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1 **Stannous chloride as a tool for mercury stripping in contaminated streams:**

2 **Experimental assessment of toxicity in an invertebrate model species**

3

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21 **Abstract**

22 The chronic toxicity of an innovative Hg water treatment system using tin (Sn) (II) chloride (SnCl₂)
23 followed by air stripping was assessed through measurements of survival, growth, and reproduction rate
24 in the freshwater cladoceran *Ceriodaphnia dubia*, a model species for toxicity testing. We first
25 calculated the concentrations of Hg causing 25% reduction in survival and reproduction (Lethal or
26 Inhibition Concentrations, or LC₂₅ and IC₂₅, for survival and reproduction, respectively) through
27 exposure to aqueous Hg at concentrations ranging from 0 to 25000 ng L⁻¹. Then, we treated media
28 (DMW and natural stream water) contaminated with Hg at LC₂₅ and IC₂₅ concentrations with SnCl₂ at a
29 Sn:Hg stoichiometric ratio of 8:1 and air stripping and exposed *C. dubia* to this Sn-amended media. Our
30 results showed that Hg significantly affected survival, reproduction rates and impaired growth. SnCl₂-
31 treatment removed 100% of the Hg from the media at all concentrations tested with no deleterious
32 effects on survival, growth and reproduction. Our results confirmed the efficacy of SnCl₂ in removing
33 aqueous Hg from stream water and showed that the added Sn did not impact *C. dubia* at the
34 concentrations tested, supporting the suitability of SnCl₂-based treatments in appropriate Hg-
35 contaminated environments.

36 **Keywords:** *Ceriodaphnia dubia*, Chronic toxicity, Hg, SnCl₂, Tin

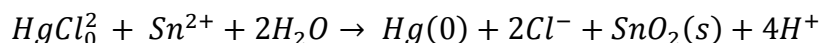
37 **1. Introduction**

38 Mercury (Hg) is extensively used in many industrial applications worldwide because of its unique
39 properties including density, volatility, redox chemistry, etc. This widespread use has led to releases into
40 the environment. In the United States alone, there are currently >800 Hg-contaminated sites on the
41 National Priorities List for remediation established by the US Environmental Protection Agency (EPA,
42 2020). Annual anthropogenic Hg emissions were estimated to about 2220-2390 tonnes in 2015 (UNEP,
43 2018; Streets et al., 2019) and this element is now listed among the “ten leading chemicals of concern”
44 (WHO, 2017) because of its toxic effects on human health (Beckers and Rinklebe, 2017) and ecological
45 effects (Boening, 2000). Mercury can be found in many forms in the environment. For example, Hg is
46 emitted into the atmosphere in its elemental form (Hg^0) which is readily transported and dispersed in the
47 environment. Elemental Hg can be oxidized in the environment to inorganic divalent mercury Hg (II), or
48 converted to methylmercury (MeHg) by anaerobic microorganisms (Parks et al., 2013). Mercury can
49 also complex with dissolved organic matter. Both the form and concentration of Hg affect its mobility,
50 bioavailability, and toxicity. At sites with large inorganic Hg releases (e.g. mining, industrial sites),
51 concentrations can be elevated enough to be acutely toxic.

52 Like other elements, inorganic mercury cannot be degraded in ecosystems, leading to its persistence in
53 the environment (Wang et al., 2020). Mercury remediation at contaminated sites is challenging in part
54 because of the transformations that mercury can undergo in the environment which may make it more or
55 less amenable to treatment or removal. Among various methods aiming to remove Hg(II) from water,
56 adsorption is the most commonly utilized approach (Abbas et al., 2018; Bao et al., 2017; Kyzas and
57 Kostoglou, 2015; Rocha et al., 2016). The sorbents usually possess high surface area as well as high
58 porosity and the formation of chelates is the major sorption mechanism (Kyzas and Kostoglou, 2015;
59 Rocha et al., 2016). New materials have been developed to capture environmental mercury including

60 nanomaterials, polymers, hydrotalcites, graphene, and covalent and metal organic frameworks (Muller et
61 al., 2019; Wang et al., 2020). Sustainable options for large scale environmental remediation must
62 consider the removal of mercury without the addition of other materials which adversely affect the
63 ecosystem.

64 A pilot study has reported the efficacy of Sn(II) chloride (i.e. stannous chloride; SnCl₂) followed by air
65 stripping (Looney et al., 2003) to remove Hg from groundwater entering a stream system in South
66 Carolina, USA (Mathews et al., 2015). This innovative treatment system was based on common
67 analytical methods for Hg (Southworth, 1996) where SnCl₂ is used as a reducing agent to reduce Hg²⁺ to
68 Hg(0), which is sparged into the gaseous phase by bubbling with air (Jackson et al., 2013), where it can
69 be removed with a sand trap (Looney et al., 2003), as follows:



70 While this treatment system was effective at decreasing aqueous and fish tissue Hg concentrations by
71 over 70%, it introduced tin into the receiving stream, leading to an increase in tin concentrations in the
72 water and resident fish(Mathews et al., 2015). Recent investigations have also shown SnCl₂ to be
73 efficient at reducing hexavalent chromium [Cr(VI)] to less toxic trivalent chromium Cr(III) in
74 groundwater (Henrie et al., 2019; Nguyen et al., 2020). While SnCl₂ appears to be promising in reducing
75 concentrations of toxic heavy metals, the longer-term impacts of adding tin to aquatic ecosystems have
76 not been evaluated.

77 The aim of the present study was to examine the effects of Sn and SnCl₂-treated Hg-containing media on
78 the aquatic invertebrate model species *Ceriodaphnia dubia*. We exposed *C. dubia* to six aqueous Hg
79 treatments, ranging from 0-25000 ng L⁻¹ in a 7-d chronic toxicity test to assess effects on survival,
80 growth, and reproduction. We then treated Hg-contaminated media with SnCl₂ and air stripping and

81 exposed *C. dubia* to this Sn-amended media at various concentrations to examine whether: 1) SnCl₂
82 reduced Hg concentrations in water, 2) SnCl₂ treatment removed Hg toxicity and 3) whether the added
83 SnCl₂ had toxic effects on *C. dubia*. Further, we compared the effect of Hg in artificial freshwater
84 medium and natural creek water to assess the relevance of our lab exposures. Our results are relevant to
85 the broader evaluation of the environmental impacts of chemical remediation of Hg in aquatic
86 ecosystems.

