cu^{2+} (Onduka et al., 2010).

The degradation pathway is in case of CuPT not straightforward due to the reverse reaction of HPT to CuPT that may occur in the presence of free Cu^{2+}. Not only for HPT, the conversion to CuPT in the presence of free Cu^{2+} was revealed for an additional CuPT's degradation product - 2,20-dithio-bis-pyridine-N-oxide (Onduka et al., 2010). The interaction of degradation products with Cu^{2+} is likely since booster biocides are commonly incorporated to copper-containing paints (Okamura and Mieno, 2006). Besides that, free Cu^{2+} is as a micronutrient naturally present in aquatic ecosystems (Grunnet and Dahllöf, 2005).

From the studies conducted up to now, only the principle basics are known regarding the behavior of CuPT and its degradation products in the presence of free Cu^{2+} in aqueous media. The behavior of organic compounds, bioavailability and toxicity may differ considerably depending on the properties of the water media. Not much is known about the influence of water properties on a toxicity of CuPT to aquatic organisms, as well as the behavior of HPT and Cu^{2+} binary mixture in different water media. During voyages it is expected that CuPT will leach out and enter different aquatic environments, with different water chemical and physical properties (DOC content, salinity, electrical conductivity and salt constitution, for instance). However, in laboratory ecotoxicity and stability experiments the organic matter free artificial water is commonly used. Since immense quantities of CuPT are still used to protect the submerged surfaces from fouling, it is mandatory to know the fate and possible adverse impacts of CuPT when it is released to the natural water ecosystems. For that reason, our study aimed to investigate the behavior and ecotoxicity of CuPT, HPT and Cu^{2+} as well as Cu^{2+}/HPT binary mixture to the brine shrimp *A. salina* in natural seawater and organic matter-free artificial seawater. Moreover, in order to elucidate the influence of ionic strength of water on CuPT toxicity, several artificial and natural seawater media were used differing in ionic strength and ion constitution.

2. Materials and methods

2.1. Test organism

A marine water crustacean *Artemia salina* was selected as a test organism to examine the effects of cupric ion (Cu^{2+}), copper pyrithione (CuPT) and its degradation product 2-mercaptopyridine-N-oxide (HPT) in different seawater media. Artemia is a favorable test organism in marine ecotoxicity studies due to its worldwide distribution, short generation time and ease of culture. As our tests investigated also the influence of salinity to CuPT toxicity, artemia was chosen as a suitable organism due to its tolerance to different water salinities (Naceur et al., 2012). For the toxicity test, commercially available dormant eggs (cysts) were used, from which the hatched nauplii are of similar age, genotype and physiological condition (Persoone et al., 1989). In natural environments, artemia plays a significant ecological role as a food source to higher trophic level aquatic invertebrates and fishes (Sorgeloos, 1980). Dried eggs of *A. salina* were harvested in Vietnam and furtherly prepared by A&A Marine LLC, USA. To our laboratory they were provided by Fujimoto Kaiyodo Co. Ltd, Japan. Before starting the test, eggs were placed in a sterile petri dish (ϕ 90 mm, H16 mm, 101VR20, Sterilin) containing artificial seawater (ASW). Then, to initiate the hatching of the eggs, the petri dish was incubated in a weather simulator (LH-55-RD/RDS, Nihon Ikakikai) with the light intensity of 8.6 klux and the temperature of 25 °C. Approximately after 15 h the nauplii started hatching, however, only the nauplii which hatched from the cysts during the 20 – 22 h of incubation were used to start the toxicity tests.

2.2. Chemicals

Analytical standards of copper pyrithione (CuPT, Hayashi Pure Chemicals), 2-mercaptopyridine-N-oxide (HPT, Tokyo Kasei Industry) and $CuSO_4 \cdot 5H_2O$ (Wako Pure Chemical Industries) were of >98.7%, >95% and > 99.5% purity, respectively. The structural formulae of copper pyrithione and its degradation product 2-mercaptopyridine-N-oxide are presented in Table 2.

DMSO (> 99.5% purity, spectroscopy grade), used as a carrier solvent in toxicity tests, was purchased from Wako Pure Chemical Industries.

All other chemicals used in chemical analysis and test media preparation were of analytical grade, with a purity of 95% or higher.

2.3. Test media

Several different types of seawater were used for toxicity testing: artificial seawater (ASW), natural seawater (NSW), three natural seawater media, each amended with different salt (NSW-Na, NSW-Ca, NSW-Mg,) and additional seven artificial seawater media differing in electrical conductivity (ASW-EC). All seawater types were freshly prepared before they were spiked with chemicals and used in toxicity tests. The results of their characterization – pH value, electrical conductivity (EC), total hardness, the concertation of dissolved organic carbon (DOC), and concentrations of selected ions (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}) are presented in Table 1. The procedure of their preparation is described below.

