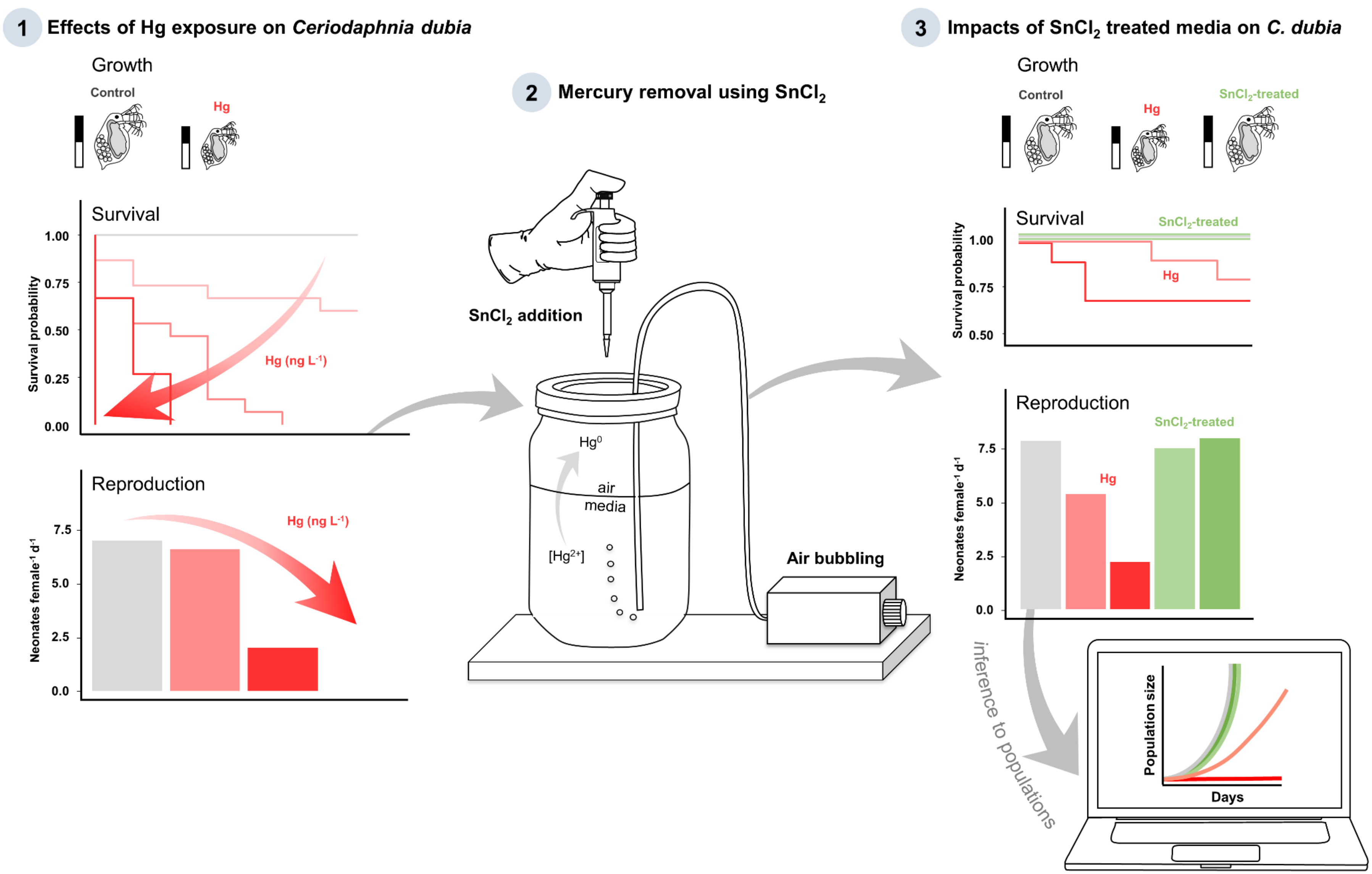
Graphical abstract



Stannous chloride as a tool for mercury stripping in contaminated streams: Experimental assessment of toxicity in an invertebrate model species

