1 **Characterization of the bioaccumulation and toxicity of copper pyrithione, an antifouling**
2 **compound, on juveniles of rainbow trout**

3

4 Charlotte Bourdon[1,2], Jérôme Cachot[2], Patrice Gonzalez[2], Patrice Couture[1]

5

6 [1] Institut National de la Recherche Scientifique – Centre Eau Terre Environnement, Québec, Québec,
7 Canada

8 [2] Univ. Bordeaux, CNRS, EPOC, EPHE, UMR 5805, F-33600 Pessac, France

9
10

11

Credit author statement:

Charlotte Bourdon: Methodology, Validation, Investigation, Writing – Original Draft. Jérôme Cachot: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft, Supervision. Patrice Gonzalez: Validation, Investigation, Writing – Original Draft, Supervision, Funding acquisition. Patrice Couture: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft, Supervision, Project administration, Funding acquisition.

19 **Abstract**

20 Since the global ban on tributyltin in antifouling paints in 2008 by the International Maritime 21 Organization, new products have been developed and brought to the market. Among them, copper 22 pyrithione (CuPT) is used, but its mechanisms of toxicity remain little known. This project aimed to identify 23 and measure the impacts of aqueous exposure to CuPT, an organic compound, and compare it to ionic Cu^{2+} 24 added in the form of its inorganic salt $CuSO_4$, in equivalent Cu^{2+} molar concentrations, on rainbow trout 25 (*Oncorhynchus mykiss*) juveniles under controlled laboratory conditions. A 24-hour acute exposure was 26 performed with nominal concentrations of 50 and 100 µg/L Cu from either $CuSO_4$ or CuPT (labelled 27 $CuSO_4$_50, $CuSO_4$_100, CuPT_50 and CuPT_100, respectively). The CuPT_100 condition induced 85 % 28 mortality in 15 hours and the CuPT_50 condition induced 5 % mortality in the same period. A chronic 29 exposure was then performed with nominal concentrations of 1 and 10 µg/L Cu from CuPT and 10 µg/L 30 Cu^{2+} from $CuSO_4$ (labelled $CuSO_4$_1, $CuSO_4$_10, CuPT_1 and CuPT_10, respectively). Measured aqueous 31 concentrations of Cu^{2+} were slightly higher than nominal concentrations for the lower concentrations, but 32 lower for the CuPT_10 condition. The 8- and 16-day toxicokinetics showed a greater accumulation of 33 copper in the gills of fish exposed to CuPT compared to fish exposed to Cu^{2+} from $CuSO_4$. The CuPT_10 34 condition induced 35 and 38 % mortality after 8 and 16 days of exposure, while no mortality was observed 35 in the $CuSO_4$_10 condition. The growth of juveniles was not impacted during the 16 days of exposure for 36 any condition. The activity of antioxidant enzymes (CAT, SOD, GPx) did not respond to exposure to either 37 contaminant. The expression of genes involved in the antioxidant response (*sod1*, *sod2*, *gpx*), detoxification 38 (*cyp1a*, *mt1x*, *mt2x*), Cu transport (*ctr1*, *ctr2*, *slc11a2*), energy metabolism (*AcoAc*, *cox*, 12S) and cell cycle 39 regulation (*bax*) strongly decreased at Day 8 in the gills and at Day 16 in the liver of CuPT-exposed fish in 40 comparison to controls at the same time point. This study clearly showed that the toxicity of Cu in the form 41 of CuPT was much higher than that of ionic Cu from $CuSO_4$ and provides new information on the compound 42 that will be useful to develop regulations concerning its use and release in the aquatic environment.

43

44 **Keywords** – Copper pyrithione; antifouling; copper sulphate; rainbow trout juveniles; lethal toxicity; sub-45 lethal toxicity; oxidative stress; gene expression

46

47

48 **1. INTRODUCTION**

49 Biofouling, *i.e.* the adhesion of organisms to any submerged surface, causes economic and
50 environmental consequences. The increase in friction forces induces fuel overconsumption (up to 40 %) and
51 increased maintenance costs (Champ, 2000). Vessels colonized by organisms can also be vectors for non-
52 native species, and these can become invasive and destabilize an entire ecosystem. The application of
53 antifouling paint helps to fight against this colonization. Since the banning of tributyltin (TBT), a widely
54 used, highly effective but very toxic compound, at the end of the 20th century, other copper (Cu)-based
55 paints (Cu_2O, CuCN) have been put on the market (Konstantinou and Albanis, 2004). One or more co-
56 biocides are generally added to act on Cu-resistant organisms (Voulvoulis, 2006). Among them, copper
57 pyrithione (CuPT), an organo-copper compound formed from two pyrithione ligands and a copper cation
58 (Cu^{2+}) in the centre, is commonly used for its antifungal and antimicrobial action (Okamura and Mieno,
59 2006), particularly in Japan (J-Check, 2021).

60 Since the first attempts to quantify metallic PTs (CuPT, ZnPT) in environmental matrices in 1999,
61 there are few data on environmental concentrations because CuPT is photosensitive and has an estimated
62 half-life of 7.1 ± 0.2 min in water (Maraldo and Dahllöf, 2004; Turley et al., 2000). The end-product of PT
63 degradation is pyridine-2-sulfonic acid (PSA), which is far less toxic than the parent compounds (Turley,
64 2000). Nevertheless, Harino *et al.* (2007) reported a concentration of 2.2 $\mu g \cdot kg^{-1}$ dw in the sediment
65 collected in a bay in Japan. In the bay of Toulon, France, Cu concentrations in the sediment have been
66 reported between 5.8 and 864 $mg \cdot kg^{-1}$ dw (Tessier, 2011). The presence of CuPT in the environment could
67 induce toxic effects on non-target species. Given the lack of information on its toxicokinetic and
68 toxicodynamic properties, ecotoxicological studies are needed. Apart from toxicity studies on microalgae
69 and crustaceans, very little research has focused on sublethal effects such as growth, reproduction, or the
70 biochemical responses of organisms to CuPT exposure (Walker, 2006; Mochida et al., 2011; Mohamat-
71 Yusuff et al., 2018). Few studies have reported on the toxicity of CuPT in fish embryos. In a recent study,
72 Shin et al. (2022) compared the toxicity of CuPT and ZnPT on embryonic flounder and reported that the
73 former had stronger effects than the latter on mortality, malformations and on the transcription levels of
74 genes related to heart, nervous system and fin development. Almond and Trombetta (2016, 2017) also
75 observed several developmental issues in zebrafish embryos exposed to CuPT.

76 The rainbow trout (*O. mykiss*) is a model species whose life cycle and physiology are well
77 documented, is easy to rear in the laboratory, and is particularly sensitive to many contaminants (Le Bihanic
78 et al., 2014; Santos et al., 2019). A few studies on metal PT toxicity have been done on the rainbow trout
79 (*O. mykiss*) (Okamura et al., 2002; Yamada, 2006). In our study, two experiments were performed on

juvenile rainbow trout in the laboratory, during which the fish were exposed to either CuPT or $CuSO_4$ in equivalent molar Cu concentrations. These exposures were aimed at characterizing the toxicity of an organo-Cu compound, CuPT, relative to ionic Cu^{2+} from $CuSO_4$ in equivalent molar Cu concentrations, from phenotypic and molecular points of view. The first exposure lasted less than 24 h with high concentrations of 50 and 100 µg $Cu^{2+}\cdot L^{-1}$ and induced rapid mortality. The second experiment exposed juveniles for 16 days to sublethal concentrations of Cu of 1 and 10 µg $Cu^{2+}\cdot L^{-1}$. These concentrations were chosen based on the results of the first experiment. Metal contamination typically leads to an overproduction of reactive oxygen species (ROS) which must be regulated by the antioxidant defence system. Toxicokinetic and toxicodynamic properties of CuPT and $CuSO_4$ were monitored by sampling fish at the beginning, halfway through the exposure, and at the end of the 16-day exposure in various tissues. Mortality and growth were the two phenotypic indicators, while from a molecular point of view, analyses of the activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) and analyses of gene expression levels were monitored. This study focused on the expression of 17 genes of interest. Genes selected included some involved in Cu transport (*ctr1* and *ctr2* (copper transporters 1 and 2) and *slc11a2* (solute carrier family 11 member 2 or divalent metal transporter)), antioxidant capacities (*gpx1* (glutathione peroxidase), *sod1* (cytoplasmic superoxide dismutase Cu/Zn), *sod2* (mitochondrial superoxide dismutase Mn) and *cat* (catalase)), detoxification (*cyp1a1* (cytochrome P450 family 1 subfamily A1), *gstA* (glutathione S- transferase A) and *mt1x* and *mt2x* (metallothionein isoforms 1X and 2X)), energy metabolism (*tgl* (triacylglycerol lipase-like), *cox1* (cytochrome c oxidase subunit 1) and *12s* (small mitochondrial ribosomal RNA)) and cell cycle regulation (*tp53* (cellular tumor protein tp53)). The objectives of this study were to (1) compare the toxicity threshold and the spectrum of sublethal effects after 8 and 16 days of exposure to CuPT or $CuSO_4$ (2) compare the accumulation of Cu in tissues and (3) compare the mechanisms of toxicity of both compounds.