87

88 **2. Materials and Methods**

89 2.1. *Ceriodaphnia dubia* culture

90 *Ceriodaphnia dubia* is a parthenogenetically reproducing freshwater microcrustacean (<1 mm length)
91 that is commonly used in toxicity testing because of its ease of culture, short life span, sensitivity to
92 water quality, and prolific reproductive cycle. *C. dubia* mass cultures were maintained in 25% Dilute
93 Mineral Water (DMW) medium (EPA, 2002) prepared by mixing Perrier[®] + Milli-Q[®] water at a ratio of
94 1:3 v/v and adding trace elements at concentrations for WC media (Guillard, 1975) without
95 ethylenediaminetetraacetic acid (EDTA). Mass cultures were kept in 1-L glass containers in an incubator
96 set at: 25 °C with a light intensity of 10-20 μmol m⁻² s⁻¹ under a 16:8 h light:dark cycle (EPA, 2002). *C.*
97 *dubia* food consisted of a combination of commercial yeast purchased from Aquatic BioSystems (Fort
98 Collins, CO, USA) and laboratory-cultured unicellular green algae *Pseudokirchneriella subcapitata* (ex
99 *Raphidocelis subcapitata*; 3-4 μm in diameter). Algae were grown in 10-L clear polycarbonate Nalgene
100 carboys containing aerated WC medium (Guillard, 1975) without EDTA at 23-24 °C; light intensity:
101 150-200 μmol m⁻² s⁻¹; light/dark: 12h/12h). Algae were harvested during their exponential growth phase
102 for feeding to *C. dubia* (i.e. after 14 ± 4 d at 11 ± 4 x 10⁶ cells mL⁻¹). Algal cell counts were done by

103 imaging flow-cytometry (FlowCam® Benchtop B3 Model). Prior to feeding, live *P. subcapitata* cells
104 were concentrated by centrifugation (5000 RPM for 5 min) and resuspended in Milli-Q® water. The use
105 of concentrated algae allowed a reduction of feeding volume (i.e. 7 $\mu\text{L mL}^{-1}$) and avoided significant
106 changes of water volume to preserve physical and chemical parameters of each tested media.
107 Throughout the experiments, special care was taken to avoid biological contamination of algal cultures;
108 all glassware used for experiments was put through a rigorous acid washing protocol and rinsed three
109 times with deionized water before drying. Photos generated by the cytometer during the cell counts
110 confirmed the absence of biological contamination.

111

112 2.2. Experimental media

113 DMW prepared as described Section 2.1 was maintained in a 20-L Nalgene carboy with an air bubbler
114 for one week to de-gas the Perrier and aerate the solution. Two mercury stocks solutions (0.0001 M and
115 0.001 M) and a tin stock solution (0.0001 M) were prepared in 1-L glass containers by dissolving
116 appropriate weights of HgCl_2 (LabChem®) or SnCl_2 dihydrate (J.T.Baker®) in ultrapure water. All
117 stock solutions were kept closed under a hood at room temperature.

118 In Experiment 1, *C. dubia* were exposed to five nominal Hg concentrations: 500, 5000, 10000, 17500
119 and 25000 ng L^{-1} and a control treatment with DMW (no Hg added). These concentrations were chosen
120 based on preliminary experiments designed to target ranges at which Hg was expected to reduce *C.*
121 *dubia* survival and reproduction by 25% to test the effectiveness of SnCl_2 as a remediator. Each
122 treatment medium was prepared daily during the experiment by spiking 300-mL DMW with appropriate
123 volumes of Hg stock solutions (i.e. 0-187 μL). In Experiment 2, *C. dubia* were exposed to nine different
124 treatments. The first two treatments tested the toxicity of Hg concentrations at the LC_{25} and IC_{25}

125 concentrations for Hg for survival and reproduction determined in Experiment 1 (i.e., 2110 and 890 ng
126 L⁻¹ respectively). Two treatments contained the same LC₂₅ and IC₂₅ Hg concentrations spiked with SnCl₂
127 at a Sn:Hg stoichiometric ratio of ~8:1 (i.e., values in the range of what has been tested in previous
128 investigations: Jackson et al., 2013; Looney et al., 2003) and bubbled for 16h (i.e. 4440 and 10550 ng Sn
129 L⁻¹, so-called [Sn1] and [Sn2] respectively). Toxicity of Sn alone was assessed in two treatments
130 prepared with the same Sn concentrations used for Hg stripping. A control treatment with DMW (no Hg
131 or Sn amendments) was used. In addition, in order to assess environmental relevance of the results from
132 artificial media (i.e. DMW based), two treatments were prepared using water from First Creek (FC), an
133 uncontaminated stream on the Oak Ridge Reservation in Oak Ridge, TN (see Table 1 for water quality).
134 First Creek water was filtered (75-µm) prior to use in order to avoid introduction of *C. dubia* predators.
135 The first of these natural water-based media was kept Hg-free while the second was spiked with Hg at a
136 nominal concentration of 5000 ng L⁻¹, a concentration which was shown in Experiment 1 to affect both
137 survival and reproduction in *C. dubia*.

138 Composition of all the media used during the experiments is provided in Table 1. Mercury
139 concentrations were measured every day in subsamples of each media. All media were kept in an
140 incubator set at 25 °C for one-hour prior to using. Water quality (i.e., pH, conductivity and O₂
141 concentration) of DMW media (prior to Hg or Sn additions, to avoid any contamination of the
142 equipment used) were monitored daily using YSI® MultiLab 4010-3W. Additional measurements
143 through titration were done for alkalinity and hardness and concentrations of NH₄⁺, NO₂⁻ and NO₃⁻ were
144 measured 3-4 times during the experiments (HACH® SL 1000).

145 2.3. Chronic test procedure

146 Prior the test, 120 adult *C. dubia* were isolated in 15 mL plastic cups and an individual monitoring of the
147 production of neonates was carried out (in “brood boards” as described in EPA, 2002). To start the test,

148 <24h old *C. dubia* neonates born within the same 8-h period were individually distributed to test vials
149 (i.e., 20 mL borosilicate Ichem vials covered by a transparent plexiglass sheet; n=10 *C. dubia* neonates
150 per treatment except as described below for growth measurements in Experiment 1) filled with 15 mL of
151 media (DMW with and without Hg and/or Sn amendments) and food (3×10^6 cells *P. subcapitata* and
152 100 μ L of yeast). Test vials were assigned to a position within a test board using a block randomization
153 procedure to keep treatments blind to the observer and avoid bias in data collection. We used an
154 additional five *C. dubia* individuals for growth measurements in Experiment 1 to determine whether the
155 additional handling would induce stress and affect survival and reproduction. Since no effect of the
156 growth measurement manipulation was observed on reproduction or survival, data for all individuals
157 were used for LC₂₅ and IC₂₅ determination (see Section 2.5). Sample media was renewed and *C. dubia*
158 were fed daily by preparing media and vials as described above and moving original *C. dubia* from old
159 vial to new vial with a glass Pasteur pipette. During media renewal, *C. dubia* were observed and data for
160 survival and reproduction (i.e. number of neonates produced) were recorded prior to transfer to the new
161 vials. Subsamples of media from each treatment were taken before and after the 24-h exposure for water
162 chemistry and contaminant analysis (see section 2.4). In Experiment 1, growth of the *C. dubia* was
163 assessed at day 0 (n = 30), 2, 4 and 7 (n= 3-15 per treatment) by measuring total length from the base of
164 the spine to the top of the eye according to Agatz et al. (2015). Measurements were performed using a
165 stereomicroscope (Leica® M80) calibrated using a 200 μ m stage micrometer under a 5x magnification.
166 Each individual was carefully transferred from its original vial onto a Petri dish lid with a glass Pasteur
167 pipette. The drop of liquid surrounding the sampled individual was small enough to prevent movement
168 of the body while avoiding contact with air. At the end of each measurement (<30 s), the individual was
169 returned to its original vial. The same procedure was repeated in Experiment 2 at day 0 (n = 30) and day
170 7 (n = 4-10 per treatment).

