

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

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**Reply to Reviewers**

<b>General revisions</b>	
Conflict of interest disclosure statement	A conflict of interest disclosure statement was added before references (new section 7)
<b>Anonymous Reviewer 1</b>	
Abstract  Line 34. It should be mentioned in the abstract that the CuSO <sub>4</sub> 10 condition did not induce mortality, what allows the statement that “the toxicity of Cu in the form of CuPT was much higher than that of ionic Cu from CuSO <sub>4</sub> ” (line 40).	Done, lines 34-35.
Introduction  Line 67-69. As mentioned by authors, very little research has focused on sublethal effects and responses of organisms to CuPT exposure. However, some references provide information on embryonic toxicity and development alterations when fish are exposed to this biofouling, that authors should add to their manuscript (for instance Shin et al., 2022, <i>Ecotoxicology and Environmental Safety</i> , 233,113337; Almond and Trombetta, 2017, <i>Ecotoxicology</i> , 26(7), pp. 855-867; Almond and Trombetta, 2016, <i>Ecotoxicology</i> , 25(2), pp. 389-398).	Thanks to the reviewer for pointing out these references. They are now included in the paragraph, lines 74-78.
Material and method  Lines 99-105. All this information on chemical preparation is not relevant without precision on concentrations of the different stock solutions. Were these solutions acidified?	The concentrations of the two stock solutions are now mentioned in Section 2.1. Solutions were not acidified.
Line 105-106. One would wonder if water quality parameters were measured before or	These lines were deleted from Section 2.1, which was renamed to concentrate on stock solution

after water renewal, what is explained p7, line 140. It should be mentioned earlier.	preparation. The information on water sampling was reorganized and placed in Section 2.2.
Line 112. How long did thermal and environmental acclimation last?	Six weeks in total. This is now indicated (line 120).
Line 139. “were used for all three analyses” instead of “were used or all”	Fixed.
Line 207. The mean housekeeping genes: was it a geometric or arithmetic mean?	As recommended by Livak and Schmittgen it is an arithmetic mean that has been calculated. This is now indicated in the text line 242.
Line 208. N=12 while authors indicate 15 fish per condition (line 138). This should be cleared up.	As indicated in the text 15 fish per condition were analyzed. Consequently, n=12 has been corrected into N=15 (line 244).
Results Figure 1 caption is not clear for Cu analyses (A). Does it correspond to the mean of values just after contamination and 12h after (only one value for both)?	The word “combined” was added to indicate that the two datasets (just after contamination and 12h after) were combined in Figure 1A.
Line 258. These are not the conditions that didn’t accumulate, but the fish exposed to these conditions. Text is quite confused in this section because of not-shown results in gills of the first experiment that are presented within those of the second experiment.	In response to this comment, we significantly improved the text of section 3.3.
Line 278. Authors write that there was 11% of mortality after 16 days of exposure to cuPT_1 but figure 3 seems to show around 20%.	Thanks to the reviewer for pointing this error, which is a value that was carried out from an earlier version from data that has been corrected. Indeed, the mortality for CuPT_1 was 20% +/- 11% for both days 8 and 16. The text was modified accordingly.
Lines 284-288. It is quite surprising to present biometric parameters (length and mass) with highest and lowest values of +/- SD each. Why not only means +/- SD?	Fixed.
Lines 290-301. Figures 4B and 4C clearly have been inverted when compared to the text. It seems that 4B presents SOD instead of GPx and vice versa, as written in the caption.	Good catch! The text and the figure have been fixed.

<p>Table 2 and related text are not always consistent:</p> <p>Line 308. <i>mt1x</i>, <i>mt2x</i> and <i>AcoAc</i> were overexpressed in gills at day 8 of CuPT_10 exposure.</p> <p>Line 311. <i>ctr1</i> was not repressed but overexpressed.</p> <p>Line 317. <i>cox</i> was not repressed in gills at day 16 when exposed to CuPT_1.</p>	<p>All these small mistakes have been corrected in the new version (Section 3.7) to be in agreement with results shown in Table 2.</p>
<p>Discussion</p> <p>Same comments as for introduction could be made concerning information on CuPT toxicity in fish embryos provided by scientific literature.</p>	<p>This point is now covered in the introduction at the suggestion of the reviewer.</p>
<p>Line 367. Authors consider that differential gene expressions may explain the difference in Cu accumulation in the liver. They should moderate this statement considering that gene expression modulations are not always coupled to modifications of functionally relevant molecules level or activity. Same remark applies for line 466.</p>	<p>We moderated the statements in the new version.</p>
<p>Line 431 to 439. Expression results of genes encoding antioxidant enzymes are presented again, but not really discussed. Authors should deepen this section.</p>	<p>This section has been deepened as requested (lines 534-538).</p>
<p><b>Anonymous Reviewer 2</b></p>	
<p>How were the concentrations chosen?</p>	<p>Concentrations from the second experiment were based on the results of the first experiment, this information is now added lines 88-89.</p> <p>Concentrations for the first exposure were inspired by our literature review.</p>
<p>49: suggestion: “causes has ... consequences”</p>	<p>We did not retain this suggestion as the sentence would not be correct English.</p>
<p>54: More information on tributyltin – in which way is it toxic (just in a few words)</p>	<p>There is a vast literature on TBT toxicity, even on Wikipedia. We do not find it relevant to review it here, as it would involve selecting some examples of modes of action (including endocrine disruption and physiological effects). It cannot be done in a few words without doing some kind of brief review.</p>