Artificial seawater (ASW) was prepared in accordance with the Standard practice for the preparation of substitute ocean water (ASTM, 2003). Ultra-pure water (production system Aquarios RFU554CA, Advantec) was used as a basis into which inorganic salts were added in proportions as instructed by the Standard. ASW is free of dissolved organic carbon (DOC) and is often used in stability studies and ecotoxicity tests with marine organisms as it standardized and therefore allows to attain the reproducible experiments.

To prepare the *natural seawater* medium (*NSW*), sea surface water was firstly collected at the Kobe University port using Van-Dorn water sampler (5026A, Rigo Co., Ltd.) and brought to the laboratory. There it was subjected through 1.0 and subsequently through 0.4 µm pore size nuclepore filter membrane (New chestnut pore membrane 111110 and 111107, respectively, Whatman). NSW contained 2.6 mg/L of DOC and had lower electrical conductivity, total hardness, as well as lower concertation of Na, Ca, Mg and K ions compared to ASW (Table 1).

Natural seawater media with added salts (*NSW-Na, NSW-Mg* and *NSW-Ca*) were prepared by adding a sufficient amount of NaCl, $MgCl_2 \cdot 6H_2O$ and $CaCl_2$, respectively, into the prepared NSW medium to

adjust the EC value of a medium to ca. 5.0 S/m. We chose this value to approach the EC value of ASW (5.06 S/m, Table 1).

ASW media of different electrical conductivity (EC) (ASW-EC) were prepared in order to investigate the influence of the reduced EC on the CuPT toxicity to crustaceans. For this, ASW with a starting EC value of 5.06 S/m was diluted in 7 consecutive steps with ultrapure water, decreasing the EC of the former sample for the value of ca. 0.5 S/m. The measured conductivity in the most diluted sample was 1.98 S/m. The pH value of each prepared water sample was adjusted to 8.2 ± 0.1 with 1M NaOH solution.

Table 1: Properties (dissolved organic carbon, pH, electrical conductivity, total hardness, and concentrations of selected ions (Na^+, Ca^{2+}, Mg^{2+}, K^+)) of seawater media used in toxicity tests

Seawater media	**DOC** (mg/L)	**pH**	**EC** (S/m)	**Total hardness** (mg/L)	**Na^+** (mg/L)	**Ca^{2+}** (mg/L)	**Mg^{2+}** (mg/L)	**K^+** (mg/L)
ASW	<0.5	8.23	5.06	7 500	17 000	480	1 500	450
NSW	2.6	8.02	3.25	4 400	7 700	290	900	280
NSW-Na	2.6	8.07	5.03	4 100	15 000	300	810	280
NSW-Ca	2.6	8.01	5.06	20 000	7 400	6 500	890	290
NSW-Mg	2.6	8.07	5.02	21 000	7 300	290	4 900	280
ASW-EC	<0.5	8.20	5.58-1.98*					

*The electrical conductivity of each subsequent dilution of ASW-EC media is lower for ca. 0.5 S/m from the preceded solution.

Abbreviations: ASW – artificial seawater, ASW-EC – artificial seawater with modified electrical conductivity, NSW – natural seawater, DOC – dissolved organic carbon, EC – electrical conductivity

2.4. Chemical analysis

ASW, NSW, NSW-Na, NSW-Ca and NSW-Mg media were characterized by determining the pH value and electrical conductivity (F-54, Horiba), dissolved organic matter (DOC, TOC analyzer Sievers InnovOx, GE) and concentrations of selected cations (Na^+, Ca^{2+}, Mg^{2+}, K^+) Cations were analyzed using a Hitachi HPLC system, employed with a TSK gel IC-Cation 1/2 HR (4.6 × 100 mm, Tosoh) column, at 40 °C. The mobile phase was 2 mM HNO_3, flowing at 0.8 mL/min rate. DOC concentrations, pH and

EC were determined also in all prepared ASW-EC media.