171 At the end of each experiment, all the surviving *C. dubia* were rinsed for 5 min in fresh DMW and
172 stored at -18 °C for further Hg analysis (see Section 2.4).

173

174 2.4. Mercury and Sn analysis

175 Water samples were taken before and after exposure for Hg analysis. Before each exposure, subsamples
176 of each media were prepared in 40-mL glass Ichem vials and preserved with BrCl (5 $\mu\text{L mL}^{-1}$) for Hg
177 analysis. After each of the 24-h exposure periods, 4 mL from each of the 10 vials per media was
178 sampled and pooled in a 40-mL glass Ichem vial as a composite sample and preserved with BrCl for Hg
179 analysis. Mercury was measured by cold vapor atomic absorption spectroscopy (CV-AAS) Zeeman
180 effect Hg analyzer (RA-915+, Ohio Lumex Company, Inc., Twinsburg, OH, USA). The instrument was
181 calibrated using $\text{Hg}(\text{NO}_3)_2$ standards with a detection limit of 10 ng L^{-1} . Duplicates were run every 2
182 samples and procedural blanks were analyzed for quality control. Blanks were typically below detection
183 limits, and duplicates were on average <5% different from their parent sample.

184 Aliquots were taken from all Sn-containing treatments and DMW control for Sn analysis at three time
185 periods during the experiment (i.e., at days 2, 4 and 6). Subsamples of 40 mL of each media were
186 prepared in 40-mL glass Ichem vials and preserved with trace metal grade nitric acid. Samples were
187 shipped to Activation Laboratories Ltd (Ontario, Canada) for analysis. Sn concentrations were assessed
188 by inductively coupled plasma mass spectrometry (ICP-MS). Standards and procedural blanks were
189 analyzed for quality control.

190 Prior to Hg analysis, *C. dubia* pooled samples (n = 1 pool of 9 to 15 individuals per treatment) exposed
191 to 0, 500 and 5000 ng Hg L^{-1} for 7 d were freeze-dried for 24 h and weighed using a microbalance. Prior
192 to measurements, the microbalance was checked with reference weights from 1 mg to 10 g prior using

193 and variations were always < 0.1 mg. The pooled samples were then digested in 100 μ L acid (70
194 HNO₃:30 H₂SO₄ v/v) and analyzed by cold vapor atomic absorption as described for water samples.
195 Procedural blanks and standard reference materials (IAEA 407 and IAEA 436) were analyzed for quality
196 control and demonstrated Hg recovery from standards >90% while blanks were below detection limits.

197

198 2.5. Data analysis

199 In Experiment 1, Hg LC₂₅ and IC₂₅ values were calculated for survival and reproduction using standard
200 methods as used in Brausch et al. 2010, Besser et al. 2009, Wang et al. 2020 and employed by the
201 spreadsheet provided by the EPA (EPA, 2019). The LC₂₅ value from linear interpolation was used to
202 determine Hg concentrations for Experiment 2, and this concentration decreased survival by 30% in
203 Experiment 2, demonstrating that this LC₂₅ value was a decent approximation. The IC₂₅ calculation for
204 reproduction was based on the total number of neonates produced for each experimental treatment
205 divided by the total number of individuals started.

206 The comparison of survival curves was based on the non-parametric log-rank test, the most widely used
207 method of comparing two or more survival curves, using the R package *survival()*.

208 Differences in reproduction rate (number of neonates surviving female⁻¹) between treatments were
209 assessed using an ANOVA followed by Tukey's post-hoc test. The reproduction rate was calculated by
210 averaging the number of neonates per female who survived over the entire test. Note that we calculated
211 IC₂₅ values for reproduction using the number of offspring reproduced per number of *initial* females, a
212 metric which combines the contaminant's effect on mortality and reproduction, but is the standard
213 metric preferred by the EPA for reproduction (EPA 2002, section 13.13.3.1). In order to more accurately

214 tease out effects of the treatments on *C. dubia* reproduction, the number of neonates per surviving
215 female was used as this metric does not combine the effects of reproduction and mortality.

216 In Experiment 1, the growth kinetics of the *C. dubia* between the different Hg treatments over time were
217 assessed using a repeated-measures ANOVA designed as a linear-mixed-effect model. “Treatment” and
218 “Time” were used as fixed factors and “Individual” was used as a random factor to consider the
219 dependency of data from the same individuals. In Experiment 2, differences in final growth of surviving
220 *C. dubia* from each media was compared by ANOVA followed by Tukey’s post-hoc test as described
221 above.

222 Assumptions of normality and heteroscedasticity were examined on plotted residuals of the models. The
223 level of significance for statistical analyses was set to $\alpha = 0.05$. All statistics were performed using R
224 version 4.0.1 (R Development Core Team, 2021) unless stated otherwise.

225

226 **3. Results**

227 3.1. Effects of Hg on survival, reproduction, and growth

228 In Experiment 1, *C. dubia* neonates were exposed to a range of nominal Hg concentrations in a 7-d
229 chronic toxicity test. Measured Hg concentrations in media were within 0-7% of nominal values (Table
230 1). Mercury body burdens in *C. dubia* were 2 ng mg^{-1} (dry weight) when exposed to 500 ng Hg L^{-1} and
231 10 ng mg^{-1} (dry weight) when exposed to $5000 \text{ ng Hg L}^{-1}$. The bioconcentration factors (BCF, i.e. the
232 ratio between Hg concentrations in *C. dubia* and in the exposure media) were 2.0×10^3 and $4.8 \times 10^3 \text{ L}$
233 kg^{-1} (dry weight) in individuals exposed to 5000 and 500 ng Hg L^{-1} , respectively (Table 2). We found
234 that Hg significantly affected all the biological endpoints considered in this study (i.e. survival,
235 reproduction and growth).