2. MATERIALS AND METHODS

2.1. Chemical preparation ~~and analysis~~

Stock solutions of $CuSO_4$ were prepared by dissolving $CuSO_4\cdot 5H_2O$ in distilled water, and by serial dilutions of the stock solution. Stock solutions of CuPT were prepared in the dark by dissolving CuPT powder in the nontoxic organic solvent dimethyl sulfoxide (DMSO, final concentration < 0.1 %) and distilled water, and by serial dilutions of the stock solution. Stock solutions were kept under dark conditions until use, to avoid photolysis. ~~Subsequently, an appropriate amount of each stock solution was dispersed in each tank after water renewal to attain a designated nominal concentration for the exposure medium. Every~~

~~two days, three quarters of the exposure medium were renewed (static renewal experiments), and water quality parameters (temperature, nitrite, nitrate, ammonium, pH) and contamination were measured.~~

2.2. Experimental conditions

Rainbow trout (*O. mykiss*) juveniles (9.9 ± 0.9 cm; 8.1 ± 1.6 g) were provided from pisciculture Saint-Alexis-des-Monts Inc. (Québec). Fish arrived at 7°C. A thermal acclimation was performed until the target temperature of 11°C was reached, at a rate of 1°C·day^{-1}. Exposures were initiated after thermal and environmental acclimation. Acclimation and exposures were performed in reconstituted water ([Ca^{2+}] 70 µM, [Cl^-] 129 µM, [K^+] 12 µM, [Mg^{2+}] 13 µM, [Na^+] 179 µM, [SO_4^{2-}] 63 µM). The first exposure included a control (0), and the molar equivalent concentration of ionic copper (Cu^{2+}), at 50 and 100 µg Cu·L^{-1} from CuPT or $CuSO_4$ to compare the range of lethal concentrations of both contaminants. There were 20 fish per tank, and one tank per condition. For the second exposure, 20 juveniles per tank were exposed during 16 days to the molar equivalent concentration of Cu^{2+}, with 0 (control), 1 and 10 µg Cu·L^{-1} from CuPT (5 and 50 µg CuPT·L^{-1}) or 10 µg Cu·L^{-1} from $CuSO_4$ (40 µg $CuSO_4$·L^{-1}) to follow sublethal parameters. ~~Every two days, three quarters of the exposure medium were renewed (static renewal experiments), and water quality parameters (temperature, nitrite, nitrate, ammonium, pH) and contamination were measured~~ Every two days, three quarters of the exposure medium were renewed (static renewal experiments), and water quality parameters (temperature, nitrite, nitrate, ammonium, pH) and contamination were measured. Appropriate amounts of each stock solution were dispersed in each tank after water renewal to attain a designated nominal concentration for the exposure medium. Water samples were taken after each water renewal (beginning of the dark period to avoid photodegradation) and the morning after (during the light period). Both samples were analyzed to monitor photodegradation.~~,~~ Water samples to quantify Cu were acidified with 0.2 (v/v) nitric acid 70 % Optima grade and kept in dark and cold at 4 °C until analysis. Water samples to quantify CuPT were kept in dark and cold at 4°C until analysis the same day. All conditions of the second experiment were in triplicate (12 tanks in total)~~ and with the same environmental parameters~~. Nominal concentrations of Cu were used to label the exposure conditions, and abbreviated as CuPT_1, CuPT_10, CuPT_50, CuPT_100, $CuSO_4$_10, $CuSO_4$_50 and $CuSO_4$_100. The experiments were carried out in oxygenated 40 L glass tanks in an environmentally controlled room (constant temperature at 11°C, light/dark cycle 14:10 h). Fish were fed daily *ad libitum* with pellets provided by the fish farmer (Nutra Fry®). Fish were checked daily for mortality and considered dead if they had no reactions after stimulation and if no movement of the mouth and the opercula could be detected. All procedures were approved by the INRS Animal care committee.

2.3. Fish ~~and water~~ sampling

The first experiment lasted less than 24 h. Dead fish were collected, and fish still alive were sacrificed. Tissues (liver, gills, and a sample of axial muscle collected above the lateral line and below the dorsal fin) of all fish were collected for Cu measurement. For the second experiment, fifteen fish were sampled at the start of the experiment (Day 0). Ten fish were also randomly sampled from all tanks (30 fish per condition) after 8 and 16 days. Fish sampled were sacrificed by a blow to the head and whole-body length and weight were recorded. Samples of gills, liver and muscle were collected to measure tissue Cu concentrations. These samples were frozen and stored at -20 °C until further analysis. Samples (20 mg) of liver and gills were kept in RNAlater® at -20 °C for genomic analyses. These two organs have been chosen for trancriptomic analyses because gills is the main route of exposure and the liver is the central internal compartment for Cu accumulation and homeostasis (Grosell *et al*., 1998). Finally, liver samples (about 10 mg) were collected and immediately frozen in liquid nitrogen and stored at -80 °C for determination of antioxidant capacities. For the determination of Cu concentration, genomic and antioxidant capacity analyses, five fish per tank (15 per condition) were selected for analysis. The same individuals were used for all three analyses.

~~Water samples were taken after each water change (beginning of the dark period to avoid photodegradation) and the morning after (during the light period). Both samples were used to monitor photodegradation. Water samples to quantify Cu were acidified with 0.2 (v/v) nitric acid 70 % Optima grade and kept in dark and cold at 4 °C until analysis. Water samples to quantify CuPT were kept in dark and cold at 4°C until analysis the same day.~~

2.4. Cu and CuPT analysis

Fish tissue Cu analysis was performed by inductively coupled plasma-mass spectrometry (ICP-MS) (Model x-7, Thermo Elemental). Frozen tissues were lyophilized for 48 h (FTS Systems TMM, Kinetics Thermal Systems, Longueuil, QC, Canada), weighed, and then digested in 1 mL of nitric acid (70 %, v/v, Optima grade, Fisher Scientific) for 48 h. Then, 0.5 mL of hydrogen peroxide (30 %, v/v, Optima grade, Fisher Scientific) was added for an additional 48 h. Finally, ultrapure water was added to stop the digestion in a final digestion volume of 10 mL. Certified standards (DOLT-5 and TORT-3, n=5) were treated along with the fish tissue samples and allowed to estimate the efficiency of the digestion procedure and analytical accuracy. Recovery rates were 99% and 93%, respectively.

Copper in water samples was analysed with an ICP-AES (Varian Vista XP Axial CCD Simultaneous ICP-AES, Agilent Technologies) or ICP-MS (Model X-7, Thermo Elemental), depending on Cu

concentration. CuPT in water samples was analysed by LC-MSMS (TSQ Quantum Access Thermo Scientific). The separation was carried out on an ACME-C18 100 mm x 2.1 mm x 3.0 µm column, with a column temperature of 40 °C, elution with 85 % methanol (0.1% formic acid) and 15% water (0.1% formic acid, 10 mM acetate) for 5 min, with a flow rate of 0.25 $mL \cdot min^{-1}$ and an injection volume of 10 µL. Samples and standards (Atrazine-D5) were diluted with the methanol/water solution using the ratio 85/15 (v/v).

2.5. Antioxidant capacities

Tissues samples were homogenized and crushed in a buffer solution prepared with 20 mM HEPES, 1 mM EDTA and 0.10 % Triton X-100. Aliquots were set aside for total protein determination by Lowry assay (Lowry *et al*., 1951). Enzyme activities included the quantification of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity. Analyses were performed using a UV/Vis spectrophotometer (Varian Cary 100, Varian Inc., Palo Alto, California, USA) on 96-well microplates at room temperature (20°C). Assay kits were purchased from Cayman Chemical Company Inc. (Ann Arbor, Michigan USA), and assays followed the manufacturer's protocols. Catalase (kit No. 707002) activity was measured at 540 nm. Superoxide dismutase (kit No. 706002) activity was measured at 450 nm. Glutathione peroxidase (kit No. 703102) activity was measured at 340 nm. Enzyme activities are expressed as nmol/min/mg protein for both CAT and GPx and as U/mg protein for SOD (one unit of SOD is defined by the manufacturer as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical). Protein concentrations were determined on liver homogenates before centrifugation using Coomassie (Bradford) Protein Assay Kit (No. 23200) at a wavelength of 595 nm and protein concentrations are expressed as mg protein per g of liver wet weight (Bradford, 1976).

2.6. RNA extraction and real-time qPCR

In this study, we measured the transcription level of 17 genes (*slc11a2*, *ctr1*, *ctr2*, *gpx1*, *sod1*, *sod2*, *cat*, *cyp1a*, *gstA*, *mt1x*, *mt2x*, *AcoAc*, *tgl*, *cox1*, 12S, t*p53* and *bax),* Accession number and specific primer pairs are shown in Table 1.