The concentrations of HPT and CuPT were measured at 0, after 24 and 48 h per each test concentration using HPLC (Waters 2695, Empower, Waters), coupled with a three-dimensional detector (Waters 2998, Waters). The separation of compounds was achieved on a C_{18} column (Develosil ODS-MG3, 2 × 100 mm, Nomura chemical) equipped with a guard column (Develosil ODS-MGS, 1.5 × 10 mm). The flow rate was set to 0.2 mL/min and injection volume to 5.0 µL. The mobile phase consisted of (A) 0.1% phosphoric acid aqueous solution and (B) MeCN. The elution was for the first 10 min isocratic, with an A : B ratio of 60% : 40%, after which the ratio of eluent B gradually increased in the following 5 min and reached 100% at the end of the sample analysis, lasting in total 15 min.

2.5. Toxicity tests

For all media and chemicals (Cu^{2+}, CuPT, HPT as well as Cu^{2+}/HPT mixture), the procedure of toxicity tests was identical. Crustaceans were chemically exposed in a 48 h toxicity test, following the guideline for artemia toxicity screening test (Artoxkit, 1990) and the procedure described in Barahona and Sánches-Fortún (1999), except where noted differently.

CuPT and HPT were introduced into seawater media from a stock solutions prepared in DMSO, while a stock solution of $CuSO_4 \cdot 5H_2O$ dissolved in ultra-pure water was used to spike the seawater with copper. DMSO was used as a carrier solvent due to high solubility of compounds in this solvent and due to low toxicity to *A. salina*, compared to some other solvents (Barahona et al., 1994). The amount of DMSO in the final test solutions was not higher than 1%. At this concentration, DMSO did not pose any toxic affect to *A. salina* in our preliminary toxicity tests.

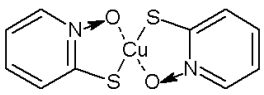
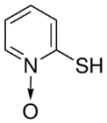
Three replicates were prepared per test concentration. For each replicate, 1 mL of a test solution was pipetted into one of a 24 well sterile micro plate (3820-024, Sansho), controls were included. The toxicity test started by introducing 10 *A. salina* nauplii into each test well. Plates were then placed into the incubator, where they were incubated in the dark at 25 °C. After 48 h, the test was terminated and the animals were examined under the stereoscopic microscope (SZ-PT, Olympus). The number of survived animals was recorded for each replicate.

To compare the sensitivity of the animals used in different tests, a toxicity test with a reference compound $K_2Cr_2O_7$ (24 mg/L) prepared in ASW was performed with each set of toxicity tests. The samples for chemical analysis contained no animals and were incubated along the test wells; they were therefore exposed to same conditions as the plates with test animals. The actual concentrations of CuPT and HPT were by HPLC determined at the beginning (t = 0 h), after 24 and after 48 h of the toxicity test. CuPT formed from a binary mixture of HPT and Cu^{2+} was in triplicates determined at the end of the test.

2.5.1. Tested concentrations

Tested nominal concentrations of single compounds and selected HPT and Cu^{2+} concentrations used in a mixture toxicity test are collected in Table 2. The effects were compared to the controls, each modified media (NSW with added salts and ASW with modified EC) had its own control. The concentration range was chosen based on our preliminary tests.

Table 2: Tested concentrations (µM) of copper pyrithione, mercaptopyridine-N-oxide and cupric ion in toxicity tests with *Artemia salina*

Compound	**Copper pyrithione (CuPT)** (µM)	**Mercaptopyridine-N-oxide (HPT)** (µM)	**Cupric ion** (µM)
Chemical formula			Cu^{2+}
Tested concentrations of single compounds in ASW (CuPT, HPT, Cu^{2+}) and in NSW (CuPT)	0 0.2 0.4 0.79 1.58 3.17	0 79 157 315 629 1258	0 16 39 79 157 315
HPT / Cu^{2+} mixture toxicity test in ASW and NSW – all combinations*		0 0.79 1.57 3.15	0 0.39 0.79 1.57
In NSW with added salts	1.58		
In ASW with modified EC	0.79		

***** Each selected concentration of HPT was tested with each Cu^{2+} concentration and vice versa, making in total 16 combinations.

Abbreviations: CuPT – copper pyrithione, HPT – 2-mercaptopyridine-N-oxide, ASW – artificial

seawater, NSW – natural seawater, EC – electrical conductivity

2.5. Data analysis

The EC_{50} values for the effect on *A. salina* survival were calculated using a logistic concentration-response model according to Haanstra et al. (1985). The effects were plotted against the actual concentrations of CuPT and HPT and against nominal ones of Cu^{2+}.

All statistical analyses were performed using GraphPad Prism 5.7.

3. Results

The toxicity tests with *A. salina* naupli to the reference toxicant $K_2Cr_2O_7$ (24 mg/L) performed along each toxicity test set caused anticipated and comparable toxic responses, indicating that the sensitivity of the animals was in a similar range in all performed toxicity test sets.