236 Survival rate was significantly affected by Hg concentrations (Figure 1A). While a 100% survival rate
237 was observed in the control and the lowest Hg concentration treatment (i.e. 0 and 500 ng Hg L⁻¹
238 respectively) after 7 d, survival rate was only 60% when *C. dubia* were exposed to 5000 ng Hg L⁻¹. At
239 ≥ 10000 ng Hg L⁻¹, no surviving *C. dubia* remained at the end of the experiment, with the last surviving
240 individuals observed at day 5, 2 and 0 at Hg concentrations of 10000, 17500 and 25000 ng L⁻¹
241 respectively (0% survival rate; Figure 1A, Table 2).

242 Mercury also significantly affected reproduction in *C. dubia* expressed as the number of neonates
243 surviving female⁻¹ d⁻¹ ($F_{(2,36)} = 22.61$, $p < 0.0001$; Figure 1B). The highest reproduction rate was
244 observed in the control treatment with 49 ± 13 neonates surviving female⁻¹, a value that was not
245 significantly different ($p = 0.781$) from the reproduction rate observed in *C. dubia* exposed to 500 ng Hg
246 L⁻¹ (i.e. 46 ± 10 neonates surviving female⁻¹). The reproduction rate in the 5000 ng Hg L⁻¹ treatment was
247 reduced by 60% with respect to the control ($p < 0.001$) with only 19 ± 10 neonates surviving female⁻¹.
248 No reproduction was observed at concentrations ≥ 10000 ng Hg L⁻¹ (Figure 1B). The total number of
249 neonates produced per surviving female throughout the Experiment 1 is provided in Table 2.

250 Hg exposure led to a reduction in *C. dubia* growth throughout the 7-d experiment. However, because
251 100% mortality was observed at concentrations ≥ 10000 ng Hg L⁻¹, growth could only be followed in
252 three experimental treatments (i.e. 0, 500 and 5000 ng L⁻¹). The growth differed among the experimental
253 treatments ($\chi^2_{(2)} = 123.80$, $p < 0.001$, Figure 2). The maximal growth was observed in the control
254 treatment and at 500 ng Hg L⁻¹ (i.e. final length of 1.12 ± 0.04 mm for the two treatments) and was
255 reduced at 5000 ng Hg L⁻¹ (0.94 ± 0.05 mm; Figure 2).

256

257 3.2. Toxicity of Sn-based Hg treatment in *Ceriodaphnia dubia*

258 In Experiment 2, we evaluated the effects of SnCl₂ treatment on Hg bioaccumulation and toxicity in *C.*
259 *dubia*, as well as the potential toxic effects of the added Sn on *C. dubia*. Our results confirmed the
260 efficiency of Hg removal from aqueous samples by SnCl₂ and air stripping, with 100% of the Hg
261 removed at both Hg concentrations examined (i.e., 890 and 2110 ng L⁻¹; Table 1). These results were
262 reflected in the biological endpoints observed in *C. dubia*.

263 When exposed to a nominal concentration of 2110 ng Hg L⁻¹ (measured values of 2020 ± 110 ng Hg L⁻¹)
264 in Experiment 2, *C. dubia* survival was reduced by 30% compared to the control treatment, supporting
265 the LC₂₅ value calculated in Experiment 1. In media with the same nominal Hg concentration treated
266 with SnCl₂ at a Sn:Hg stoichiometric ratio of ~8:1 ([Sn1], Table 2), no mortality was observed (Figure
267 3A, Table 2). The same observation was made in *C. dubia* exposed to Sn alone at the same concentration
268 ([Sn1]; Table 2). In the [Sn2] treatment, one adult was found dead, tangled in its molt, on the last day of
269 the experiment.

270 In Experiment 2, when the *C. dubia* were exposed to the calculated IC₂₅ for reproduction from
271 Experiment 1 (i.e., 890 ng L⁻¹; measured values of 850 ± 70 ng Hg L⁻¹), a 25% decrease in reproduction
272 rate was observed compared to the control treatment (26 ± 8 vs. 35 ± 4 neonates surviving female⁻¹),
273 supporting the IC₂₅ values for reproduction estimated in Experiment 1. *C. dubia* exposed to the same Hg
274 concentrations treated with SnCl₂ had a reproduction rate similar to the control treatment ($p = 0.999$)
275 with 36 ± 3 neonates surviving female⁻¹ d⁻¹. Exposure to SnCl₂ alone did not affect the reproduction rate
276 in either of the tested Sn concentrations ($p \geq 0.984$, Figure 3B). The total number of neonates produced
277 per surviving female throughout the Experiment 1 is provided in Table 2.

278 *C. dubia* growth was not affected by SnCl₂ ($p \geq 0.965$), with similar total length measurements observed
279 between the SnCl₂-treated Hg, SnCl₂ and control treatments at the end of Experiment 2 (i.e. 1.03 ± 0.03
280 and 1.05 ± 0.01 mm) for all the Sn-containing media and the DMW control, respectively).

281

282 3.3. Hg toxicity: comparison between artificial medium and stream water

283 While standard toxicity tests often use artificial growth media, Hg speciation and therefore toxicity may
284 be significantly different in natural waters (Miller et al., 2009). Survival of *C. dubia* exposed to 5000 ng
285 L⁻¹ Hg in FC water and DMW decreased 50 and 60%, respectively, compared to the control media (i.e.,
286 FC water and Hg-free DMW; Figure 1A and Figure 3A). We observed similar results on *C. dubia*
287 growth, with decreases of 13% and 16% when exposed to the same concentrations of Hg in FC water
288 and DMW, respectively, compared to the respective control treatments (Figure 4). Effects of Hg on
289 reproduction rate were greater in DMW than in FC with decreases of 61% for DMW vs. 50% in FC
290 relative to the controls (Figure 1B and Figure 3B).

291

292 4. Discussion

293 4.1. Toxicity of Hg on *Ceriodaphnia dubia* in artificial media and stream water

294 We observed that Hg exposure affected the fitness of *C. dubia* with chronic effects found on survival,
295 production of neonates and growth. Survival was significantly affected by Hg: survival decreased in *C.*
296 *dubia* exposed at 5000 ng Hg L⁻¹ with no survival observed at the end of the chronic test at
297 concentrations ≥ 10000 ng Hg L⁻¹. Previous studies experimentally assessed the effects of aqueous Hg in
298 Daphniidae (Biesinger and Christensen, 1972; Kim et al., 2017; Rodrigues et al., 2013; Tsui and
299 Wang2006). While the LC₂₅ value we report for survival in *C. dubia* was similar to the one we estimated
300 from Biesinger et al. (1982) data for and 21-day chronic exposure of Hg to *D. magna* (~1700 ng Hg L⁻¹,
301 calculated via linear interpolation using EPA (2019)), effects of Hg on Daphniidae survival are highly
302 dependent of the testing conditions (e.g., temperature, population, body size, and pre-exposure, Tsui and

303 Wang 2006). For example, Tsui and Wang (2006) found the median lethal concentration (LC₅₀) after 24
304 or 48h of exposure to aqueous Hg for *D. magna* to be highly variable, ranging from 5 to $\geq 250 \mu\text{g Hg L}^{-1}$
305 across different daphnid populations, sizes, pre-exposures, and temperatures. From the initial 48h of the
306 chronic assay performed in Experiment 1, the LC₅₀ in *C. dubia* is $7.04 \mu\text{g Hg L}^{-1}$ (calculated using EPA
307 (2019)), suggesting that Hg has similar effects on survival of *C. dubia* and *D. magna* (Biesinger and
308 Christensen, 1972; Kim et al., 2017; Rodrigues et al., 2013; Tsui and Wang 2006).