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Abstract

The chronic toxicity of an innovative Hg water treatment system using tin (Sn) (II) chloride ($SnCl_2$) followed by air stripping was assessed on the freshwater cladoceran *Ceriodaphnia dubia*, a species model for toxicity testing, through the measurements of survival, growth, and reproduction rate. We first calculated the concentrations of Hg causing 25% reduction in survival and reproduction (Inhibition Concentration, or IC_{25}) through exposure to aqueous Hg at concentrations ranging from 0 to 25000 ng L^{-1}. Then, we treated media (DMW and natural stream water) contaminated with Hg at IC_{25} concentrations with $SnCl_2$ at a Sn:Hg stoichiometric ratio of 5:1 and air stripping and exposed *C. dubia* to this Sn-amended media. Our results showed that Hg significantly affected survival (IC_{25}: 2109 ng Hg L^{-1}), reproduction rates (IC_{25}: 888 ng Hg L^{-1}) and impaired growth. $SnCl_2$-treatment removed 100% of the Hg from the media at all concentrations tested while no deleterious effects on survival, growth and reproduction were recorded in the $SnCl_2$ treated media. Our results confirmed that $SnCl_2$-treatment at the concentrations tested present no toxicity, at individual and population levels, in a sensitive invertebrate species suggesting no impairment for aquatic biota by using such treatment systems to remediate Hg in contaminated sites.

Keywords: *Ceriodaphnia dubia*, Chronic toxicity, Hg, $SnCl_2$, Tin

1. Introduction

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

Like other heavy metals, inorganic mercury cannot be degraded in ecosystems, leading to its persistence in the environment (Wang et al., 2020). Mercury remediation at contaminated sites is challenging in part because of the transformations that mercury can undergo in the environment which may make it more or less amenable to treatment or removal. Among various methods aiming to remove Hg(II) from water, adsorption is the most commonly utilized approach (Abbas et al., 2018; Bao et al., 2017; Kyzas and Kostoglou, 2015; Rocha et al., 2016). The sorbents usually possess high surface area as well as high porosity and the formation of chelates is the major sorption mechanism (Kyzas and Kostoglou, 2015; Rocha et al., 2016). New materials have been developed to capture environmental mercury including nanomaterials, polymers, hydrotalcites, graphene, and covalent and metal organic frameworks (Muller et al., 2019; Wang et al., 2020). Sustainable options for large scale environmental remediation must consider the removal of mercury without the addition of other materials which adversely affect the ecosystem.

A pilot study has reported the efficacy of Sn(II) chloride (i.e. stannous chloride; $SnCl_2$) followed by air stripping (Looney et al., 2003) to remove Hg from groundwater entering a stream system in South Carolina, USA (Mathews et al., 2015). This innovative treatment system was based on common analytical methods for Hg (Southworth, 1996) where $SnCl_2$ is used as a reducing agent to reduce Hg^{2+} to Hg0, which is sparged into the gaseous phase by bubbling with air (Jackson et al., 2013), according to the following equation:

$$HgCl_0^2 + Sn^{2+} + 2H_2O \rightarrow Hg(0) + 2Cl^- + SnO_2(s) + 4H^+$$

While this treatment system was effective at decreasing aqueous and fish tissue Hg concentrations by over 70%, it introduced tin into the receiving stream, leading to an increase in tin concentrations in the water and resident fish (Mathews et al., 2015). Recent investigations have also shown $SnCl_2$ to be efficient at reducing hexavalent chromium [Cr(VI)] to less toxic trivalent chromium Cr(III) in groundwater (Henrie et al., 2019; Nguyen et al., 2020). While $SnCl_2$ appears to be promising in reducing concentrations of toxic heavy metals, the longer-term impacts of adding tin to aquatic ecosystems have not been evaluated.

The aim of the present study was to examine the effects of Sn and $SnCl_2$-treated Hg-containing media on the reproduction, growth, survival, and predicted population-level effect of an aquatic invertebrate model species *Ceriodaphnia dubia*. We exposed *C. dubia* to six aqueous Hg treatments, ranging from 0-25000 ng L^{-1} in a 7-d chronic toxicity test to assess effects on survival, growth, and reproduction. We then treated Hg-contaminated media with $SnCl_2$ and air stripping and exposed *C. dubia* to this Sn-amended media at various concentrations to examine whether: 1) $SnCl_2$ reduced Hg concentrations in water, 2) $SnCl_2$ treatment removed Hg toxicity and 3) whether the added $SnCl_2$ had toxic effects on *C. dubia*. Further, we compared the effect of Hg in artificial freshwater medium and natural creek water to assess the relevance of our lab exposures. Our results are relevant to the broader evaluation of the environmental impacts of chemical remediation of Hg in aquatic ecosystems.