3.1. Toxicity tests with single compounds in artificial and natural seawater

The actual CuPT and HPT concentrations in ASW measured at the beginning, after 24 and after 48 h very well matched with the nominal ones and were stable along the toxicity tests (Table S1 and S2), however one outlier was recognized at 0.79 µM CuPT. Also in NSW, measured CuPT values were consistent with the nominal ones, however slightly lower values were obtained for CuPT 3.17 µM nominal concentration (Table S1).

The mean survival of the controls for all tested compounds in ASW and NSW was above 96.7%. The highest CuPT toxicity was observed in ASW, with a 48 h EC_{50} of 0.79 µM (95% CI: 0.73 – 0.86 µM) (250 µg/L, 95% CI: 231 – 272 µg/L). The derived EC_{50} for Cu^{2+} was 99.74 µM (95% CI: 82.53 – 120.5 µM) (6338 µg/L, 95% CI: 5245 – 7657 µg/L) and for HPT 682.8 µM (95% CI: 571.8 – 780.8 µM) (86824 µg/L, 95% CI: 72710 – 99287 µg/L). The concentration–response curves obtained for each compound tested on *A. salina* in ASW are plotted in Figure 1.

In NSW, CuPT appeared to be significantly less ($P < 0.05$) toxic than in ASW (Figure 1). The calculated EC_{50} for CuPT in NSW is 1.76 µM (95% CI: 1.62 – 1.90 µM) (556 µg/L, 95% CI: 512 – 600 µg/L).

237 *3.2. Toxicity test of binary mixture (Cu^{2+} and HPT) in artificial and natural seawater*

238 The chemical analysis showed that Cu^{2+} and HPT in a binary mixture toxicity test react together in 1 : 2
239 stoichiometry, and form copper pyrithione (CuPT). In Table 3, the measured concentrations of formed
240 CuPT, analyzed at the end (t = 48 h) of the binary mixture toxicity test in ASW and NSW are recorded.
241 For the comparison, the theoretical values of CuPT produced for each combination of chosen nominal
242 Cu^{2+} and HPT concentrations are supplemented to the table. It can be observed that in ASW in sufficient
243 amounts of Cu^{2+} and HPT, CuPT was formed in accordance with the Cu^{2+} : HPT 1 : 2 stoichiometry. In
244 NSW however, the measured concentrations were in all cases lower compared to the theoretical values.
245 For example, the concentration combination of HPT and Cu^{2+} 0.79/0.39 µM, respectively, yielded 0.181
246 µM CuPT, which represents approximately 46 % of the nominal value.
247
248

Table 3: Theoretical and measured concentrations (µM) of copper pyrithione formed from a binary mixture of 2-mercaptopyridine-N-oxide (HPT) and Cu^{2+} after 48 h, together with the mortality (M, %) of *Artemia salina* exposed for 48 h to a binary mixture in artificial (ASW) and natural seawater (NSW)

		HPT nominal (µM)								
		0		0.79		1.57		3.15		
		CuPT (µM)	M (%)	CuPT (µM)	M (%)	CuPT (µM)	M (%)	CuPT (µM)	M (%)	
Cu^{2+} nominal (µM)	0	0	-	0	-	0	-	0	-	**Theoretical**
		n.d.	0±0	n.d.	0±0	n.d.	7.4 ±12.8	n.d.	0±0	**In ASW**
		n.d.	0±0	-	-	-	-	-	-	**In NSW**
	0.39	0	-	0.39	-	0.39	-	0.39	-	**Theoretical**
		n.d.	0±0	0.352 ±0.009	17.4 ±6.5	0.384 ±0.018	16.7 ±11.5	0.485 ±0.042	19.4 ±10.1	**In ASW**
		n.d.	-	0.181± 0.001	0±0	-	-	-	-	**In NSW**
	0.79	0	-	0.39	-	0.79	-	0.79	-	**Theoretical**
		n.d.	0±0	0.345 ±0.037	23.3 ±5.8	0.883 ±0.024	26.7 ±5.8	0.886 ±0.036	32.2 ±11.3	**In ASW**
		n.d.	-	-	-	0.509± 0.003	3.3 ±5.8	-	-	**In NSW**
	1.57	0	-	0.39	-	0.79	-	1.57	-	**Theoretical**
		n.d.	3.3 ±5.8	0.360 ±0.018	20.7 ±10.1	0.949 ±0.024	38.2 ±16.0	1.573 ±0.068	79.3 ±1.3	**In ASW**
		n.d.	-	-	-	-	-	1.164 ±0.005	3.3±5.8	**In NSW**

Concentrations (CuPT µM) are mean actual concentrations of copper pyrithione, formed from a binary mixture of each HPT : Cu^{2+} combination. The ± values represent the standard deviation (n = 3).