309 The IC₂₅ value we calculated for reproduction in *C. dubia* was lower than the LC₂₅ for survival. Our
310 findings corroborate Biesinger et al. (1982) where IC₂₅ for reproduction in *D. magna* was estimated at
311 $\sim 1150 \text{ ng L}^{-1}$ for inorganic Hg (calculated via linear interpolation using EPA (2019)). While acute or
312 lethal toxicity may occur at elevated Hg concentrations, in natural systems, Hg concentrations are likely
313 to be much lower than the lethal concentrations found in this study. Because sublethal responses on
314 reproduction are more likely to govern population responses than lethal responses measured at
315 environmentally unrealistic high toxic exposure levels, reproduction toxicity tests are among the few
316 sensitive ecotoxicological tests that provide the basis for legislative decisions (Breitholtz et al., 2006;
317 Rudén et al., 2017). In our study, the reproduction rate during the chronic assay was significantly
318 reduced by increasing Hg concentrations of 500 and 5000 ng L^{-1} while no reproduction was observed at
319 10000 ng Hg L^{-1} with the last female dead after 5 days. Negative effects of Hg on reproduction were also
320 found in *Daphnia* sp. with a reduction in the production of neonates (e.g. Biesinger and Christensen,
321 1972; Biesinger et al., 1982; Tsui and Wang, 2005a) and deleterious effects on embryonic development
322 (Khangarot and Das, 2009).

323 While growth was similar at 0 and 500 ng Hg L^{-1} , final total length was 16% less than controls at 5000
324 ng Hg L^{-1} , showing that Hg also affected *C. dubia* growth (Figure 4). Animals under Hg stress direct
325 more energy towards detoxification and recovery at the expense of other mechanisms such as feeding,

326 somatic growth and reproduction (Fong et al., 2019; Issa et al., 2021). Past studies also highlighted that
327 metal exposure may change cellular energy allocation in *D. magna* (Bossuyt and Janssen, 2004; Tsui
328 and Wang, 2004a) while a direct relationship between energy reserves and the tolerance of *D. magna* to
329 acute metal toxicity was found by Canli (2005). It could be hypothesized that mercury stress can
330 decrease the amount of resources available to *C. dubia* by decreasing their filtration activity and also by
331 impairing their swimming ability through oxidative stress and thus alter feeding behavior as shown in
332 *Daphnia* sp (Bownik, 2017; Lopes et al., 2014). As a consequence, reduced food intake can
333 subsequently result in deleterious effects on the endpoints we looked at in the present study.

334 *Daphnia* are common freshwater zooplankton species in temperate lakes making the link between
335 primary producers like phytoplankton and high trophic levels organisms. They are relatively large in size
336 and can carry higher Hg body burdens than other taxa (Watras et al., 1998). Understand the pathways of
337 Hg bioaccumulation of Hg at the base of the food networks is crucial for the subsequent transfer to
338 higher trophic levels. In the present study in which we exposed *C. dubia* to Hg primarily through water,
339 we found a BCF of 4.8×10^3 and 2.0×10^3 L kg⁻¹ in *C. dubia* with respective exposures of 500 and 5000
340 ng Hg L⁻¹. The BCF values we measured in *C. dubia* are in accordance with BCF values reported for *D.*
341 *magna* (Tsui and Wang, 2004a). Tsui and Wang (2004a) found that water exposure represents 31–96%
342 of inorganic Hg bioaccumulation in *D. magna*, depending on the variation of Hg BCF in ingested food.
343 Although mechanisms of Hg bioaccumulation in Daphniidae are not fully elucidated yet, a recent
344 experimental *in vivo* study provided new insights of inorganic Hg bioaccumulation in *D. carinata*. Using
345 aggregation-induced emission fluorogen, He et al. (2019) found that the carapace and eye are the major
346 recipient organs of aqueous Hg, which may have implications for observed effects. Previous works has
347 shown that the exposure route to contaminants can affect bioaccumulation and toxicity in zooplankton

348 taxa (e.g. Hook and Fisher, 2001; Wang et al., 2018). Future work should investigate how exposure
349 affects mercury bioaccumulation and toxicity in daphnids.

350 To assess the environmental relevance of the experimental approach we used, we compared effects in *C.*
351 *dubia* when exposed to artificial medium and stream water (i.e., FC water). We found slight differences
352 in the controls: survival was 80% in control FC water while no mortality was observed in the control
353 DMW. However, when the media were spiked with Hg, the toxicity response relative to the respective
354 control media was similar between DMW and FC water. These findings suggest that our conclusions
355 based on DMW medium may be environmentally relevant. Another study (Muna et al. 2018) also
356 compared the toxicity of heavy metals to zooplankton in natural versus artificial freshwaters and, unlike
357 our study, found that the toxicity changed with different media. Muna et al. (2018) studied the combined
358 effects of dietary algae and test media and found that the toxicity of CuSO₄ and ZnSO₄ in *D. magna*
359 were mitigated by algal addition in the natural lake water media but not in the artificial medium. Such
360 results were not expected and mechanisms underlying such differences remain largely unknown.
361 Further, Cu and Zn may react differently in freshwaters than Hg, which may also explain the different
362 results found here compared to Muna et al. (2018).

363

364 4.2. Sn-based treatment: Hg removal and the effects of *Ceriodaphnia dubia* fitness

365 Environmental concentrations for Hg are generally much lower than the Hg concentrations used in the
366 present study (0 - 22 ng L⁻¹ in average based on a worldwide survey of published measurements
367 collected in rivers flowing into estuaries; Amos et al., 2014). However, at industrially contaminated sites
368 (e.g., mining etc.) concentrations can be up to thousand times greater than maximum baseline
369 concentrations for total Hg (Wentz et al., 2014; Brooks and Southworth, 2011). We experimentally used