2. Materials and Methods

2.1. *Ceriodaphnia dubia* culture

Ceriodaphnia dubia is a parthenogenetically reproducing freshwater microcrustacean (<1 mm length) that is commonly used in toxicity testing because of its ease of culture, short life span, sensitivity to water quality, and prolific reproductive cycle. *C. dubia* cultures were maintained in 25% Dilute Mineral Water (DMW) medium in 1-L glass containers in an incubator 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. dubia* food consisted of a combination of commercial yeast purchased from Aquatic BioSystems (Fort Collins, CO, USA) and laboratory-cultured unicellular green algae *P. subcapitata* (ex *Raphidocelis subcapitata*; 3-4 μm in diameter). Algae were grown in 10-L clear polycarbonate Nalgene carboys containing aerated WC medium (Guillard, 1975) without ethylenediaminetetraacetic acid (EDTA) at 23-24 °C; light intensity: 150-200 µmol m^{-2} s^{-1}; light/dark: 12h/12h). Algae were harvested during their exponential growth phase for feeding to *C. dubia* (i.e. after 14 ± 4 d at 11 ± 4 x 10^6 cells mL^{-1}). Algal cell counts were done by imaging flow-cytometry (FlowCam® Benchtop B3 Model). Prior to feeding, live *P. subcapitata* cells were concentrated by centrifugation (5000 RPM for 5 min) and resuspended in Milli-Q® water. The use of concentrated algae allowed a reduction of spike volume (i.e. 7 µL mL^{-1}) and avoided significant changes of water volume to preserve physical and chemical parameters of each tested media. Throughout the experiments, special attention has been paid to avoid contamination of algae cultures and all glassware used for experiments was put through a rigorous acid washing protocol and rinsed three times with deionized water before drying. The absence of contamination was confirmed by observations made during cell counts.

2.2. Experimental media

DMW (EPA, 2002) was prepared by mixing (Perrier® + Milli-Q® water at a ratio of 1:3 v/v) and adding trace elements at concentrations for WC media (Guillard, 1975) without ethylenediaminetetraacetic acid (EDTA). DMW was maintained in a 20-L Nalgene carboy with an air bubbler for one week to de-gas the Perrier and aerate the solution. Two mercury stocks solutions (0.0001 M and 0.001 M) were prepared in 1-L glass containers by dissolving appropriate weights of $HgCl_2$ (LabChem®) in ultrapure water. A tin stock solution (0.0001 M) was prepared as described above using $SnCl_2$ dihydrate (J.T.Baker®) in ultrapure water. All stock solutions were kept closed under a hood at room temperature.

In Experiment 1, *C. dubia* were exposed to five different nominal Hg concentrations: 500, 5000, 10000, 17500 and 25000 ng L^{-1} and a control treatment with DMW (no Hg added). Each treatment medium was prepared daily during the experiment by spiking 300-mL DMW with appropriate volumes of Hg stock solutions (i.e. 0-187 µL). Spike volumes were maintained as low as possible to avoid significant changes in pH that we confirmed through measurements.

In Experiment 2, *C. dubia* were exposed to nine different treatments. The first two treatments tested the toxicity of Hg concentrations at the IC_{25} concentrations for Hg for survival and reproduction determined in Experiment 1 (see section 2.5 for details on IC_{25} calculations). Two treatments contained the same IC_{25} Hg concentrations spiked with $SnCl_2$ at a Sn:Hg stoichiometric ratio of 5:1 (Jackson et al., 2013; Looney et al., 2003) and bubbled for 16h (i.e. 4440 and 10545 ng Sn L^{-1}, so-called [Sn1] and [Sn2] respectively). Toxicity of Sn alone was assessed in two treatments prepared with the same Sn concentrations used for Hg stripping. A control treatment with DMW (no Hg or Sn amendments) was used. In addition, in order to assess environmental relevance of the results from artificial media (i.e. DMW based), two treatments were prepared using water from First Creek (FC), an uncontaminated stream on the Oak Ridge Reservation in Oak Ridge, TN (see Table 1 for water quality). First Creek water was filtered (75-µm) prior to use in order to avoid introduction of *C. dubia* predators. The first of these natural water-based media was kept Hg-free while the second was spiked with Hg at a nominal concentration of 5000 ng L^{-1}, a concentration which was shown in Experiment 1 to affect both survival and reproduction in *C. dubia*.

Composition of all the media used during the experiments is provided in Table 1. Mercury concentrations were measured every day in subsamples of each media. All media were kept in an incubator set at 25 °C for one-hour prior to using. Water quality (i.e. pH, conductivity and O_2

concentration) of DMW media (prior to Hg or Sn additions, to avoid any contamination of the equipment used) were monitored daily using YSI® MultiLab 4010-3W. Additional measurements through titration were done for alkalinity and hardness and concentrations of NH_4^+, NO_2^- and NO_3^- were measured 3-4 times during the experiments (HACH® SL 1000). Values of water parameters are summarized in Table 1.