M (%) is an average mortality (n = 3) of *Artemia salina*, exposed binary mixture.

Abbreviations: CuPT – copper pyrithione, HPT - 2-mercaptopyridine-N-oxide, M – mortality, ASW – artificial seawater, NSW – natural seawater

The average mortality (%) of *A. salina* is for each tested Cu^{2+}-HPT combination in ASW and NSW recorded in Table 3. The survival (% initial animals) is for the mixture and pure CuPT in both media plotted in Figure 1. In binary mixture toxicity test, the survival was 100% in all ASW and NSW control replicates. When one or both of the reagents (Cu^{2+} or HPT) were missing, no CuPT was formed and the survival was in all cases above 92.6%. In ASW, the mortality increased with the increased concentration of produced CuPT from the mixture. The highest mortality recorded was 79%, which occurred at the highest measured CuPT concentration, 1.57 µM. The estimated EC_{50} for Cu^{2+}-HPT combination in ASW

was 1.11 µM (95% CI: 0.89 – 1.32 µM) (350.6 µg/L; 95% CI: 281.1 - 417.0 µg/L). In NSW mixture toxicity test the mortality was in all cases low. While very high mortality (79%) occurred at Cu^{2+}/HPT 1.57 /3.15 µM in ASW, in NSW the mortality was only 3.3% for the same tested combination. The CuPT concentration that was measured in this solution was 1.16 µM.

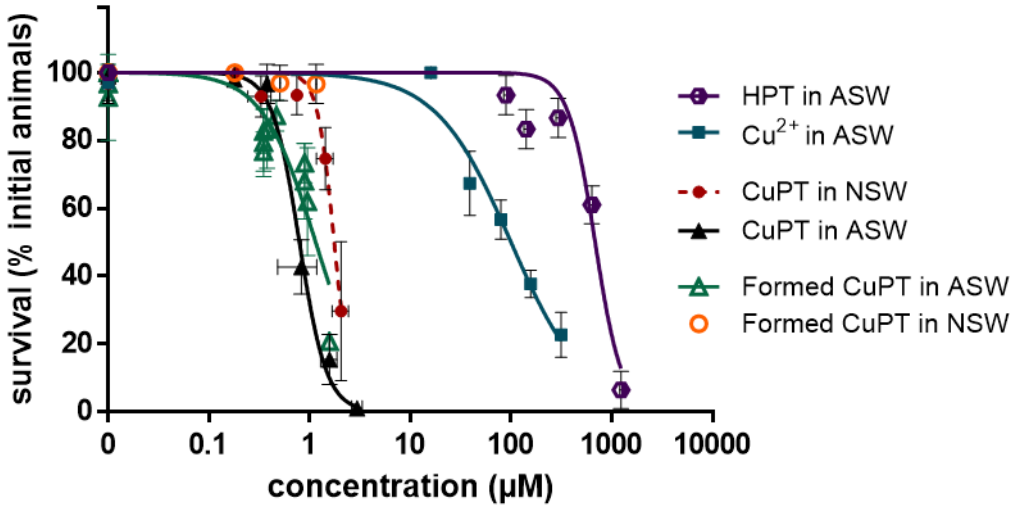


Figure 1: Survival (% initial animals) of *Artemia salina* exposed to Cu^{2+} and 2-mercaptopyridine-N-oxide (HPT) in artificial seawater as well as pure copper pyrithione (CuPT) and CuPT formed from a HPT/Cu^{2+} binary mixture in artificial and natural seawater for 48 h. The logistic curves represents the fitted concentration−response relationships. Error bars (in x and y) represent the standard deviation (n = 3 for the effect and the concentration).

Abbreviations: CuPT – copper pyrithione, HPT - 2-mercaptopyridine-N-oxide, ASW – artificial seawater, NSW – natural seawater

3.3. Influence of ionic strength on CuPT toxicity

3.3.1. Toxicity test of CuPT in natural water with added salts

The electrical conductivity (EC) of NSW used in our experiments was 3.25 S/m, therefore lower than in ASW (EC = 5.06 S/m). By adding salts, we adjusted the EC of NSW to around 5.0 S/m in order to investigate the influence of EC on a toxicity of CuPT (1.58 µM nominal). This concentration was chosen as the EC_{50} of CuPT in NSW was around that value (EC_{50} = 1.76 µM, Figure 1). Adding the salts did not

285 significantly change the pH of the NSW solutions (Table 1).