370 SnCl₂ treatment to remove Hg from DMW media. Previous field pilot studies have demonstrated the
371 efficiency of SnCl₂ for Hg stripping from water with abatement of ~90% (Mathews et al., 2015;
372 Southworth, 1996; Southworth et al., 2009). In the present study, SnCl₂ used at a Sn:Hg stoichiometric
373 ratio of 8:1 and coupled with air bubbling air was effective for Hg stripping with 100% of Hg removed
374 after a 16-h treatment regardless of the initial concentrations of Hg used (i.e., 890 and 2110 ng L⁻¹).
375 While SnCl₂ appeared to be effective in reducing concentrations of toxic heavy metals in field trials, the
376 effects of the added Sn to aquatic ecosystems had not been previously evaluated. The present study
377 demonstrates the absence of toxicological effects of Sn at concentrations of 2.1 ± 0.4 and 10.9 ± 0.6 µg
378 L⁻¹ in the model invertebrate *C. dubia*. Previous studies assessing toxicity of SnCl₂ reported high values
379 for LC₅₀ (i.e. 22-55 mg L⁻¹; Biesinger and Christensen, 1972; Khangarot et al., 1987) in *D. magna*.
380 Okamoto et al., (2014) found IC₅₀ values for SnCl₄ of 6.2 mg L⁻¹ based on acute immobilization in the
381 same species while, more recently, Okamoto et al. (2021) found EC₅₀ for reproductive toxicity of 6.5 mg
382 L⁻¹ for the same compound in *C. dubia*. As a comparison, Mathews et al. (2015) found that pilot SnCl₂-
383 based treatment system increased aqueous Sn concentrations from <0.5 to ~12 µg L⁻¹ in treated stream.
384 Interestingly, in addition to the absence of toxicological effects of Sn on growth, survival and
385 reproduction, we also demonstrated that the stripping of Hg using SnCl₂ allowed a full recovery of the
386 three investigated endpoints. Nevertheless, in the present study, the bioaccumulation of Sn was not been
387 measured in *C. dubia* and the potential long-term impacts of an Sn enrichment at low trophic levels in
388 freshwater ecosystems remain unknown (Mathews et al., 2015) and need to be investigated before SnCl₂
389 can be considered a treatment to remediate Hg in natural environments.

390

391 **5. Conclusion**

392 Our experimental results indicate that inorganic Hg causes chronic effects on *C. dubia*. Increasing
393 aqueous concentrations of Hg induced mortality, reproduction inhibition, reduced growth, and decreased
394 long term population growth rates. Toxicity of Hg has been confirmed both in artificial media and
395 stream water. We demonstrated that SnCl₂ treatment is efficient to remove Hg from the water column
396 and also removes the toxicological effects of Hg on survival, reproduction and growth. Our findings
397 indicate that at concentrations relevant for Hg removal, the addition of Sn does not cause any
398 toxicological effects on *C. dubia*. The effectiveness of SnCl₂-based remediation of Hg has already been
399 demonstrated in the field but the toxicological risks of this remediation treatment were previously
400 unknown.

401

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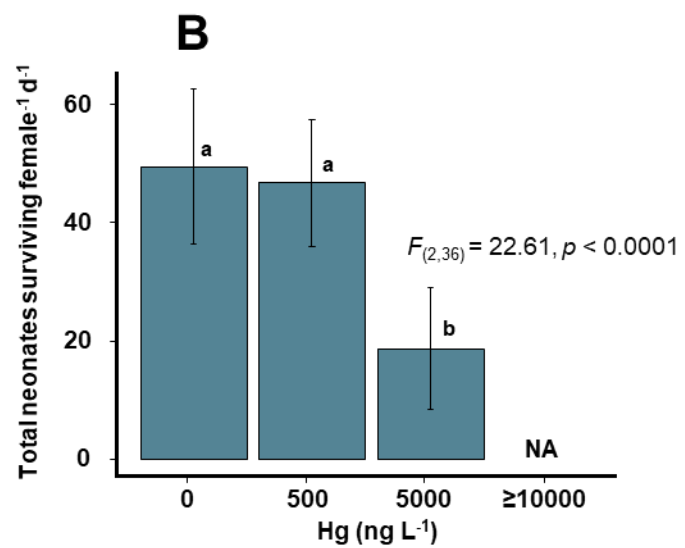
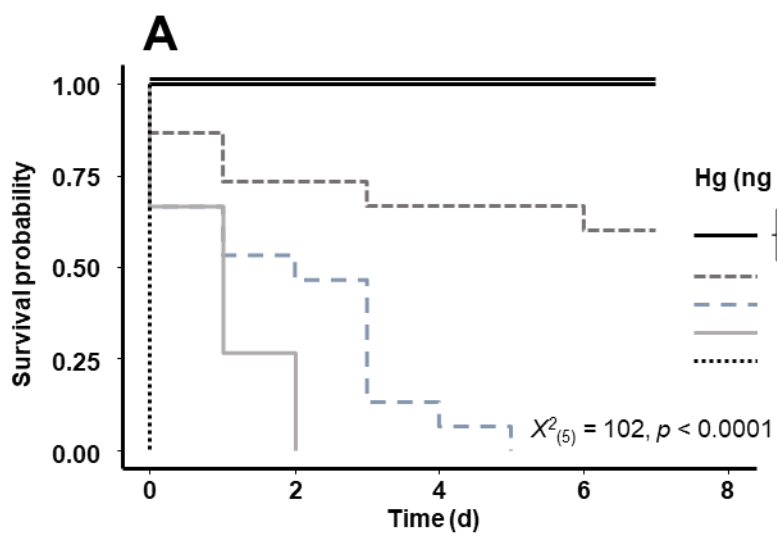
588 **Captions to Figures**

589 Figure 1. Survival kinetics (A) and reproduction rates (expressed as the number of neonates surviving
590 female⁻¹, Means ± SD, see Section 2.5 for details) (B) of *Ceriodaphnia dubia* (n = 10-15 per condition)
591 exposed for 7 days to DMW media containing a gradient of Hg concentrations. Letters denote significant
592 differences.

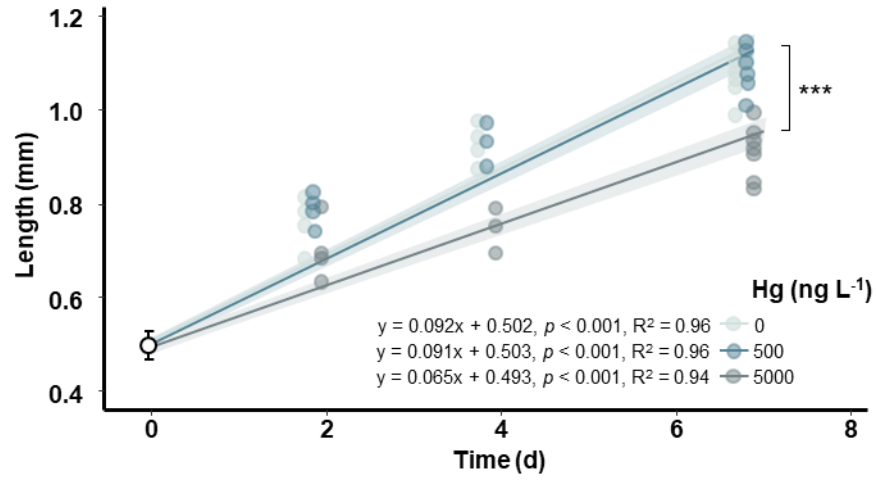
593 Figure 2. Growth kinetics (expressed as the length from the top of the eye to the base of the spine;
594 (Agatz et al., 2015) of *Ceriodaphnia dubia* (n = 3-15 per condition) exposed for 7 days to DMW media
595 containing a gradient of Hg concentrations. Symbol denotes significant differences ($p < 0.001$).