Gills and liver from 15 individuals per experimental condition were used for RNA extraction. Extraction was conducted with the kit Promega SV Total RNA Isolation System assay. Briefly, phenol-chloroform-isoamyl alcohol (25:24:1) was added to the homogenate, vigorously shaken and centrifuged (13 500 rpm for 5 min at room temperature). The upper aqueous layer (about 500 µL) was transferred to a new tube without disrupting the interface and one volume of ethanol 75% was added. DNA digestion was performed with the RNase-Free DNase I set for 15 min at 37°C. Reaction was stopped with the DNase Stop

203 Solution and samples were washed with the RNA Wash Solution provided by the kit. RNase-free water was
204 added to the spin column at the end of the process to eluate total RNA from the column. Total RNA quality
205 and concentration for each extract were determined using absorbance measures at 260 and 280 nm with the
206 spectrometer Epoch plate reader (Take3, Biotek) and analysed with the Gen5 software. The quality of RNA
207 extracts with a 260/280 ratio upper than 1.8 was considered satisfactory.

208 Reverse transcription was conducted with the Promega GoScript Reverse Transcription System
209 assay. One microliter of oligo dT at 1µM and hexa-primers at 1µM were added to 1 µg of total RNA with
210 RNase-free water and incubated in thermocycler 5 min at 70°C then 5 min at 4°C. Then 8 µL of a mix
211 (GoScript reaction buffer containing $MgCl_2$, PCR Nucleotides, Recombinant RNasine Ribonuclease
212 Inhibitor, GoScript Reverse Transcriptase) provided by the kit was added to start the reverse transcription
213 reaction in the thermocycler (Eppendorf Flexide Mastercycler Nexus) for 5 min at 25°C, 1 h at 42°C and
214 then kept at 4°C. Samples were kept at -20°C until real-time qPCR (rt qPCR). Rt qPCR was conducted with
215 Promega GoTaqR qPCR Master Mix. Each well of the 384 wells plate has been filled with 3 µL of samples
216 ~~contained~~ containing 30 ng of cDNA and 9 µL of master mix with SYBR green, reverse and forward primers
217 pair at 2 µM each for one of the 17 selected genes and RNase-free water for a final volume of 12 µL. Real-
218 time quantitative PCR (qPCR) analysis was performed using a LightCycler®480 (Roche), with a first cycle
219 at 95°C for 2 min, followed by 45 cycles at 95°C for 15 sec and at 60°C for 1 min.. The reaction specificity
220 was determined for each reaction from the dissociation curve of the PCR product. It was obtained by
221 following the SyberGreen fluorescence level during a gradual heating of the PCR products from 60 to 95°C
222 for 2 min. The relative quantification of each gene expression level was normalized ~~against~~ to the arithmetic
223 mean housekeeping genes *actb* and *rpl7*, and ~~changes in~~ ΔCt values were recorded. From this comparison,
224 fold-change factors were obtained for each gene by comparing each mean (n=~~12~~15) value observed in the
225 contaminated conditions with that of the corresponding control according to $2^{-\Delta\Delta Ct}$ method (Livak and
226 Schmittgen, 2001). ~~Difference between control and exposed conditions were considered significant if p-~~
227 ~~value was below 0.05.~~

Mis en forme : Police :Symbol

228 **Table 1: Accession number and specific primer pairs of 19 selected genes from the rainbow trout *O. mykiss***

Fonction	Gene	Primer (5' - 3')	Accession number
Reference genes	*rpl7*	F: GGTCGCTCTCACAGACAACA R :TTATGTCCGTCCCTCTGGGT	NM_001160672.2
	actb	F : GCCCCATGTTACAGAACGC R :ACTGGAACGGTGAAACAGC	NM_001124235.1
Copper transport	*slc11a2*	F : TGCTCTGATCCCCATCCTGA R :ACCGAGGGCTATCAGACACT	NM_001172513.1
	ctr1	F : GCTCATACAGGGCATTTGCG R : TATGACCCCCATCGGACAGT	XM_021590711.2
	ctr2	F : TCATACCGACCCTGTTGCAC R : TGTGCTCGATCACTCCCCTA	XM_021590778.2
Antioxidant response	*gpx1*	F : ACCCCAAGTGCATCATCTGG R : CTCCCCACCATTGCTTCTGT	NM_001124525.1
	sod1	F: TGATTGGGGAGATCTCGGGT R : CGGGTCCAGTGAGAGTCAAC	NM_001124329.1
	sod2	F : CAACGCAGAGATCATGCAGC R : CCGCCATTTGGAGAGAGGTT	XM_021612540.2
	cat	F : CAGGTGTCTTTCTTGTTCAG R : GTCCAGGATGGGAAGTTGC	FJ226382.1
Detoxication	*cyp1a*	F : ACCATCAGCACAGCTCTGTC R : AACGGCAGGAAGGAAGAGTG	AF015660.1
	gstA	F : CAGGTGTCTTTCTTGTTCAG R : GTCCAGGATGGGAAGTTGC	BT073173.1
	mt1x	F : GTGGATCCTGCAAGTGCTCA R : GTAATGCACCAGGCCTCACT	M18104.1
	mt2x	F : ATCTTGCAACTGCGGTGGAT R : CCTCACTGACAACAGCTGGT	XM_021597409.1
Energy metabolism	*AcoAc*	F : CACACAATTGGCCAAGGTGG R : AAAGGTGTGCAAACCCTCCA	XM_036990264.1
	tgl	F : GTCCCAGTTGTGTCTGAGGG R : CCTGAACACTGCAGCCCATA	XM_036980805.1
	cox1	F : TCGTTTGAGCCGTGCTAGTT R : CTTCTGGGTGGCCGAAGAAT	KP013084.1
	12S	F : GCGCCAGCTTAAAACCCAAA R : GCCCATTTCTTCCCACCTCA	KY798500.1
Cell cycle regulation	*tp53*	F : CCTACCAATGAGTAAGACAT R : CCCGAAACATCCCGCT	NM_001124692.1
	bax	F : CAGAAAACCCAGGGAGGCAT R : AGAACACATCCTGGGCACAG	BT074328.1

229

2.7. Statistical analyses

Normality (Shapiro-Wilks) and variance homoscedasticity of residuals (Levene) were verified with a p-value set at 0.05. Since these assumptions of normality were not met, non-parametric tests were performed, as indicated in figure and table legends. Statistical analyses were performed using Excel's statistical functions and Statistica. Difference between control and exposed conditions were considered significant if p-value was below 0.05.

3. RESULTS

3.1. Cu concentration in the water

The Cu concentrations in water for the two compounds are shown in figure 1A. The concentration in the control aquaria was ~~generally~~ between 0.2 and 1.4 $\mu g \cdot L^{-1}$ (with an outlier of 3.3 $\mu g \cdot L^{-1}$). The measured concentrations in aquariums of the condition CuPT_1 were between 0.9 and 2.8 $\mu g \cdot L^{-1}$, with 50 % of the values between 1.3 and 2.2 $\mu g \cdot L^{-1}$. The measured concentrations in aquariums of the condition CuPT_10 were between 1.1 and 14.4 $\mu g \cdot L^{-1}$, with 50 % of the values between 2.4 and 4.4 $\mu g \cdot L^{-1}$. The measured concentrations in tanks of the condition $CuSO_4$_10 were between 1.3 and 12.2 $\mu g \cdot L^{-1}$, with 50 % of the values between 6.1 and 10.0 $\mu g \cdot L^{-1}$. Since there was a Cu background value in the controls, the exposure values of the CuPT_1 condition were higher than the nominal value. On the other hand, the exposure values of the CuPT_10 condition were strongly below the nominal value, with ~~a yield of~~ 50 % of the values between only 24 and 44 %, while the values of the $CuSO_4$_10 condition were quite close to the nominal value (50 % of the yield between 60 and 100 %).

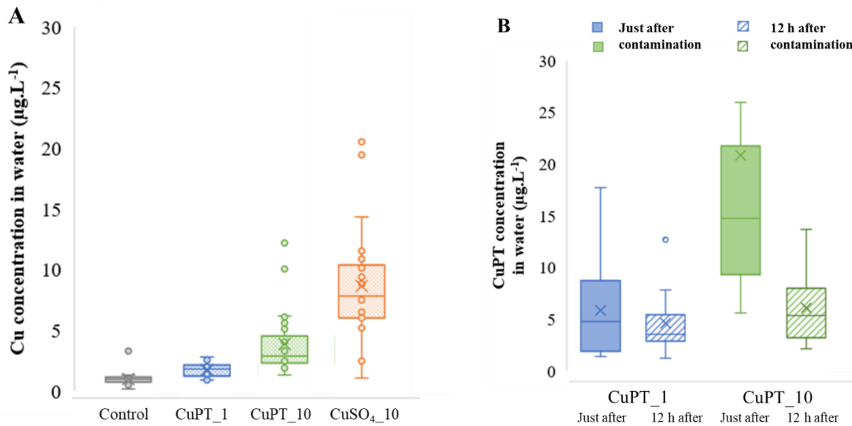


Figure 1: Boxplot of the gross values of Cu (A, n=15) and CuPT (B, n=10) concentration in water (µg·L^{-1}) on the sampling days for the different conditions of exposure to CuPT and $CuSO_4$. For Cu analyses: boxplot of all the values (just after contamination and 12 h after contamination, combined) for control (grey, n=24), CuPT_1 (blue, n=30), CuPT_10 (green, n=26) and $CuSO_4$ (orange, n=26). For CuPT analyses: boxplot of the values just after contamination (n=13) and 12h after contamination (n=13), same colours for CuPT_1 (n=14) and CuPT_10 (n=14).