286 Results on measured concentrations of CuPT and % mortality for the controls and test solutions are for
287 each test media presented in Table 4. In the controls of ASW, NSW, NSW-Mg and NSW-Ca the *A. salina*
288 survival was 96.7% or higher. Some higher mortality (9.7%) was observed in NSW-Na control. This
289 could be an artifact, as the concentrations of all ions measured in NSW-Na were still lower than in ASW
290 (Table 1). As the basis of the media (NSW) is also suitable for *A. salina* (survival of NSW control was
291 100%), it is unlikely that the combination of Na^+ in NSW would be the reason for higher mortality.
292 Chemical analysis showed that salts present in water did not cause any chemical changes to CuPT. In
293 all cases, CuPT concentrations were comparable to the NSW and ASW controls (Table 4).
294 Striking differences were observed in *A. salina* survival among different test media (Table 4). At 1.58
295 µM CuPT nominal concentration, the mortality after 48 h was in ASW 93%, but only 5.6% in NSW.
296 The addition of salts added to NSW had a remarkable influence on CuPT toxicity. Toxicity was the
297 highest in NSW amended with Na^+, followed by Mg^{2+} and Ca^{2+}. In contrast to the observed low toxicity
298 in NSW (5.6%), the addition of Na^+ to NSW to adjust the EC to ca. 5.0 S/m escalated the mortality to
299 90%. NSW amended with Mg^{2+} caused 78% mortality while the addition of Ca^{2+} to NSW resulted in
300 58% mortality.

301

302 **Table 4:** Measured concentrations and the mortality (%) of *Artemia salina* after 48 h exposure to
303 copper pyrithione (1.58 µM) in artificial and natural seawater, and natural seawater amended with
304 salts.

Medium	**EC** (S/m)	**pH**	**CuPT** (µM)		**Mortality** (%)	**Mortality control** (%)
			nominal	**actual**		
ASW	5.06	8.23	1.58	1.27	93	0
NSW	3.25	8.02	1.58	1.41	5.6	0
NSW$^+$Na	5.03	8.07	1.58	1.43	90	9.7
NSW$^+$Mg	5.02	8.07	1.58	1.39	78	0
NSW$^+$Ca	5.06	8.01	1.58	1.38	58	3.2

305 Abbreviations: CuPT – copper pyrithione, EC – electrical conductivity, ASW – artificial seawater, NSW
306 – natural seawater. For media characterization refer to Table 1.

307

308 *3.3.2. Influence of modified EC to CuPT toxicity in ASW*

309 The electrical conductivity (EC) values measured in each ASW dilutions are reported in Table 5. The
310 EC value of each subsequent dilution was for about 0.5 S/m lower from the preliminary solution. The
311 pH value was comparable between different media samples, ranging from 8.13 – 8.3. The actual mean
312 measured concentrations of CuPT very well matched with the targeted nominal one (0.79 µM) in all
313 sample solutions (Table 5).
314 No or slight mortality was observed in controls, with the highest average mortality (6.7%) in sample 3
315 (EC = 4.5 S/m).
316 The highest CuPT toxicity (61% mortality) occurred in undiluted ASW sample (sample 1), with
317 measured EC 5.58 S/m. The mortality remained over 50% also in the next two samples with higer EC
318 value, but it dropped to 23% with further dilution (EC 3.99 S/m). In the following diluted samples (EC
319 = 3.49 – 1.98 S/m), the mortality was in all cases below 3.3%. The results suggest that the toxicity of
320 CuPT is induced with higher salt concentration.
321
322 **Table 5:** Measured electrical conductivity, pH, copper pyrithione concentrations and mortality of
323 *Artemia salina* after 48 h exposure to copper pyrithione (0.79 µM) in artificial seawater and seven
324 artificial seawater dilutions.

Sample	**EC** (S/m)	**pH**	**CuPT** (µM)		**Mortality** (%)	**Mortality control** (%)
			Nominal	**Actual**		
1	5.58	8.13	0.79	0.80	61	0
2	4.99	8.26	0.79	0.84	53	3.3
3	4.50	8.30	0.79	0.85	56	6.7
4	3.99	8.26	0.79	0.84	23	0
5	3.49	8.24	0.79	0.84	0	0
6	3.00	8.28	0.79	0.80	2.8	3
7	2.49	8.26	0.79	0.79	3.3	0
8	1.98	8.18	0.79	0.77	0	0

325 Abbreviations: CuPT – copper pyrithione, EC – electrical conductivity, ASW – artificial seawater
326
327 ***4. Discussion***
328 Due to the rapid photodegradation, CuPT can hardly be detected in the natural environments (Maraldo
329 and Dahllöf, 2004), however some prolonged exposure could be possible during the night or in the sea
330 depths. In our research, CuPT was chosen as a model compound in order to investigate the influence of

different seawater properties to the toxicity of CuPT to *A. salina*.