2.3. Chronic test procedure

To start the test, <24h old *C. dubia* neonates born within the same 8-h period were individually distributed to test vials (i.e. 20 mL borosilicate Ichem vials covered by a transparent plexiglass sheet; n=10 *C. dubia* neonates per treatment except as described below for growth measurements in Experiment 1) filled with 15 mL of media (DMW with and without Hg and/or Sn amendments) and food ($3x10^6$ cells *P. subcapitata* and 100 µL of yeast). Test vials were assigned to a position within a test board using a block randomization procedure to keep treatments blind to the observer and avoid bias in data collection. We used an additional five *C. dubia* individuals for growth measurements in Experiment 1 to determine whether the additional handling would induce stress and affect survival and reproduction. Since no effect of the growth measurement manipulation was observed on reproduction or survival, all the data were used for IC_{25} determination (see Section 2.5). Then, 10 individuals per treatment were used in Experiment 2.

Sample media was renewed and *C. dubia* were fed daily by preparing media and vials as described above and moving original *C. dubia* from old vial to new vial with a glass Pasteur pipette. During media renewal, *C. dubia* were observed and data for survival and reproduction (i.e. number of neonates produced) were recorded prior to transfer to the new vials prepared with fresh media and food as described above. Subsamples of media from each treatment were taken before and after the 24-h exposure for water chemistry and contaminant analysis (See section 2.3). In Experiment 1, growth of the *C. dubia* was assessed at day 0 (n = 30), 2, 4 and 7 (n= 3-15 per treatment) by measuring total length from the base of the spine to the top of the eye according to (Agatz et al., 2015). Measurements were performed using a stereomicroscope (Leica® M80) calibrated using a 200 µm stage micrometer under a 5x magnification. The same procedure was repeated in the Experiment 2 at day 0 (n = 30) and day 7 (n = 4-10 per treatment).

At the end of each experiment, all the surviving *C. dubia* were measured for length as described above and rinsed for 5 min in fresh DMW and stored at -18 °C for further Hg analysis (see Section 2.5).

2.4. Mercury and Sn analysis

Before each exposure, 40 mL subsamples of each media were prepared in 40-mL glass Ichem vials. A small volume (i.e. 10-300 µL) was directly analyzed for checking Hg initial concentrations, while the remaining volume was preserved with BrCl (5 µL mL^{-1}). Mercury was measured by cold vapor atomic absorption spectroscopy (CV-AAS) Zeeman effect Hg analyzer (RA-915+, Ohio Lumex Company, Inc., Twinsburg, OH, USA). The instrument was calibrated using $Hg(NO_3)_2$ standards with a detection limit of 10 ng L^{-1}. Duplicates were run every 2 samples and procedural blanks were analyzed for quality control. Blanks are typically below detection limits, and duplicates are on average <5% different from their parent sample.

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

Prior to Hg analysis, *C. dubia* pooled samples (n = 1 pool of 9 to 15 individuals per treatment) exposed to 0, 500 and 5000 ng Hg L^{-1} for 7 d were freeze-dried for 24 h, weighed using a microbalance (± 0.1 mg accuracy), digested in acid in 100 µL (70 HNO_3:30 H_2SO_4 v/v), and analyzed by cold vapor atomic absorption as described for water samples. Procedural blanks and standard reference materials (IAEA 407 and IAEA 436) were analyzed for quality control and demonstrated Hg recovery from standards >90% while blanks were below detection limits.

2.5. Data analysis

In Experiment 1, Hg IC_{25} were calculated for survival and reproduction using the linear interpolation method as described in the EPA chronic manual according to the following equation:

$$IC_{25} = C_j + \left[M_1 \left(1 - \frac{25}{100}\right) - M_j\right] \frac{C_{j+1} - C_j}{M_{j+1} - M_j}$$

Where C_j and C_{j+1} are the test concentrations whose observed mean response is greater and less than M_1 (1 - 25/100), M_1, M_j and M_{j+1} are the smoothed mean responses for the control, concentrations j and j+1 respectively. IC_{25} 95% bootstrapped confidence intervals using standard methods developed by the U.S. Environmental Protection Agency (EPA, 2019). The IC_{25} calculation for reproduction was based on the total number of neonates produced for each experimental treatment divided by the total number of individuals started.

Differences in reproduction rate (number of neonates surviving female^{-1} d^{-1}) between treatments were assessed using an ANOVA followed by Tukey's post-hoc test. The reproduction rate was calculated by averaging the number of neonates per surviving female per observation day. As an example, the reproduction rate of a female who died on day 5 would be calculated as the total of total number of neonates over the 5-day period divided by the surviving time (here 5 days). Assumptions of normality and heteroscedasticity were examined on plotted residuals. Survival curves were compared among experimental treatments with Kaplan-Meier Survival Analysis.