3.2. CuPT concentration in the water

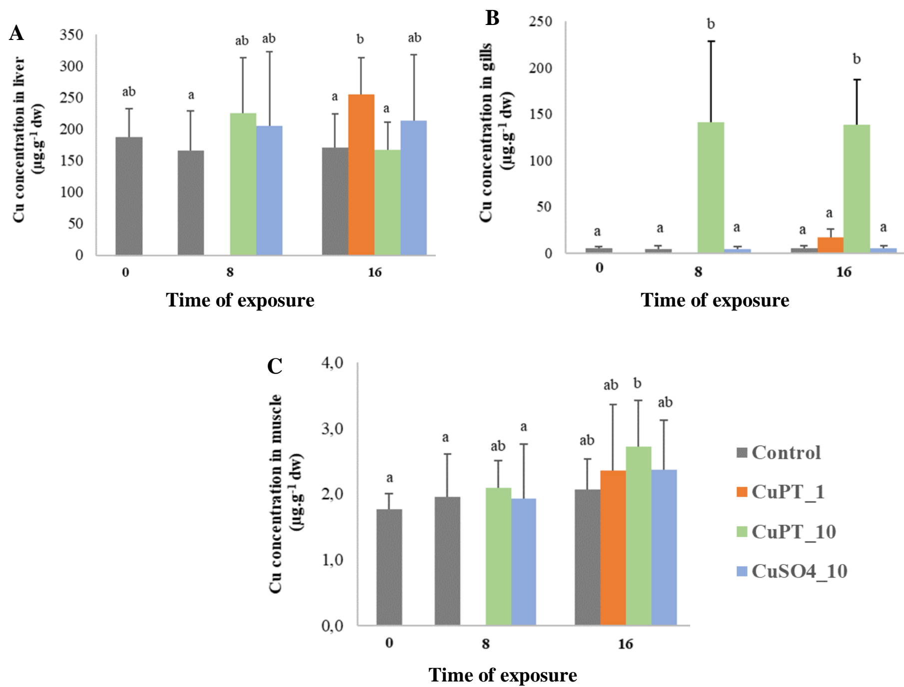
The CuPT concentrations in water just after contamination, and 12 h after contamination for the two ~~compounds~~ concentrations are shown in figure 1.B. The concentration in aquariums of condition CuPT_1 was between 2.7 and 12.0 µg·L^{-1} just after contamination with 50 % of the values between 3.3 and 8.0 µg·L^{-1}, and between 2.5 and 14.0 µg·L^{-1} 12 h after contamination, with 50 % of the values between 4.2 and 5.8 µg·L^{-1}. The concentration in aquariums of the condition CuPT_10 was between 6.9 and 27.2 µg·L^{-1} (outlier of 59.0 and 66.0 µg·L^{-1}), just after contamination with 50 % of the values between 11.3 and 21.5 µg·L^{-1}, and between 3.4 and 15.0 µg·L^{-1} 12 h after contamination, with 50 % of the values between 4.8 and 9.0 µg·L^{-1}. The concentrations of CuPT obtained for the CuPT_1 condition coincide with the nominal concentration of CuPT (condition of 1 µg Cu·L^{-1} equals to 5.0 µg CuPT·L^{-1}) while the values for the CuPT_10 condition are below the nominal concentration of CuPT (condition of 10 µg Cu·L^{-1} equal to 50.0 µg CuPT·L^{-1}).

3.3. Cu accumulation in tissues

The mean Cu concentration in the liver of juveniles ranged from 165 ± 63 to 255 ± 58 µg·g^{-1} dw (figure 2A). No accumulation kinetics were visible between the beginning and the end of the exposure. ~~Cu~~ Copper accumulation in liver was not compound-dependent. Only the fish in the CuPT_1 condition at Day

272 16 showed a significant increase of Cu accumulation in liver compared to the control. There was no
273 significant difference ~~for~~ in the liver of fish from the other conditions. In contrast, gill Cu accumulation
274 varied among treatments (Figure 2B). Fish from t~~T~~he control and $CuSO_4$_10 conditions at the three days of
275 sampling did not accumulate Cu in their gills (5 to 6 $\mu g \cdot g^{-1}$ dw). Conversely, fish from the CuPT_10
276 condition showed an accumulation of 141 ± 87 $\mu g \cdot g^{-1}$ dw on day 8 and 138 ± 49 $\mu g \cdot g^{-1}$ dw after 16 days.
277 Gill Cu content in fish from the CuPT_1 condition did not differ from the controls. ~~Finally~~Remarkably, in
278 fish exposed to the CuPT_50 and CuPT_100 conditions (first exposure), we observed ~~an~~ a significantly
279 greater accumulation of Cu in the gills ~~significantly greater~~ than in the control after less than 24 h of
280 exposure, with 72 ± 20 $\mu g \cdot g^{-1}$ dw for CuPT_50 and 68 ± 15 $\mu g \cdot g^{-1}$ dw for CuPT_100 (data not shown).
281 Finally, the Cu contents in the muscle of fish exposed to CuPT_1, CuPT_10 or $CuSO_4$_10 for 8 or 16 days
282 were all very low, between 1.8 and 2.7 $\mu g \cdot g^{-1}$ dw, and did not differ from their controls (figure 2.C).

283



284

285 **Figure 2: Cu concentrations in liver (A), gills (B) and muscle (C) according to the duration of the exposure to CuPT or**
286 **$CuSO_4$ ($\mu g \cdot g^{-1}$ dw) (n = 15, mean + SE). Different letters indicate a significant difference among conditions**
287 **(Kruskal-Wallis test followed by Dunn's test; p <0.05).**

3.4. Mortality

During the daily water change, dead fish were counted and removed from the tanks. In the first experiment, there was 85 % mortality for the CuPT_100 and almost 5 % mortality for the CuPT_50 condition after less than 15 h of exposure (data not shown). No mortality was observed for the $CuSO_4$_50 or $CuSO_4$_100 condition during that period. The first experiment was stopped after these observations, which allowed to set the highest concentration for the second exposure (16-day chronic exposure) to 10 µg/L. For the second experiment, the percentage of cumulative mortality for CuPT_10 condition was significantly different from the control after 8 and 16 days of exposure with 38 % and 43 %. There was ~~11~~ 20 % of mortality after ~~16~~ 8 days of exposure to CuPT_1 and this value was maintained after 16 days. There was no mortality for control, and $CuSO_4$_10 conditions after 16 days of exposure (Figure 3).

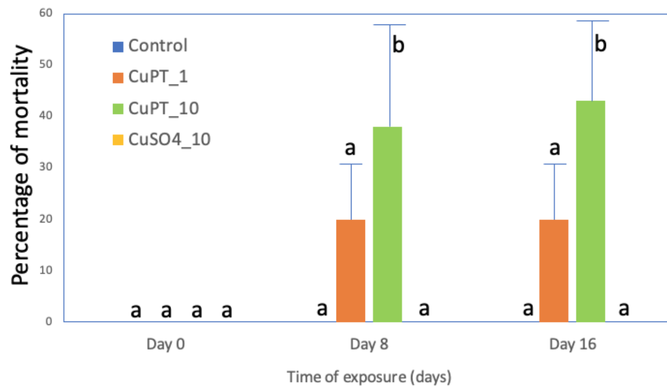


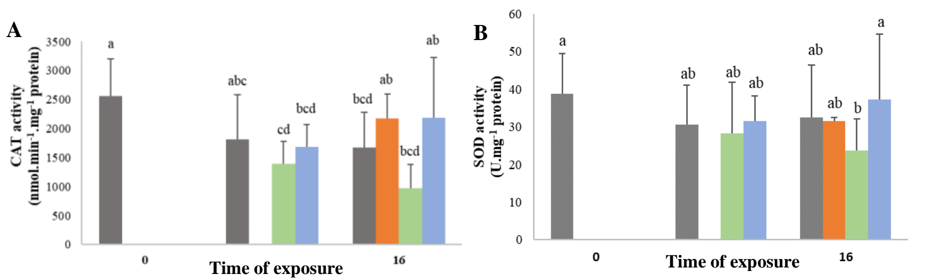
Figure 3: Percentage of mortality of juvenile depending on the time of exposure for all conditions (no mortality observed for control and $CuSO_4$_10; Dunnett's test, $p<0.05$; mean +SE; n=3 aquariums per condition).

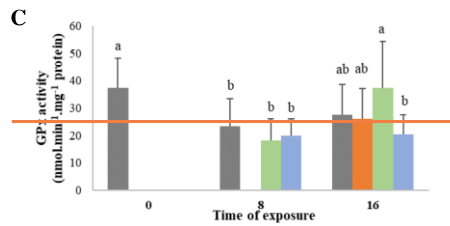
3.5. Biometric parameters

The fish sampled on Day 0 had an average length of 9.8 ± 0.9 cm and an average mass of 8.1 ± 1.6 g wet weight. After the 16 days of exposure, all fish had grown, reaching an average length of ~~between~~ ~~10.0~~10.2 ± ~~0.8~~0.2 ~~and 10.6 ± 0.7~~ cm and an average mass of ~~between 9.9~~10.4 ± ~~2.0~~0.2 ~~and 10.9 ± 1.8~~ g wet weight. No significant difference in growth was observed among the conditions after the 16 days of exposure to ~~both~~ either contaminant~~s~~.