596 Figure 3. Survival kinetics (A) and reproduction rates (expressed as the number of neonates surviving
597 female⁻¹, Means ± SD, see Section 2.5 for details) (B) of *Ceriodaphnia dubia* (n = 10 per condition)
598 exposed for 7 days to experimental media containing SnCl₂ and/or Hg based on DMW or First Creek
599 water. Letters denote significant differences.

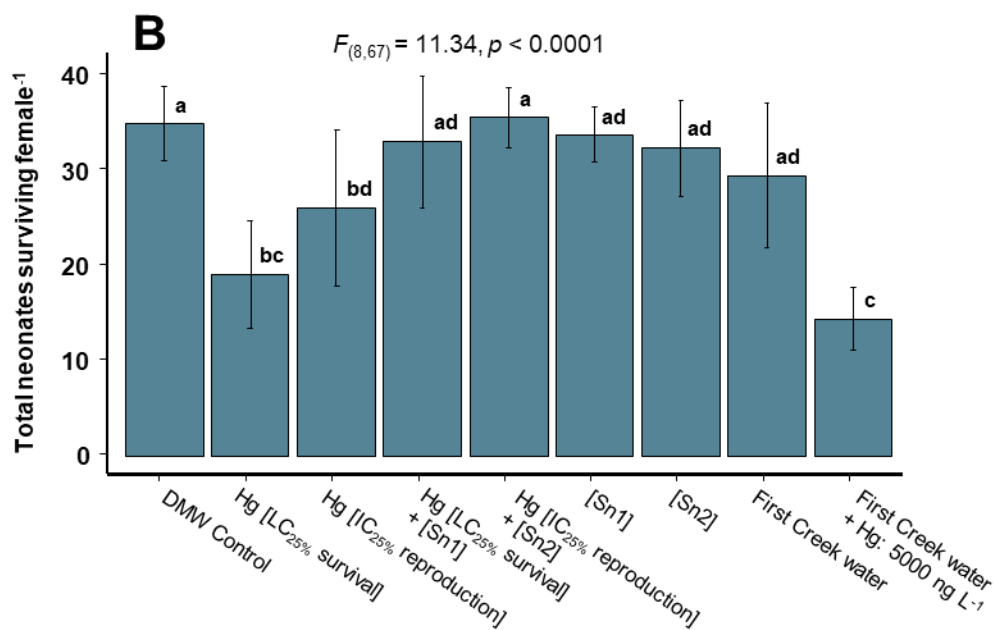
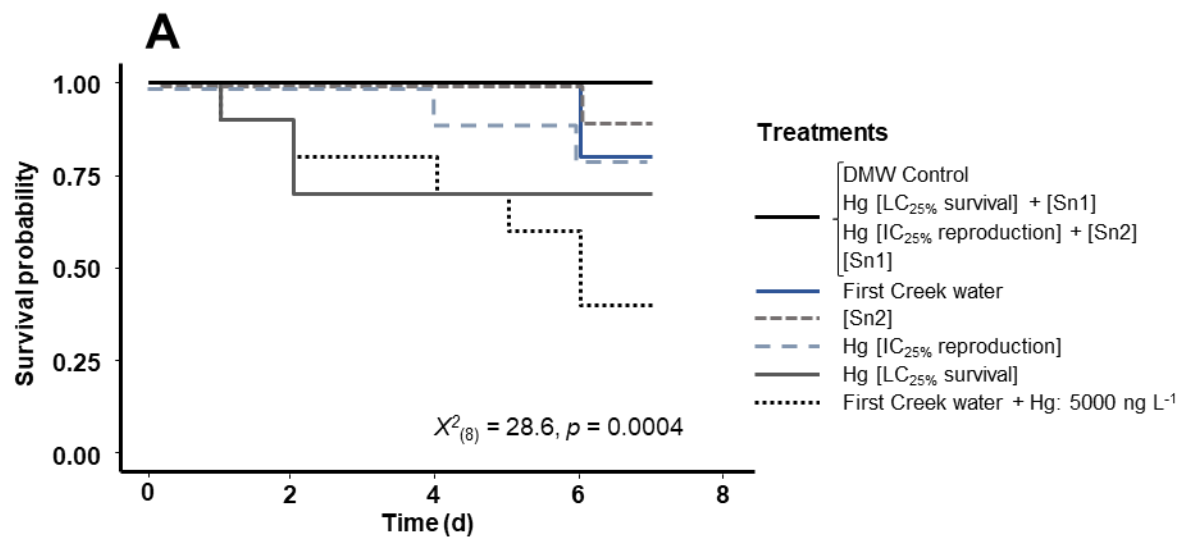
600 Figure 4. Final length (in mm from the top of the eye to the base of the spine; (Agatz et al., 2015) of
601 *Ceriodaphnia dubia* (n = 4-10 per condition) exposed for 7 days to experimental media containing SnCl₂
602 and/or Hg based on free-EDTA DMW or First Creek water. Letters denote significant differences.