In Experiment 1, the growth kinetics of the *C. dubia* between the different Hg treatments were best fitted using a linear model. Model constants were estimated by iterative adjustment of the model. In order to statistically assess the effects of Hg on *C. dubia* growth, ANCOVA followed by Tukey's test were applied on length data to identify differences between regression slopes. In Experiment 2, differences in final growth of surviving *C. dubia* from each media was compared by ANOVA followed by Tukey's post-hoc test as described above. The level of significance for statistical analyses was set to $\alpha = 0.05$.

We estimated the intrinsic rate of increase (r) of a Daphniidae population exposed to each treatment using the Euler equation:

$$1 = \sum_{x=0}^{k} e^{-rx} l(x) b(x)$$

Methods for these calculations are outlined in Stevenson et al. (2017) but briefly: *l(x)* represents survival from birth to age x, and *b(x)* represents the average number of neonates born per day to a female of age x (also called the fecundity schedule); both are calculated daily from the start (x = 0) until the end of the experiment (x = k). We numerically solved the Euler equation to find the value of *r* for our entire data set using the uniroot function in R. We resampled the data with replacement 1000 times using a bootstrapping technique and recalculated values for *r* based on resampled data sets to estimate the uncertainty around these values. We calculated standardized mean differences (Cohen's *d* values) and their confidence intervals (Nakagawa and Cuthill, 2007) to compare the *r* values to each other using the compute.es package in R. All statistics were performed using R version 4.0.1 (R Development Core Team, 2021) unless stated otherwise.

3. Results

3.1. Effects of Hg on survival, reproduction, growth, and long-term population growth rate

In Experiment 1, *C. dubia* neonates were exposed to a range of nominal Hg concentrations (i.e. 0, 500, 5000, 10000, 17500 and 25000 ng Hg L^{-1}) in a 7-d chronic toxicity test. Measured Hg concentrations in media were within 0-7% of nominal values (Table 1). Mercury body burdens in *C. dubia* were 2.3 ng mg^{-1} (dry weight) when exposed to 500 ng Hg L^{-1} and 10 ng mg^{-1} (dry weight) when exposed to 5000 ng Hg L^{-1}. The bioconcentration factors (BCF, i.e. the ratio between Hg concentrations in *C. dubia* and in the exposure media) were 2005 and 4812 L kg^{-1} (dry weight) in individuals exposed to 5000 and 500 ng Hg L^{-1}, respectively (Table 2). We found that Hg significantly affected all the biological endpoints considered in this study (i.e. survival, reproduction and growth).

Survival rate was significantly affected by Hg concentrations ($\chi^2_{(5)} = 102$, $p < 0.0001$; Figure 1A). While a 100% survival rate was observed in the control and the lowest Hg concentration treatment (i.e. 0 and 500 ng Hg L^{-1} respectively) after 7 d, survival rate was only 60% when *C. dubia* were exposed to 5000 ng Hg L^{-1}. At 10000 ng Hg L^{-1}, no surviving *C. dubia* remained at the end of the experiment, with the last surviving individuals observed at day 5, 2 and 0 at Hg concentrations of

10000, 17500 and 25000 ng L^{-1} respectively (0% survival rate; Figure 1A, Table 2). The calculated IC_{25} for survival was 2019 ng Hg L^{-1} (1186-5065 ng L^{-1} 95% confidence intervals).

Mercury also significantly affected reproduction in *C. dubia* expressed as the number of neonates surviving $female^{-1}$ d^{-1} ($F_{(3,49)} = 65.87$, $p < 0.0001$; Figure 1B). The highest reproduction rate was observed in the control treatment with 7.0 ± 1.8 neonates $female^{-1}$ d^{-1}, a value that was not significantly different ($p = 0.883$) from the reproduction rate observed in *C. dubia* exposed to 500 ng Hg L^{-1} (i.e. 6.6 ± 1.5 neonates surviving $female^{-1}$ d^{-1}). The reproduction rate in the 5000 ng Hg L^{-1} treatment was reduced by 70% with respect to the controls ($p < 0.001$) with only 2.0 ± 1.6 neonates $female^{-1}$ d^{-1}. No reproduction was observed at concentrations ≥ 10000 ng Hg L^{-1} (Figure 2B). The IC_{25} for reproduction was estimated at 888 ng Hg L^{-1} (64-1269 ng L^{-1} 95% confidence intervals). The total number of neonates produced per surviving female throughout the Experiment 1 is provided in Table 2.