3.6. Antioxidant capacities

At the start of exposure (Day 0), mean CAT and ~~GPx~~ SOD activities were 2562 ± 641 ~~nmol·min^{-1}·mg^{-1} proteins~~ and 39 ± 11 nmol·min^{-1}·mg^{-1} proteins~~U·mg^{-1} proteins~~, respectively. There was no significant difference in CAT and ~~GPx~~ SOD activity among exposure days and exposure conditions (Figures 4A and B). The mean ~~SOD~~ GPx activity on Day 0 was 37 ± 11 nmol·min^{-1}·mg^{-1} proteins. The ~~SOD~~ GPx activity was significantly reduced for all conditions on Day 8 and ranged from 18 ± 8 to 23 ± 10 nmol·min^{-1}·mg^{-1} proteins. The mean ~~SOD~~ GPx activity at Day 16 was not significantly different from the values at Day 0 and Day 8 (Figure 4C).





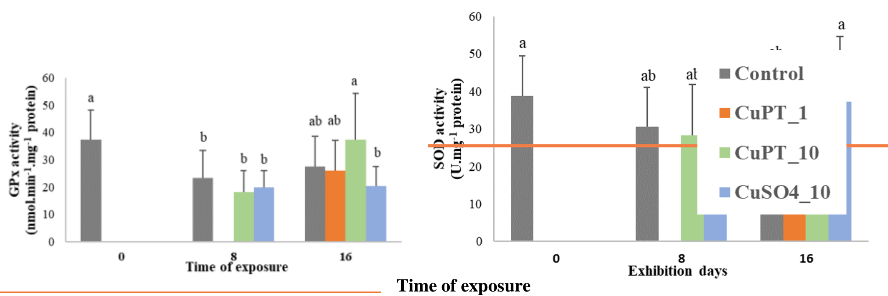


Figure 4: Enzyme activities in liver of rainbow trout in nmol·min^{-1}·mg^{-1} proteins for CAT (A), ~~and~~ SOD (B), and ~~in U·mg^{-1} proteins for~~ GPx (C) depending on the duration of exposure to CuPT or $CuSO_4$ (n = 15, mean + SE). Different letters indicate a significant difference among conditions (Kruskal-Wallis followed by Mann-Whitney U test with Bonferroni correction, p <0.05), no data for CuPT_1 at D~~d~~ay 8.

3.7. Gene expression by real-time qPCR

After 8 and 16 days of exposure to CuPT and $CuSO_4$, the expression of several genes varied significantly compared to the control (Table 2). There are no results for condition CuPT_1 at Day 8 because no fish were sampled. Genes *cat*, *gstA*, *tp53* and *tgl* showed no significant variation in their expression following exposure to the two contaminants, in either tissue (data not shown). CuPT_10 strongly repressed gene expression in gills at Day 8 for genes *sod1*, *sod2*, *gpx1*, *cyp1a*, *cox1*, 12S, *bax*, *ctr1*, *ctr2*. In contrast, several genes were overexpressed in the liver after the same exposure time in particular *gpx1*, *mt1x*, *mt2x*, *cox1*, *ctr1* and *AcoAc*. Also at Day 8, for $CuSO_4$_10 in liver, the gene expression profiles were different to those of fish exposed to CuPT_10. There was indeed overexpression of *mt1x* and *mt2x* (like CuPT_10), *ctr1*, but there was also a repression of ~~*ctr1*,~~ *slc11a2* and *AcoAc*. In the gills of fish exposed to $CuSO_4$_10, ~~gene expression tended to be more like CuPT_10, but on fewer genes. T~~there was ~~also~~ overexpression of *mt1x* and *mt2x*, and repression of *gpx1*, *cox1*, *ctr1* and *ctr2*. After 16 days of exposure, the expression levels showed widely different trends than after 8 days. We observed a repression of all genes in the liver for all three exposure conditions CuPT_1, CuPT_10 and $CuSO_4$ (except *ctr1* ~~exposed to~~during CuPT_10 exposure condition). In the gills, fewer genes responded compared to the liver. For $CuSO_4$_10, only one gene, *mt2x*, was repressed compared to the control. For CuPT_1, the *cyp1a* ~~, *cox*~~ and *AcoAc* genes were repressed, and the *mt1x*, *mt2x* and *bax* genes were overexpressed. For CuPT_10, only the *cyp1a* gene was repressed, while the *mt1x*, *mt2x* and *ctr1* genes were overexpressed. Overall, our data show a rapid response in the gills following Cu contamination, with transcription levels of genes related to oxidative stress, detoxification and Cu transport functions altered, and these responses decreased at the end of the experiment. Conversely, the response was delayed in the liver, starting with overexpression of a few genes, then repression of most of the genes at the end of the exposure.

Table 2: Expression factor of genes of interest in gills and liver of juveniles of rainbow trout exposed to CuPT or $CuSO_4$ (n=15). Only significantly different results from the control are shown (t test, p<0.05), overexpression and repression are indicated with + and – signs.

	Gills					Liver				
	Day 8		Day 16			Day 8		Day 16		
	CuPT_10	$CuSO_4$_10	CuPT_1	CuPT_10	$CuSO_4$_10	CuPT_10	$CuSO_4$_10	CuPT_1	CuPT_10	$CuSO_4$_10
sod1	-0.42	/	/	/	/	/	/	-0.64	-0.17	/
sod2	-0.49	/	/	/	/	/	/	-0.47	-0.35	-0.61

gpx	-0.17	-0.30	/	/	/
cyp1a	-0.07	/	-0.55	-0.41	/
mt1x	+3.09	+1.38	+2.32	+13.42	/
mt2x	+8.06	+1.77	+2.16	+16.64	-0.73
ctr1	-0.60	-0.68	/	+1.45	/
ctr2	-0.33	-0.77	/	/	/
AcoAc	+1.51	/	-0.59	/	/
12s	-0.18	/	/	/	/
cox	-0.25	-0.47	/	/	/
bax	-0.41	/	+574	/	/

gpx	+1.69	/	-0.50	-0.45	-0.56
cyp1a	/	/	-0.42	-0.07	/
mt1x	+4.42	+2.38	-0.69	/	-0.46
mt2x	+3.32	+2.37	-0.71	/	-0.55
ctr1	+1.63	+1.62	-0.49	+2.57	/
ctr2	/	/	-0.52	-0,23	/
slc11a2	-0.58	-0.77	-0.46	/	/
AcoAc	+2.49	-0.43	/	-0.32	/
cox	+1.51	/	/	-0.31	-0.47
12s	/	/	/	-0.21	-0.42
bax	/	/	-0.59	-0.39	-0.65

4. DISCUSSION

The objectives of this study were to (1) compare the toxicity ~~threshold~~ and the spectrum of sublethal effects after 8 and 16 days of exposure to CuPT or $CuSO_4$; (2) compare the accumulation of Cu in tissues; and (3) compare the mechanisms of toxicity of both compounds by enzymatic biomarkers of antioxidant capacity and transcriptional response of selected genes.

4.1. Tissue accumulation of Cu and toxicity

The concentrations of Cu and CuPT in water were measured during the experiment and several phenomena were observable. Considering the process of photodegradation, the CuPT concentration measured just after tank contamination (in the dark) was at the desired targeted level following this contamination for the CuPT_1 condition (nominal CuPT concentration of 5.0 $\mu g \cdot L^{-1}$). The concentration remained close to target values 12 h later. The concentration of the CuPT_10 condition was lower than the nominal concentration (50.0 $\mu g \cdot L^{-1}$) just after the contamination and even lower 12 h later. The CuPT_1 condition met expectations of nominal Cu concentration. For the CuPT_10 condition, Cu concentrations did not reach 50 % of the nominal concentration. The $CuSO_4$_10 condition had Cu values very close to the nominal concentration. Therefore, juveniles exposed to CuPT_1 and $CuSO_4$_10 were exposed to the desired nominal concentrations, while those exposed to the CuPT_10 condition instead had exposure levels equivalent to 5 μg $Cu^{2+} \cdot L^{-1}$. We hypothesize that CuPT had a strong tendency to adhere to inorganic and

organic surfaces, such as aquarium and filter components and fish gills, a phenomenon that could be exacerbated at the higher concentration, leading to lower measured values than nominal aqueous concentrations.