The measured concentrations of CuPT were in NSW and in ASW comparable but the toxicity of CuPT to *A. salina* was in NSW significantly lower (EC_{50} in NSW = 1.76 µM, EC_{50} in ASW = 0.79 µM). The main differences between ASW and NSW are DOC (2.6 mg/L in NSW, < LOD in ASW) and concentration of salts, which influence the conductivity and the total hardness of the water (Table 1). Although without DOC content, ASW contains a mixture of dissolved mineral salts in ratios that simulate the seawater (ASTM, 2003). It has been long known that DOC has the ability to bind or adsorb the organic chemicals and heavy metals (Maoz and Chefez 2010, Manceau and Matynia 2010). Since only chemicals that are freely dissolved in water are assumed to be taken up by an organisms, the adsorption to DOC decreases their bioavailability and toxicity to the exposed organisms (Day, 1991). The adsorption of CuPT to DOC present in NSW was in our case likely and may be the reason for a decreased toxicity in this medium. Day (1991) observed that the accumulation of the pesticide deltamethrin by the water flea *Daphnia magna* was significantly reduced already at 2.6 mg/L DOC, at the same amount of DOC measured in our NSW medium. Our study investigated the influence of only one DOC value (2.6 mg/L), which was naturally present in NSW, in comparison with organic matter-free ASW. However, the toxicity of compounds may differ depending on the quantity of DOC in water. For example, Deruytter et al. (2015) investigated the influence of DOC content in seawater, ranging from 0.56 to 4.66 mg/L, on a toxicity of Cu^{2+} to *Mytilus galloprovincialis* larvae. Their results showed a decrease in Cu^{2+} toxicity with increased DOC content.

In a binary mixture toxicity study the measured CuPT concentrations were for same Cu^{2+}/HPT ratios lower in NSW than in ASW. This could be attributed to DOC present in NSW (2.6 mg/L, Table 1) and would suggests a strong affinity of DOC to bind the reagents. We believe that due to the chelation to DOC, the concentration of Cu^{2+} and HPT in their free form decreased, which subsequently led to decreased production of CuPT. Since the concentrations of Cu^{2+} and HPT were in this experiment not followed, we are unable to compare the binding affinity of DOC to both compounds, however a strong binding of Cu^{2+} by DOC has been observed and described frequently in the literature (see for example Manceau and Matynia, 2010). It can be observed that the yields of produced CuPT are in a binary mixture toxicity test in NSW higher when concentrations of reagents were higher (Table 3). By

359 increasing the concentration of each reagent in a fixed volume, the probability that the molecules will
360 meet and interact increases. Other reason could be that the concentration of DOC or its affinity is limited
361 to bind higher concentrations of reagents.
362 While Cu^{2+} and HPT were not particularly toxic to artemia, in combination they form a more hazardous
363 compound, CuPT. Toxicity responses in ASW positively correlated with the amount of produced CuPT.
364 In NSW, the mortality was low (3%) also at the highest Cu^{2+}/HPT combination which produced 1.16
365 µM CuPT. This low mortality of formed CuPT in NSW again suggests that the toxicity is in NSW
366 suppressed. In accordance to results obtained in ASW in our study, Onduka et al. (2010) found the
367 toxicity of HPT in the presence of Cu^{2+} similar to the toxicity of CuPT alone when tested on marine
368 algae *S. costatum*. In their 72-h toxicity test an f/2 medium prepared from a filtered natural seawater was
369 used, however the authors do not state the characteristics of the medium.
370 The experiment in NSW with added salts revealed that not only DOC present in water but also ion
371 strength has an influence on toxicity of CuPT to *A. salina*. While the average conductivity in the seawater
372 is 5 S/m (such as in ASW), EC can however vary in different areas of the ocean (Forchhammer, 1865).
373 In our case, the EC in NSW was 3.25 S/m. The addition of salts to NSW in an amount to adjust the EC
374 from 3.25 to around 5 S/m caused an increase of toxicity. In accordance to that, decreasing the EC values
375 of ASW by dilution with an ion free water resulted in lower toxicity. One explanation for the increased
376 toxicity at higher salt concentration could be that the uptake of compound is increased, when the salinity
377 is increased. Such observations, but also observations in contrast to this one have been observed in
378 previous studies with different pollutants and different test species. For instance, Deruytter et al. (2015)
379 found an increase of Cu^{2+} toxicity to mussel larvae with an increase in salinity. Brecken-Folse et al.
380 (1994) observed an increased toxicity of industrial chemical 2,4- dinitrophenol to a grass shrimp
381 *Palaemonetes* spp. with an increased salinity. Contrary, when tested on the sheepshead minnow
382 *Cyprinodon vanegatus*, the toxicity of 2,4- dinitrophenol decreased when salinity increased. Toxicity
383 was also lower in water with higher salinity for 4 – nitrophenol, tested on both species. Another example
384 was a study on Japanese medaka embryos, where the toxicity of L-selenomethionine was induced by
385 salinity in fish embryos (Lavado et al., 2012). The influence of salinity to the toxicity of compounds
386 therefore varies on the chemical species tested as well as the testing organism. Brine shrimps are known