Hg exposure led to a reduction in *C. dubia* growth, expressed as the length from the top of the eye to the base of the spine according to Agatz et al. (2015) throughout the 7-d experiment. However, because 100% mortality was observed at concentrations ≥ 10000 ng Hg L^{-1}, growth could only be followed in three experimental treatments (i.e. 0, 500 and 5000 ng L^{-1}). The slopes of the linear growth curves differed among the experimental treatments ($F_{(2,152)} = 14.61$, $p < 0.001$, Figure 2). The maximal growth was observed in the control treatment and at 500 ng Hg L^{-1} (i.e. final length of 1.12 ± 0.04 mm for the two treatments). Growth kinetics were significantly and negatively affected by Hg when the *C. dubia* were exposed to 5000 ng Hg L^{-1} (Figure 2) with individuals, on average, 16% shorter than the ones from the other treatments (0.94 ± 0.05 mm).

The effect of Hg exposure on survival and reproduction also manifested to predicted impacts at the population level, as estimated by the calculation of the long-term population growth rate (r) of

C. dubia exposed to these treatments (Figure 5A). Populations with $r > 0$ are predicted to grow exponentially while populations for which r values cannot be calculated (due to lack or reproduction or survival) or $r < 0$ are predicted to decline to extinction. Concentrations higher than 10000 ng Hg L^{-1} are predicted to cause extinction of populations of *C. dubia* (Figure 5A) and concentrations as low as 5000 ng Hg L^{-1} significantly decrease the long-term population growth rate compared to the control (Cohen's d = -6.32; Table 3) and also dramatically increase the variability in these estimates (shown through the larger error bars; Figure 5A and Table 3).

It is worth noting that although there is technically a statistically significant difference between the control and 500 ng Hg L^{-1} (Table 3), the difference in r values and the corresponding standardized mean difference are very small (Table 3). Technically, all of the r values of the treatments are statistically significantly different from their respective controls ($p < 0.05$; Table 3), however we argue that the differences in actual r values are so small they are not biologically significant (e.g. DMW Control Experiment 1 vs. 500 ng Hg L^{-1} have r values of 0.78 and 0.77 d^{-1}, respectively, Table 3). This is probably due to the fact that, with some notable and interesting exceptions (mostly the highest [Hg] treatments for which we could calculate r), the variability between individuals in the data set was low resulting in low variability in the population estimates, making small differences in r values statistically but not necessarily biologically significant. Therefore, we will focus on discussing differences in the r values themselves and standardized mean differences (Cohen's d values) rather than whether or not an effect is significantly different statistically. We maintain that this is more appropriate for the r value bootstrapped estimates since these data are simulated in order to get an estimate of variability. We tested the effect of the number of bootstrapped iterations on estimates of Cohen's d and corresponding confidence intervals and statistical significance, and we found that differences between the treatments using 100 or 250 iterations instead of 1000 were still all statistically significant even with small differences in calculated *r* values (data not shown).

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

In Experiment 2, we evaluated the effects of $SnCl_2$ treatment on Hg bioaccumulation and toxicity in *C. dubia*, as well as the potential toxic effects of the added Sn on *C. dubia*. Our results confirmed the efficiency of Hg removal from aqueous samples by $SnCl_2$ and air stripping, with 100% of the Hg removed at both Hg concentrations examined (i.e. 888 and 2109 ng L^{-1}; Table 1). These results were reflected in the biological endpoints observed in *C. dubia*.

When exposed to a nominal concentration of 2109 ng Hg L^{-1} (measured values of 2023 ± 113 ng Hg L^{-1}) in Experiment 2, *C. dubia* survival was reduced by 30% compared to the control treatment, validating the IC_{25} value calculated in Experiment 1. In media with the same nominal Hg concentration treated with $SnCl_2$ at a Sn:Hg stoichiometric ratio of 5:1 ([Sn1], Table 2), no mortality was observed (Figure 3A, Table 2). The same observation was made in *C. dubia* exposed to Sn alone at the same concentration ([Sn1]; Table 2). In the [Sn2] treatment, one adult was found dead, tangled in its molt, on the last day of the experiment.

In Experiment 2, similar to results seen for survival, when exposed to the calculated IC_{25} for reproduction from Experiment 1 (i.e. 888 ng L^{-1}; measured values of 854 ± 71 ng Hg L^{-1}), a 30% decrease in reproduction rate was observed (3.5 ± 1.2 neonates surviving female^{-1} d^{-1}), validating the IC_{25} values for reproduction estimated in Experiment 1. Also similar to results seen for survival, *C. dubia* exposed to the same Hg concentrations treated with $SnCl_2$ had a reproduction rate similar to the control treatment *(p* = 0.999) with 5.1 ± 0.4 neonates female^{-1} d^{-1} vs. 5.0 ± 0.6

neonates female^{-1} d^{-1} for the control. Exposure to $SnCl_2$ alone did not affect the reproduction rate in either of the tested Sn concentrations ($p \geq 0.963$, Figure 3B). The total number of neonates produced per surviving female throughout the Experiment 1 is provided in Table 2.