Although the exposure to $CuSO_4$_10 corresponded to the targeted concentration, there was no Cu accumulation in the different tissues of the juveniles. Conversely, although fish from the CuPT_10 condition were exposed to lower than expected CuPT concentration, there was an accumulation of Cu in the gills, with significantly higher levels compared to the control and the $CuSO_4$_10 conditions. This difference in Cu accumulation between the two compounds imply that Cu is more bioavailable in the form of CuPT than in its ionic Cu^{2+} form from $CuSO_4$. In their study, Borg and Trombetta (2010) showed that an exposure of juvenile brook trout (*Salvelinus fontinalis*) for 2 h at 16, 32 and 64 $\mu g \cdot L^{-1}$ of CuPT was sufficient to induce a significant accumulation of Cu in the gills. This very short exposure induced morphological modifications of the gills with the fusion of the secondary gill lamellae, induction of œdemas, loss of microridge structure and epithelial exfoliation. The authors observed the swelling of chloride cells and mitochondria and rupture of the lipid membranes. Another study reported severe damage to the gills of red sea bream (*Pagrus major*) after an exposure to CuPT (and ZnPT), including a dilation and fusion of secondary gill lamellae, necrosis and vacuolization as well as an expansion of the epithelial cells of the branchial cavity (Mochida et al., 2006).

In the liver of juveniles from our study, only the CuPT_1 condition induced a significant Cu accumulation after 16 days of exposure, while fish from the CuPT_10 and $CuSO_4$_10 conditions did not accumulate Cu in this organ. The expression of the genes involved in Cu transport (*ctr1*, *ctr2* and *slc11a2*) and detoxification (*mt1*, *mt2* and *cyp1a*) in the liver were all repressed for the CuPT_1 condition while there was less response for the CuPT_10 and $CuSO_4$_10 conditions. These differential gene expressions, if it is followed in the call by an increase in the quantity of the corresponding protein and its activity, ~~may~~ could explain the difference in Cu accumulation in the liver. Following waterborne exposure, teleost fish normally accumulate Cu in the gills (the main route of exposure) and the liver is the central internal compartment for Cu accumulation and homeostasis (Grosell *et al*., 1998). In juvenile rainbow trout, an exposure of 10 days to 20 and 100 $\mu g \cdot L^{-1}$ of $CuSO_4$ induced accumulation of Cu in the gills but not in the liver, which does not coincide with the observations of our study (Shaw *et al*., 2012). Muscle is not a target organ for Cu storage, so it is not surprising that none of the exposure conditions studied led to an accumulation. Due to the low solubility of CuPT in water, it is expected to adsorb to suspended matter and food pellets. It would have been interesting to measure the Cu concentration in the digestive tract of our juveniles, to examine a potential accumulation of Cu by gut. Indeed, the relative efficiency of Cu uptake from food appears to be

~~like~~ similar to the efficiency of Cu uptake from water ~~that~~ filtered by the gills (Clearwater et al., 2002). In addition, it might have been interesting to follow the Cu levels in the tissues during a depuration period. These observations would have made it possible to know whether CuPT is rapidly eliminated from the gills, or whether it is instead transferred to the liver or metabolized and cleared from the body.

Only the CuPT_10 condition induced mortality after 8 and 16 days of exposure, while for $CuSO_4$_10 no mortality was observed during the 16 days of the exposure. Cu toxicity has been studied in several fish species. It is acutely toxic to rainbow trout with a 96-h LC_{50} of 210 $\mu g \cdot L^{-1}$ (De Boeck *et al.*, 2004). This result could explain the absence of mortality in the second experiment with $CuSO_4$ since the exposure concentration was 10 $\mu g \cdot L^{-1}$. The difference of mortality between CuPT and $CuSO_4$ can be related to differences in the levels of Cu accumulation in the tissues. The bioavailability of Cu for the fish exposed to CuPT directly impacted their survival. This observation is strongly supported by the results of the first experiment, where in less than 24 h, the gills of the juveniles of the CuPT_100 condition had already accumulated 68 ± 15 $\mu g \cdot g^{-1}$ dw of Cu (compared to 5.53 ± 1.9 $\mu g \cdot g^{-1}$ dw for the controls) which had induced 85 % of mortality. In contrast to mortality, growth was not affected in our study under any experimental condition over the 16 days of exposure. We can assume that the growth of the juveniles did not have time to be impacted by the contaminants in 16 days compared to the control conditions. Our experimental design does not allow to calculate the lethal concentration 50 % of CuPT (LC50) after 8 or 16 days. In the literature, several studies have focused on the acute toxicity of CuPT, mainly on microalgae and crustaceans. Among these studies, growth inhibition after 72-h (72-h EC_{50}) for the microalgae *Dunaliella tertiolecta* was 7.3 $\mu g \cdot L^{-1}$, while the values for the microalgae *Tetraselmis tetrathele* was 12 $\mu g \cdot L^{-1}$, for the microalgae *Chaetoceros calcitrans* was 3.2 $\mu g \cdot L^{-1}$ and 1.5 $\mu g \cdot L^{-1}$ for the diatom *Skeletonema costatum* (Onduka et al., 2010). Crustacean mortality after 24 to 96 h of exposure to CuPT has been studied on several species. Values reported were 830 $\mu g \cdot L^{-1}$ (24-h LC_{50}) for the artemia *Artemia salina* (Koutsaftis and Aoyama, 2007) and 250 $\mu g \cdot L^{-1}$ (48-h LC_{50}) for the same species (Lavtizar et al., 2018). Data for the *Tigriopus japonicus* copepod give 24-h $LC_{50} = 41$ $\mu g \cdot L^{-1}$ (after Yamada 2006) and 96-h $LC_{50} = 30$ and 32.7 $\mu g \cdot L^{-1}$ (Bao et al., 2014, 2011). Studies on the acute toxicity of CuPT on fish give 96-h $LC_{50} = 7.67$ and 9.3 $\mu g \cdot L^{-1}$ on *Pagrus major* (from Yamada, 2006; Mochida *et al.*, 2006) and 96-h $LC_{50} = 4.3$ and 2.6 $\mu g \cdot L^{-1}$ on the fathead minnow *Pimephales promelas* (from Yamada 2006; Regulation (EU) No. 528/2012, 2014). Finally, Okamura *et al.* (2002) carried out a toxicity test on rainbow trout larvae (24 h post-hatch) with CuPT and other biocides (ZnPT, Irgarol 1051, diuron, Sea-Nine 211) for 28 days, at concentrations of 0, 1.0, 2.0, 4.0, 8.0, 16 $\mu g \cdot L^{-1}$. The order of toxicity of the compounds (based on nominal concentrations) on the trout larvae after 28 days of exposure was CuPT> ZnPT> Sea-Nine 211> KH101> diuron> Irgarol 1051. The authors evaluated 4 values of LC_{50}, at 7, 14, 21 and 28 days, which gives respectively for CuPT exposure 7.6; 3.0; 1.7 and 1.3 $\mu g \cdot L^{-1}$, *i.e.,* 1000

times more toxic than for Irgarol 1051 and diuron. On marine medaka *Oryzias melastigma* larvae, the 96-h LC_{50} was reported to be~~=~~ 8.2 µg·L^{-1} (Bao et al., 2011). All these studies show to what extent aquatic species at all stages of life are sensitive to CuPT. In our study, juvenile rainbow trout were more tolerant than the aquatic species cited above. It could be interesting to carry out the same experiment on the larval stage of rainbow trout to have a comparison of these two life stages.

4.2. Oxidative stress and molecular responses

Free Cu generates hydroxyl radicals which are the source of ROS. To fight against ROS, antioxidant molecules (ascorbic acid, glutathione) ~~can trap them, or antioxidant~~and enzymes (SOD, GPx, CAT, etc.) can eliminate them. ~~If~~ When ROS levels increase ~~too much~~beyond the capacities of these antioxidant mechanisms, ~~the defence system is no longer sufficient to neutralize them leading~~this leads to oxidative stress. The 16-days exposure to CuPT and $CuSO_4$ did not induce an increase in the activity of antioxidant enzymes, suggesting an absence of oxidative stress in the liver of these juvenile fish. Sanchez *et al*. (2005) showed a significant increase in SOD and CAT activities after 4 days of exposure to $CuSO_4$ on the three-spine stickleback, at a concentration of 25 µg·L^{-1}, then it returned to baseline after 8 days. Regarding GPx activity, only the 200 µg·L^{-1} condition induced an increase after 12 days of exposure in the same study. If, in our study, juveniles were sampled after 4 days of exposure, we may have observed a response of these antioxidant enzymes like that of Sanchez *et al*. (2005). Borg and Trombetta (2010) have shown that TBARS levels in the brook trout *Salvelinus fontinalis* gills were significantly increased following CuPT exposure conditions at 16, 32 and 64 µg·L^{-1}. In parallel, there was a significant decrease of 10 and 25 % respectively in total antioxidant capacity (TAC) for conditions 32 and 64 µg·L^{-1} (Borg and Trombetta, 2010).