for their very high osmotolerancy as they can be found in brackish as well as supersaturated aquatic environments, however they are mostly found in salinities ranging 45-200 psu (Naceur et al., 2012). Although the adjusted EC values of NSW media amended with salts were same (5 S/m), as well as the tested concentration of CuPT, toxicity differed depending on the salt that was used to adjust the EC. While differences in proportion of main salt constituents in seawater are not likely (Forchhammer, 1865), the concentrations of salts and their proportions in freshwater bodies vary and are constantly changing, in both time and place (Golterman, 2004). Our study showed that the concentrations of individual constituents and their proportions in water are important in the expression of toxicity of compounds, which can be highly relevant especially for biota living in freshwater environments.

When CuPT was tested on a crustacean *T. japonicus* (Onduka et al., 2010) the EC_{50} obtained after a 24 h exposure to CuPT was 0.073 µM (23 µg/L). *T. japonicus* therefore showed to be more sensitive to CuPT compared to *A. salina* (48 h EC_{50} = 0.79 µM). As in our study, HPT posed lower risk than the mother compound with a 24-h EC_{50} > 98.0 µM. Accordingly, high CuPT toxicity was observed also on duckweed *Lemna gibba* with an EC_{50} of 10 µg/L for the effect on growth rate, while much lower toxicity was noted for HPT (EC_{50} = 46 µg/L) (Okamura et al., 2012). In a study of Koutsaftis and Aoyama (2007), the 24-h exposure of *A. salina* to CuPT in a standard artificial seawater resulted in a toxicity with an EC_{50} of 830 µg/L. The duration of the toxicity test was in our case longer (48 h) and the EC_{50} in ASW obtained is expectedly lower (EC_{50} = 250 µg/L). In their study, CuPT was shown to be the most toxic among the tested antifoulant biocides, as well as in a study of Okamura et al. (2002) on salmon cell line CHSE-sp. High toxicities of CuPT were observed for CuPT by Mochida et al. (2006) on fish sea bream *Pagrus major* (96-h LC_{50} = 9.3 µg/L) and toy shrimp *Heptacarpus futilirostris* (96-h LC_{50} = 2.5 µg/L). Marine algae (*S. costatum, T. tetrahele, C. calcitrans* and *D. tertiolecta*) showed high sensitivity to CuPT with an 72-h EC_{50} ranging from 1.5 – 12 µg/L as well as fish *P. major*, with an 96-h LC_{50} = 9.3 µg/L (Onduka et al., 2010).

5. Conclusions

In the present study, the toxicity of copper pyrithione (CuPT), its degradation product HPT and binary mixture of HPT and Cu^{2+} was investigated on a brine shrimp *A. salina* in different water media, differing

in dissolved organic matter (DOC), salinity and salt constitution. In a DOC-free artificial seawater (ASW, EC ~ 5 S/m), the toxicity of CuPT was considerably higher than in natural seawater (NSW) with 2.6 mg/L DOC and EC of 3.25 S/m. HPT was shown to be considerably less toxic to *A. salina* than the parent compound. In the presence of copper however, HPT converted back to CuPT. The amount of produced CuPT from Cu^{2+}/HPT binary mixture as well as its toxicity was in NSW lower than in ASW, suggesting the binding of the reagents to DOC. Salts had a remarkable influence on CuPT toxicity on *A. salina*. In water with an increased salinity, the toxicity of CuPT was higher than in low salinity water. The toxicity was also influenced by the constitution and proportion of ions contributing to the ionic strength of the medium. Among salts tested, the CuPT toxicity was the highest in a medium with elevated Na ions.

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