C. dubia growth was not affected by $SnCl_2$ ($p \geq 0.965$), with similar total length measurements observed between the $SnCl_2$-treated Hg, $SnCl_2$ and control treatments at the end of Experiment 2 (i.e. 1.03 ± 0.03 and 1.05 ± 0.01 mm) for all the Sn-containing media and the DMW control, respectively).

Exposure of *C. dubia* to media where Hg was stripped out of solution with Sn did not show predicted population level impacts (Figure 5B). At initial concentrations of Hg that otherwise have a large effect on *r* values (both through decreased predicted population growth and increased variability in population growth; Cohen's *d* = -4.89 and -5.19 for Hg[IC_{25} survival] and Hg[IC_{25} reproduction], respectively, compared to the control; Table 3) treatment with $SnCl_2$ removed this threat from predicted population-level effects (Cohen's *d* = -1.01 and -0.2 for Hg[IC_{25} survival]+[Sn1] and Hg[IC_{25} reproduction]+[Sn2], respectively, compared to the control; Table 3). Further, $SnCl_2$ itself is not predicted to have a large effect on populations of *C. dubia* (Cohen's *d* = -1.26 and -1.76 for [Sn1] and [Sn2], respectively, compared to the DMW control; Table 3).

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

While standard toxicity tests often use artificial growth media Hg speciation and therefore toxicity may be significantly different in natural waters (Miller et al., 2009). We observed similar effects on *C. dubia* survival when exposed to 5000 ng L^{-1} Hg in DMW and FC water with respective decreases of 60 and 50% of survival compared to the control media (i.e. Hg-free DMW and FC;

Figure 3A). We observed similar results on *C. dubia* growth, with decreases of 16% and 13% when exposed to the same concentrations of Hg in DMW and FC water, compared to the respective control treatments (Figure 4). Effects of Hg on reproduction rate were greater in FC than in DMW with decreases of 72% in FC vs. 53% for DMW relative to the controls (Figure 3B).

The calculated *r* values also indicate that our lab experiments may be relevant for estimation of the ecological risk of Hg in natural systems (Figure 6). First, the DMW control *r* values (r = 0.78 and 0.71 day^{-1} from Experiments 1 and 2, respectively) are a little higher but similar to the value found for *C. dubia* in FC (r = 0.65 day^{-1}; Figure 6). Further, the *r* values are similar in *C. dubia* exposed to 5000 ng Hg L^{-1} in DMW and FC (r = 0.42 and 0.31 day^{-1}; Cohen's *d* value = -1.30), however again there is a greater negative response in FC compared to DMW but this may be due to experimental differences (Figure 6). *C. dubia* in general had lower *r* values for comparable treatments in Experiment 2 compared to Experiment 1 (Cohen's *d* value = -5.53 comparing DMW Controls from Experiment 2 to Experiment 1), which could indicate some subtle difference in the individuals in the different experiments. This could explain the decreased *r* value for *C. dubia* exposed to 5000 ng Hg L^{-1} in FC in Experiment 2 compared to the *r* value for *C. dubia* exposed to 5000 ng Hg L^{-1} in DMW in Experiment 1. Overall, these data support the comparison between the predicted effects of Hg in artificial medium (DMW) and stream water (First Creek Water) on *C. dubia* populations.

4. Discussion

We observed that Hg exposure affected the fitness of *C. dubia* with chronic effects found on survival, production of neonates and growth. Survival was significantly affected by Hg. Indeed, a decrease in survival was observed in *C. dubia* exposed at 5000 ng Hg L^{-1} while no survival was observed at the end of the chronic test at concentrations ≥ 10000 ng Hg L^{-1} with an estimated IC_{25} of 2109 ng L^{-1}. Previous studies experimentally assessed the effects of aqueous Hg in Daphniidae (Biesinger and Christensen, 1972; Kim et al., 2017; Rodrigues et al., 2013; Tsui and Wang, 2005, 2006). While the IC_{25} value we report for survival in *C. dubia* was similar to the one we estimated from Biesinger et al. (1982) data for *D. magna* (~1700 ng Hg L^{-1}), effects of Hg on Daphniidae survival are highly dependent of the testing conditions (e.g. temperature, population, body size, and pre-exposure). For example, Tsui and Wang (2006) found the dose causing 50% of mortality (LC_{50}) after 24 or 48h of exposure to aqueous Hg for *D. magna* to be highly variable, ranging from 5 to ≥ 250 μg Hg L^{-1}. From the initial 48h of the chronic assay performed in Experiment 1, the LC_{50} in *C. dubia* is 7.04 µg Hg L^{-1} suggesting that Hg has similar effects on survival of *C. dubia* and *D. magna*.