Given the absence of response at the level of antioxidant enzyme activity, the levels of gene expression observed in the liver provide interesting insights. Like for the CAT enzyme activity, there was no response from the *cat* gene transcription over the entire exposure period. The *sod1* gene did not show differential expression on Day 8, whereas on Day 16 it was significantly less expressed in the conditions CuPT_1 and CuPT_10 but was not deregulated in the condition $CuSO_4$_10. The *sod2* gene was repressed for all exposure conditions on the 16th day of exposure. The *gpx1* gene was overexpressed only in the CuPT_10 condition, in contrast to the GPx enzyme which did not vary in comparison to the control and the other conditions. On day 16, the three exposure conditions induced a decrease in the expression of the *gpx 1* gene in contrast to the activity of the corresponding enzyme which did not vary. The lack of induction of enzymes involved in the response to oxidative stress could be counterbalanced by the expression of metalothioneins.~~The overexpression of *mt* genes probably contributed to the control of oxidative stress and~~

463 limited the change in enzyme levels Indeed, these proteins have been described as being involved in the
464 response to oxidative stress in a number of organisms (Ruttkay-Nedecky et al., 2013). Thus, the
465 overexpression of *mt* genes probably contributed to the control of oxidative stress and limited the change in
466 enzyme levels. The *nfe2.1* gene (nuclear factor erythroid 2 related factor 1) is a precursor of oxidative stress
467 response and would have been interesting to study in addition to the *sod1*, *sod2*, *cat*, *gst* and *gpx1* genes to
468 better understand the extent of oxidative stress that our exposure conditions induced.

469 The levels of gene expression show differential responses among tissues and conditions, and over
470 time. In the gills, gene expression was predominantly repressed at Day 8 for the CuPT_10 condition, while
471 in fish exposed to $CuSO_4$_10 fewer gene expression levels were altered. For both conditions, the antioxidant
472 response genes and Cu transport genes were repressed, while the *mt1x* and *mt2x* genes were overexpressed.
473 These early responses for CuPT_10 coincided with Cu accumulation in the gills, while molecular responses
474 still occurred despite the absence of accumulation of Cu in the gills for the $CuSO_4$ condition. Still in the
475 gills, on Day 16, the levels of gene expression returned to the baseline for the three exposure conditions,
476 except for the detoxification genes (*mt1x*, *mt2x*, and *cyp1a*) and pro-apoptotic gene (*bax*).

477 In juvenile trout, differential mechanisms between the absorption and metabolism of Cu are induced
478 between the two Cu species. Indeed, there was an increase in the levels of transcription of the *mt1x* and *mt2x*
479 genes induced by the two compounds, yet the accumulation of Cu in the gills was observed only for the
480 conditions exposed to CuPT. Metallothioneins (MTs) are cysteine-rich proteins involved in maintaining
481 sufficient intracellular supplies of certain essential metals such as Cu and Zn and detoxifying excess
482 intracellular metals. The overexpression of these genes clearly shows that Cu has induced a molecularly
483 impacted the molecular responses (Amiard *et al*., 2006). Exposure to CuPT, but not to $CuSO_4$, induced a
484 repression of the *cyp1a* gene in the gills and the liver. Cytochromes P450 are a multigene family of heme-
485 containing proteins that oxidize, hydrolyse, or reduce hydrophobic chemicals by inserting an oxygen atom
486 to the substrates during the reaction cycle to increase their water solubility. They are present mainly in the
487 liver of fish, but also in their gills and digestive tract (Varanasi 1989). In our study, detoxification seems to
488 have been managed mainly by MTs rather than by cytochromes P450.

489 Regarding the regulation of the cell cycle, *p53* gene expression was not modified. On the other hand,
490 the *bax* gene for CuPT_1 at day 16 was particularly overexpressed (+574), suggesting a peak of gill cell
491 apoptosis at the end of the experiment. We hypothesise that the gills were initially the target organ, but that
492 they were able to adapt by setting up molecular defence mechanisms against this contamination at the start
493 of exposure. However, the significant increase in the *bax* gene after 16 days seems to indicate cellular
494 damage in this organ at the end of the experiment.We hypothesize that the gills were initially the target

Mis en forme : Police :Italique

~~organ but were able to adapt and defend themselves against this contamination at the end of the exposure~~. In the liver, the molecular response was quite different. There were fewer genes with an altered level of expression following exposure to our experimental conditions. The CuPT_10 condition predominantly caused overexpression of genes for detoxification, oxidative stress, and energy metabolism. At the same time, the $CuSO_4$_10 condition instead induced the repression of the Cu transport genes and an overexpression of the detoxification genes. Despite a lack of quantifiable accumulation of Cu in the liver, it still had measurable responses for both contaminants at the molecular level. Finally, on Day 16, almost all genes were differentially expressed under all exposure conditions and were mostly repressed. Our data indicate that in the liver, there was probably a massive cytotoxicity after 16 days of exposure, and that juveniles were no longer able to defend themselves against contamination, for both contaminants studied.

5. CONCLUSIONS

This study supports the greater toxicity of CuPT compared to $CuSO_4$, for equivalent Cu concentrations, on juvenile rainbow trout. The major phenotypic response that we observed was the mortality of juveniles exposed to CuPT_10 (35 and 38 % on Days 8 and 16), CuPT_50 (5% in 15 h) and CuPT_100 (85 % in 15 h), while no mortality was observed for $CuSO_4$ exposures up to 10 $\mu g \cdot L^{-1}$. The 16-days exposure to CuPT and $CuSO_4$ did not affect the growth of the juveniles. Our study allows to suggest a concentration range for which CuPT is toxic to juvenile rainbow trout (from 1 to 100 $\mu g\ Cu^{2+} \cdot L^{-1}$) while this is not the case for $CuSO_4$. The higher toxicity of CuPT could be explained by the higher bioavailability of Cu in CuPT compared to $CuSO_4$, as supported by the strong and rapid accumulation of Cu in the gills of fish exposed to CuPT. The activities of antioxidant enzymes (CAT, SOD, GPx) were not significantly altered, making it difficult to conclude on the oxidative stress generated by our exposures. Nevertheless, gene expression analyses showed the adaptive responses of juveniles to CuPT and $CuSO_4$ in the gills, while the liver experiences cytotoxic effects at the end of the exposure. The mechanisms of action of CuPT have yet to be investigated through additional studies. Our study confirms the toxicity of CuPT in antifouling paints for juveniles of rainbow trout, a non-target species. This is particularly worrying since CuPT will naturally adsorb to suspended particles and settle to the sediment. Accumulation of CuPT in the sediment can impact species with at least one benthic life stage, which is the case for rainbow trout embryos and larvae. Further studies are clearly needed to evaluate the toxicity and the risk of CuPT on early life stages of fish.

6. AKNOWLEDGEMENTS

The authors express their gratitude to J. Perreault and J.F. Dutil for performing the quantification of Cu and in waters and in juvenile tissue, and S. Prémont and S. Moïse for the development of an analytical method to quantify CuPT in water. Also, we thank the instrumental platform of Molecular Biology for the formation to extraction and RT-qPCR (EPOC Laboratory, University of Bordeaux-Arcachon). This study was funded by a Discovery grant from the Natural Sciences and Engineering Research Council of Canada to P. Couture and by funds from the EPOC laboratory. The authors also wish to thank an anonymous reviewer who provided a critical review of an earlier version of this paper.

7. CONFLICT OF INTEREST DISCLOSURE

The authors declare they have no conflict of interest relating to the content of this article. Patrice Couture is a recommender for PCI Ecotox Env Chem.

8. REFERENCES

Almond, K.M., Trombetta, L.D., 2016. The effects of copper pyrithione, an antifouling agent, on developing zebrafish embryos. Ecotoxicology 25:389-398. https://doi.org/10.1007/s10646-015-1597-3.

Almond, K.M., Trombetta, L.D., 2017. Copper pyrithione, a booster biocide, induces abnormal muscle and notochord architecture in zebrafish embryogenesis. Ecotoxicology 26:855-867. https://doi.org/10.1007/s10646-017-1816-1.

Amiard, J., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P., 2006. Metallothioneins in Aquatic Invertebrates: Their Role in Metal Detoxification and Their Use as Biomarkers. Aquatic Toxicology 76 (2): 160-202. https://doi.org/10.1016/j.aquatox.2005.08.015.

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. https://doi.org/10.1016/j.marpolbul.2011.02.041

Bao, V.W.W., Lui, G.C.S., Leung, K.M.Y., 2014. Acute and chronic toxicities of zinc pyrithione alone and in combination with copper to the marine copepod *Tigriopus japonicus*. Aquatic Toxicology 157, 81–93. https://doi.org/10.1016/j.aquatox.2014.09.013

Biocidal Product 2014: Regulation (EU) n ° 528/2012 concerning the making available on the market and the use of biocidal products: report on CuPT (EU Regulation n ° 528/2012, 2014)

552 Borg, D.A., Trombetta, L.D., 2010. Toxicity and bioaccumulation of the booster biocide copper pyrithione,
553 copper 2-pyridinethiol-1-oxide, in gill tissues of *Salvelinus fontinalis* (brook trout). Toxicology and
554 Industrial Health 26, 139–150. https://doi.org/10.1177/0748233710362381

555 Champ, M.A., 2000. A review of organotin regulatory strategies, pending actions, related costs and benefits.
556 Science of The Total Environment 258, 21–71. https://doi.org/10.1016/S0048-9697(00)00506-4

557 Clearwater, S.J., Farag, A.M., Meyer, J.S., 2002. Bioavailability and toxicity of dietborne copper and zinc
558 to fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 132, 269–313.
559 https://doi.org/10.1016/S1532-0456(02)00078-9

560 De Boeck, G., Meeus, W., De Coen, W., Blust, R., 2004. Tissue-Specific Cu Bioaccumulation Patterns and
561 Differences in Sensitivity to Waterborne Cu in Three Freshwater Fish: Rainbow Trout (*Oncorhynchus*
562 *mykiss*), Common Carp (*Cyprinus carpio*), and Gibel Carp (*Carassius auratus* Gibelio). Aquatic Toxicology
563 70 (3): 179-88. https://doi.org/10.1016/j.aquatox.2004.07.001

564 Grosell, M.H., Hogstrand, C., Wood, C.M., 1998. Renal Cu and Na excretion and hepatic Cu metabolism
565 in both Cu acclimated and non acclimated rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 40,
566 275–291. https://doi.org/10.1016/S0166-445X(97)00026-X

567 Hiroya, H., Yamamoto, Y., Eguchi, S., Kawai, S., Kurokawa, Y., Arai, T., Ohji, M., Okamura, H.,
568 Miyazaki,. N., 2007. Concentrations of Antifouling Biocides in Sediment and Mussel Samples Collected
569 from Otsuchi Bay, Japan. Archives of Environmental Contamination and Toxicology 52 (2): 179-88.
570 https://doi.org/10.1007/s00244-006-0087-2.