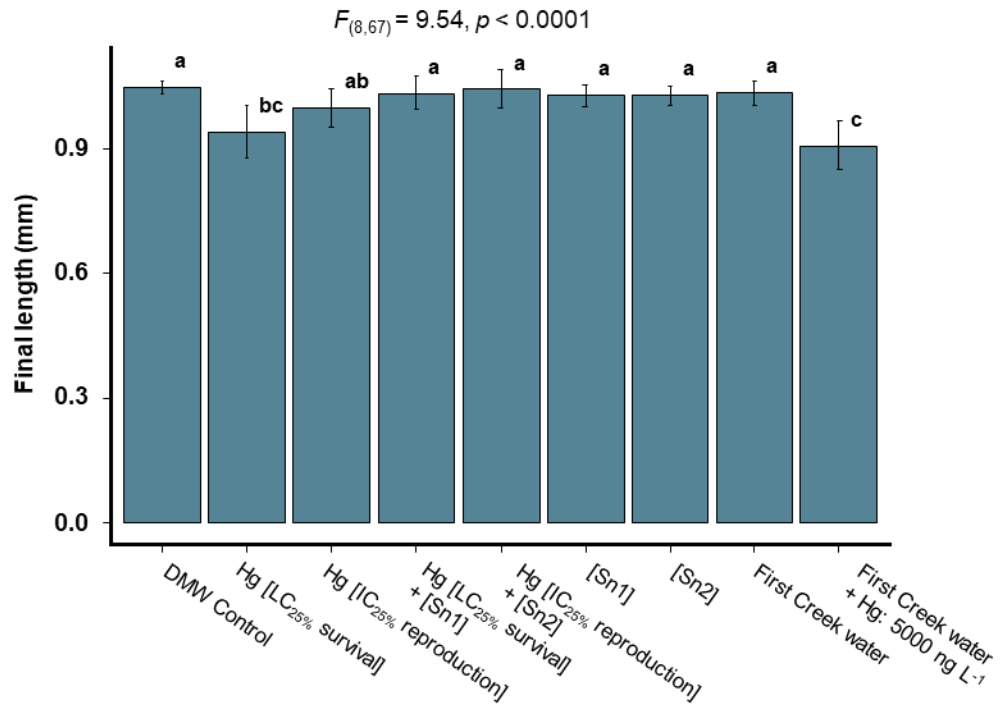
603 Figure 1



604 Figure 2



605 Figure 3



606 Figure

Table 1. Composition and water quality of the different media used in (1) Experiment 1 assessing the effects of Hg on survival, reproduction and growth in *Ceriodaphnia dubia* and (2) in Experiment 2 investigating potential toxicity of SnCl₂-treated media. Values are Means \pm SD (n = 3-13). In Experiment 1 and Experiment 2, letters denote significant differences for each of the measured water parameters among the different media.

Media	Hg (ng L ⁻¹)	Sn (μ g L ⁻¹)	pH	Conductivity (μ S cm ⁻¹)	Oxygen (mg L ⁻¹)	Alkalinity (mg CaCO ₃ L ⁻¹)	Hardness (mg CaCO ₃ L ⁻¹)	NH ₄ ⁺ (mg L ⁻¹)	NO ₂ ⁻ (mg L ⁻¹)	NO ₃ ⁻ (mg L ⁻¹)
Experiment 1										
DMW Control	0 \pm 0 ^a	-	8.37 \pm 0.05	150 \pm 3	8.41 \pm 0.14	55 \pm 1	68 \pm 1	0.04 \pm 0.02	0.004 \pm 0.003	0.36 \pm 0.11
Hg: 500 ng L ⁻¹	501 \pm 17 ^b	-	-	-	-	-	-	-	-	-
Hg: 5000 ng L ⁻¹	4791 \pm 145 ^c	-	-	-	-	-	-	-	-	-
Hg: 10000 ng L ⁻¹	9825 \pm 435 ^d	-	-	-	-	-	-	-	-	-
Hg: 17500 ng L ⁻¹	17119 \pm 282 ^e	-	-	-	-	-	-	-	-	-
Hg: 25000 ng L ⁻¹	23279 \pm 841 ^f	-	-	-	-	-	-	-	-	-
Experiment 2										
DMW Control	0 \pm 0 ^a	0.2 \pm 0 ^a	8.33 \pm 0.11 ^a	156 \pm 3 ^a	8.53 \pm 0.19 ^a	56 \pm 2 ^a	70 \pm 2 ^a	0.03 \pm 0.03 ^a	0.004 \pm 0.002 ^a	0.44 \pm 0.03 ^a
Hg [LC ₂₅ survival]	2023 \pm 113 ^b	-	-	-	-	-	-	-	-	-
Hg [IC ₂₅ reproduction]	854 \pm 71 ^c	-	-	-	-	-	-	-	-	-
Hg [LC ₂₅ survival] + [Sn1]	0 \pm 0 ^a	8.6 \pm 0.6 ^b	-	-	-	-	-	-	-	-
Hg [IC ₂₅ reproduction] + [Sn2]	0 \pm 0 ^a	3.1 \pm 0.6 ^c	-	-	-	-	-	-	-	-
[Sn1]	0 \pm 0 ^a	10.9 \pm 0.6 ^b	8.33 \pm 0.05 ^a	156 \pm 3 ^a	8.51 \pm 0.25 ^a	57 \pm 1 ^a	69 \pm 2 ^a	0.05 \pm 0.03 ^a	0.005 \pm 0.003 ^a	0.36 \pm 0.04 ^a
[Sn2]	0 \pm 0 ^a	4.3 \pm 0.8 ^c	8.34 \pm 0.05 ^a	156 \pm 3 ^a	8.53 \pm 0.23 ^a	58 \pm 2 ^a	69 \pm 1 ^a	0.04 \pm 0.05 ^a	0.005 \pm 0.003 ^a	0.036 \pm 0.05 ^a

First Creek water	0 ± 0^a	-	8.39 ± 0.26^a	301 ± 3^b	8.25 ± 0.16^b	161 ± 1^b	162 ± 1^b	0.04 ± 0.02^a	0.002 ± 0.002^a	0.14 ± 0.02^b
First Creek water + Hg: 5000 ng L ⁻¹	4530 ± 1045^d	-	-	-	-	-	-	-	-	-

Table 2. Chronic toxicity and bioconcentration of Hg by *Ceriodaphnia dubia* in (1) Experiment 1 assessing the effects of Hg on survival, reproduction and growth and (2) in Experiment 2 investigating potential toxicity of SnCl₂-treated media.

Media	Hg in media (ng L ⁻¹)	Survival (%)	Reproduction (neonates surviving female ⁻¹)	Hg in <i>C. dubia</i> (ng mg ⁻¹ , DW)	BCF (L kg ⁻¹)
Experiment 1					
DMW Control	0 ± 0	100	49 ± 13	0	-
Hg: 500 ng L ⁻¹	501 ± 17	100	46 ± 10	2.3	4800
Hg: 5000 ng L ⁻¹	4791 ± 145	60	19 ± 10	10.0	2000
Hg: 10000 ng L ⁻¹	9825 ± 435	0	0 ± 0	-	-
Hg: 17500 ng L ⁻¹	17119 ± 282	0	0 ± 0	-	-
Hg: 25000 ng L ⁻¹	23279 ± 841	0	0 ± 0	-	-
Experiment 2					
DMW Control	0 ± 0	100	35 ± 4	-	-
Hg [LC ₂₅ survival]	2023 ± 113	70	19 ± 6	-	-
Hg [IC ₂₅ reproduction]	854 ± 71	80	26 ± 8	-	-
Hg [LC ₂₅ survival] + [Sn1]	0 ± 0	100	33 ± 7	-	-
Hg [IC ₂₅ reproduction] + [Sn2]	0 ± 0	100	36 ± 3	-	-
[Sn1]	0 ± 0	100	34 ± 3	-	-
[Sn2]	0 ± 0	90	32 ± 5	-	-
First Creek water	0 ± 0	80	30 ± 8	-	-
First Creek water + Hg: 5000 ng L ⁻¹	4530 ± 1045	40	15 ± 3	-	-

BCF: Bioconcentration factor