<p>57: Why/how is PT making copper more bioavailable? Can you mention briefly the modes of action of copper and pyrithione in the introduction? Why is addition of pyrithione important? Is CuPT also used in the paint? Is PT the co-biocide ? This might be evident for readers that know about that compound but otherwise some precisions could be useful.</p>	<p>We added this information lines 59-62.</p>
<p>99: please provide the Chemical Abstracts Service (CAS) numbers for the chemicals and where you purchased them</p>	<p>CAS numbers were added and the source of chemicals is now mentioned in section 2.1.</p>
<p>102: Is the exposure water tap water or distilled water?</p>	<p>This information is already provided lines 121-122.</p>
<p>106: if the compound is supposed to be rapidly degraded by light, is renewing <math>\frac{3}{4}</math> of the exposure medium enough?</p>	<p>The protocol is now clearer about this point. We explain lines 130-132 that the compound was added at the beginning of the dark period immediately followed by water sampling, and that another water sample was collected the following morning to monitor photodegradation.</p>
<p>106: what are the outcomes of the water quality parameters? Were they stable and in an acceptable range?  Are there known degradation products, can they be measured, and do you expect toxicity from those products?</p>	<p>This section was moved to section 2.2. Regarding general water quality parameters (temperature, nitrite, nitrate, ammonium, pH), they were monitored only to ensure that they were stable and within an acceptable range, but are not reported, except for temperature (11°C).  At the suggestion of the reviewer, we added a statement, lines 65-67, about the degradation of CuPT and the toxicity of the end-product, PSA.</p>
<p>120: environmental parameters: could you be more specific</p>	<p>This statement was deleted as it is no longer relevant, given the additional information added in section 2.2.</p>
<p>135: briefly mention why you chose to analyse the liver and gills for gene expression</p>	<p>This point is now added in the revised version (lines 163-165).</p>
<p>142: do you provide the results or an estimation for photodegradation?</p>	<p>Yes this information is presented in Figure 1B and in the related text.</p>
<p>160: why atrazine?</p>	<p>Because this the appropriate standard to use for validation of LC-MSMS data (there is no standard PT).</p>
<p>183: 500 ml or rather microliters?</p>	<p>Changed (line 219). Good catch!</p>

200: containing?	Changed (line 227).
207: against the mean of the housekeeping genes actb and rpl7?	We agree that the term “against” is not appropriate. This sentence has been modified to be clearer (line 242).
207: how were the housekeeping genes selected? Did you verify that there was no effect of the treatments on their expression?	Housekeeping genes selected are classical ones for qPCR. We checked that their level of expression was stable over time, regardless of the treatments used.
207: you get only one Ct value per gene and sample. But you do not get changes in Ct values, right? Or what do you mean by changes? or do you mean differences in Ct values?	We get a single Ct value per gene and sample. A delta Ct is then obtained by normalizing to the average Ct of the reference genes for each sample. To be clearer this sentence has been modified (lines 242-243).
208: n=12 but you had 15 individuals (line 181)?	As indicated in the text, 15 fish per condition were analyzed. Consequently n=12 has been corrected into n=15 (line 244).
211: include this information in the next paragraph about statistical analysis.	Done.
Did you test the efficiency of the primers?	Efficiency of the primers was determined. Each time, it was above 98%, validating their use.
216: did you try transformation of the data? Did you apply a correction for multiple testing?	Yes, but that generally did not allow using parametric tests. The tests are described in each figure, including tests that are designed to consider multiple testing, when relevant.
221: Afterwards you mention 50 percent of the values between ... and ... . Could you write this information for the controls (instead of “generally”)?	To fix this confusion, we just deleted the word “generally”.
229: with a yield of 50 % with 50 % of the yield?	To fix this confusion, we just deleted the words “a yield of”.
235: Why are Cu concentrations after contamination and 12h after pooled in the same boxplot and not shown separately as for CuPT?	Because Cu does not break down, unlike CuPT. The purpose of Figure 1B was to illustrate the rapid degradation of CuPT.
242: mention which compounds. Or do you mean concentration? I only see CuPT in Fig1B	Yes indeed we meant concentration. Fixed.

288: did you calculate the condition factor (although I do not expect to see an effect either if there was no effect on size and weight)?	No since as the reviewer indicated, if neither weight nor length changed, then condition factor would not vary either.
298: it seems B and C are switched in the legend compared to the graphs? I see a significant reduction in the activity at day 8 in the fig 4B (GPx) but in the text you mention SOD.	This error was also reported by Reviewer 1 and is now fixed.
312: “tended to be more like”, but on a fewer genes... please reformulate, not very clear/scientific.	This sentence has been modified to be clearer (lines 383-385).
316: ctrl exposed to CuPT_10?	Replaced by “ <i>ctrl</i> during CuPT_10 exposure condition” (lines 387-388).
330: is it really a threshold if you test only two or three concentrations? – you cannot calculate a LC50 or EC50 as mentioned at line 393	In the two experiments combined, we tested 4 concentrations, but the reviewer is right that the approach did not allow comparing the thresholds for the two compounds. We fixed this concern by simply deleting the word “threshold” (line 408).
377: like that filtered by the gills: reformulate 411: reformulate sentence with marine medaka	We reformulated the sentence, which now reads “Indeed, the relative efficiency of Cu uptake from food appears to be similar to the efficiency of Cu uptake from water filtered by the gills” (line 457). We reformulated the sentence (lines 501-502).
418: trap, eliminate, increase too much: maybe reformulate in a more scientific language	These two sentences were rewritten (lines 507-508).
459: molecularly, molecular reponse: repetition. Maybe: ...that Cu has induced a molecular response	We took the suggestion for modification (line 561).
468-469, 477: the gills were able to adapt and defend themselves, ... “quite” different: reformulate in more scientific language	This paragraph has been modified as requested (lines 570-573).