571 IMO, 2001. International convention on the control of harmful antifouling systems on ships. International
572 Maritime Organisation (IMO).
573 http://www.imo.org/en/OurWork/Environment/AntifoulingSystems/Pages/Default.aspx (Accessed
574 30.02.2020)

575 J-Check, 2021. Japan CHEmicals collaborative knowledge database. Substance data: Bis(1-hydroxy-1H-
576 pyridine-2-thionato-O,1-hydroxy-1H-pyridine-2-thionato-O,S)copper.
577 ⟨http://www.safe.nite.go.jp/jcheck/detail.action?Cno=14915-37-8&mno=5-6271&request_locale=en⟩
578 (Accessed 08.07.2021).

579 Konstantinou, I.K., Albanis, T.A., 2004. Worldwide occurrence and effects of antifouling paint booster
580 biocides in the aquatic environment: a review. Environment International 30, 235–248.
581 https://doi.org/10.1016/S0160-4120(03)00176-4

582 Koutsaftis, A., Aoyama, I., 2007. Toxicity of four antifouling biocides and their mixtures on the brine shrimp
583 *Artemia salina*. Science of The Total Environment 387, 166–174.
584 https://doi.org/10.1016/j.scitotenv.2007.07.023

585 Lavtizar, V., Kimura, D., Asaoka, S., Okamura, H., 2018. The influence of seawater properties on toxicity
586 of copper pyrithione and its degradation product to brine shrimp *Artemia salina*. Ecotoxicology and
587 Environmental Safety 147, 132–138. https://doi.org/10.1016/j.ecoenv.2017.08.039

588 Le Bihanic, F., Clérandeau, C., Le Menach, K., Morin, B., Budzinski, H., Cousin, X., Cachot, J., 2014.
589 Developmental toxicity of PAH mixtures in fish early life stages. Part II: adverse effects in Japanese
590 medaka. Environmental Science and Pollution Research 21, 13732–13743. https://doi.org/10.1007/s11356-
591 014-2676-3

592 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein Measurement with the Folin Phenol
593 Reagent. J. Biol. Chem. 193, 265–275.

594 Maraldo, K., Dahllöf, I., 2004. Indirect estimation of degradation time for zinc pyrithione and copper
595 pyrithione in seawater. Marine Pollution Bulletin 48, 894–901.
596 https://doi.org/10.1016/j.marpolbul.2003.11.013

597 Mochida, K., Amano, H., Onduka, T., Kakuno, A., Fujii, K., 2011. Toxicity and metabolism of copper
598 pyrithione and its degradation product, 2,2′-dipyridyldisulfide in a marine polychaete. Chemosphere 82,
599 390–397. https://doi.org/10.1016/j.chemosphere.2010.09.074

600 Mochida, K., Ito, K., Harino, H., Kakuno, A., Fujii, K., 2006. Acute toxicity of pyrithione antifouling
601 biocides and joint toxicity with copper to red sea bream (*Pagrus major*) and toy shrimp (*Heptacarpus*
602 *futilirostris*). Environmental Toxicology and Chemistry 25, 3058. https://doi.org/10.1897/05-688R.1

603 Mohamat-Yusuff, F., Sarah-Nabila, Ab.G., Zulkifli, S.Z., Azmai, M.N.A., Ibrahim, W.N.W., Yusof, S.,
604 Ismail, A., 2018. Acute toxicity test of copper pyrithione on Javanese medaka and the behavioural stress
605 symptoms. Marine Pollution Bulletin 127, 150–153. https://doi.org/10.1016/j.marpolbul.2017.11.046

606 Okamura, H., Watanabe, T., Aoyama, I., Hasobe, M., 2002. Toxicity evaluation of new antifouling
607 compounds using suspension-cultured fish cells. Chemosphere 46, 945–951. https://doi.org/10.1016/S0045-
608 6535(01)00204-1

609 Okamura, Mieno, 2006. Present Status of Antifouling Systems in Japan: Tributyltin Substitutes in Japan,
610 in: Konstantinou, I.K. (Ed.), Antifouling Paint Biocides. Springer Berlin Heidelberg, pp. 201–212.
611 https://doi.org/10.1007/698_5_055

612 Onduka, T., Mochida, K., Harino, H., Ito, K., Kakuno, A., Fujii, K., 2010. Toxicity of Metal Pyrithione
613 Photodegradation Products to Marine Organisms with Indirect Evidence for Their Presence in Seawater.
614 Archives of Environmental Contamination and Toxicology 58, 991–997. https://doi.org/10.1007/s00244-
615 009-9430-8

616 Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., Stiborova, M., Adam,
617 V., Kizek, R., 2013. The Role of Metallothionein in Oxidative Stress. International Journal of Molecular
618 Sciences 14, 6044–6066. https://doi.org/10.3390/ijms14036044

619 Sanchez, W., Palluel, O., Meunier, L., Coquery, M., Porcher, J.-M., Aït-Aïssa, S., 2005. Copper-Induced
620 Oxidative Stress in Three-Spined Stickleback: Relationship with Hepatic Metal Levels. Environmental
621 Toxicology and Pharmacology 19 (1): 177-83. https://doi.org/10.1016/j.etap.2004.07.003.

622 Santos, S.W., Cachot, J., Gourves, P.-Y., Clérandeau, C., Morin, B., Gonzalez, P., 2019. Sub-lethal effects
623 of waterborne copper in early developmental stages of rainbow trout (*Oncorhynchus mykiss*). Ecotoxicology
624 and Environmental Safety 170, 778–788. https://doi.org/10.1016/j.ecoenv.2018.12.045

625 Shaw, B.J., Al-Bairuty, G., Handy, R.D., 2012. Effects of waterborne copper nanoparticles and copper
626 sulphate on rainbow trout, (*Oncorhynchus mykiss*): Physiology and accumulation. Aquatic Toxicology 116–
627 117, 90–101. https://doi.org/10.1016/j.aquatox.2012.02.032

628 Shin, D., Choi, Y., Soon, Z.Y., Kim, M., Kim, D.J., Jung, J.H., 2022. Comparative toxicity study of
629 waterborne two booster biocides (CuPT and ZnPT) on embryonic flounder (Paralichthys olivaceus).
630 Ecotoxicology and environmental safety 233:113337. https://doi.org/10.1016/j.ecoenv.2022.113337

631 Tessier, E., 2011. Study of the spatial and historical distribution of sediment inorganic contamination in the
632 Toulon bay (France). Marine Pollution Bulletin 12.

633 Turley, P.A., Fenn, R.J., Ritter, J.C., 2000. Pyrithiones as antifoulants: Environmental chemistry and
634 preliminary risk assessment. Biofouling 15, 175–182. https://doi.org/10.1080/08927010009386308

635 Varanasi, U., Nishimoto, M., Baird, W. M., Smolarek, T. A., 1989. Metabolic activation of PAH in
636 subcellular fractions and cell cultures from aquatic and terrestrial species. *In* Metabolism of Polycyclic
637 Aromatic Hydrocarbons in the Aquatic Environment. pp. 203–250. Edited by U. Varanasi. CRC Press, Boca
638 Raton.

639 Voulvoulis, N., 2006. Antifouling Paint Booster Biocides: Occurrence and Partitioning in Water and
640 Sediments, in: Konstantinou, I.K. (Ed.), Antifouling Paint Biocides, The Handbook of Environmental
641 Chemistry. Springer Berlin Heidelberg, pp. 155–170. https://doi.org/10.1007/698_5_053

642 Walker, C.H., 2006. Ecotoxicity testing of chemicals with particular reference to pesticides. Pest
643 Management Science 62, 571–583. https://doi.org/10.1002/ps.1218

644 Yamada, H., 2006. Toxicity and Preliminary Risk Assessment of Alternative Antifouling Biocides to
645 Aquatic Organisms. *In* Antifouling Paint Biocides, The Handbook of Environmental Chemistry. Springer
646 Berlin Heidelberg, pp. 213–226. Edited by Konstantinou, I.K.. https://doi.org/10.1007/698_5_056

647