The IC_{25} value we calculated for reproduction in *C. dubia* (888 ng Hg L^{-1}) was lower than that for survival (2109 ng Hg L^{-1}). Our findings corroborate Biesinger et al. (1982) where IC_{25} for reproduction in *D. magna* was estimated at ~1150 ng L^{-1} for inorganic Hg. While acute or lethal toxicity may occur at elevated Hg concentrations, in natural systems, Hg concentrations are likely to be much lower than the lethal concentrations found in this study. Because sublethal responses on reproduction are more likely to govern population responses than lethal responses measured at high toxic exposure levels, reproduction toxicity tests are among the few sensitive ecotoxicological tests that provide the basis for legislative decisions (Breitholtz et al., 2006; Rudén et al., 2017). In our study, the reproduction rate during the chronic assay was significantly reduced by increasing Hg concentrations of 500 and 5000 ng L^{-1} while no reproduction was observed at 10 000 ng Hg L^{-1} with the last female died after 5 days. Indeed, these concentrations are predicted to have population-level impacts based on our calculations of the long-term growth rate (*r* values; Figure 5). Negative effects of Hg in reproduction was also found in *Daphnia* sp. with a reduction in the production of neonates (e.g. Biesinger and Christensen, 1972; Biesinger et al., 1982; Tsui and Wang, 2005a) and deleterious effects on embryonic development (Khangarot and Das, 2009).

While growth was similar at 0 and 500 ng Hg L^{-1}, final total length was 16% less than controls at 5000 ng Hg L^{-1}, showing that Hg also affected *C. dubia* growth (Figure 4). Animals under Hg stress direct more energy towards detoxification and recovery at the expense of other mechanisms such as feeding, somatic growth and reproduction (Fong et al., 2019; Issa et al., 2021). Past studies also highlighted that metals exposure may change cellular energy allocation in *D. magna* (Bossuyt and Janssen, 2004; Tsui and Wang, 2004a) while a direct relationship between energy reserves and the tolerance of *D. magna* to acute metal toxicity was found by (Canli, 2005). Mercury stress can decrease the amount of resources available to *C. dubia* by decreasing their filtration activity and also by impairing their swimming ability through oxidative stress and thus alter feeding behavior as shown in *Daphnia* sp (Bownik, 2017; Lopes et al., 2014). As a consequence, reduced food intake can subsequently result in deleterious effects on the endpoints we looked at in the present study.

Tsui and Wang (2004a) found that inorganic Hg predominantly accumulated in *D. magna* via the dissolved pathway. In the present study, we found a BCF of 4812 and 2005 L kg^{-1} in *C. dubia* with respective exposures of 5000 and 500 ng Hg L^{-1}. The BCF values we measured in *C. dubia* are in accordance with BCF values reported for *D. magna* (Tsui and Wang, 2004a) but remained at least 1 or 2 order of magnitude lower than the BCF usually observed for wild freshwater zooplankton (BCF of 10^3 to 10^6; Back and Watras, 1995) suggesting Hg uptake in *C. dubia* did not reach steady state within the 7-d experiment. Because we found that the uptake of Hg by *C. dubia* was directly proportional to the ambient concentrations we can reasonably assume that Hg bioaccumulation in *C. dubia* in our study was mostly driven by passive uptake. Although mechanisms of Hg bioaccumulation in Daphniidae are not fully elucidated yet, a recent experimental *in vivo* study provided new insights of inorganic Hg bioaccumulation in *D. carinata*. Using aggregation-induced emission fluorogen, He et al. (2019) found that the carapace and eye are the major recipient organs of aqueous Hg, presumably due to their chitin content, followed by the digestive (intestine) and excretory (shell gland) organs (Barriada et al., 2008).

Muna et al. (2018) studied the combined effects of dietary algae and test media and found that the toxicity of $CuSO_4$ and $ZnSO_4$ in *D. magna* were mitigated by algal addition in the natural lake water media but not in the artificial medium. Such results were not expected and mechanisms underlying such differences remain largely unknown. To assess the environmental relevance of the experimental approach we used, we compared effects in *C. dubia* when exposed to artificial medium and stream water (i.e. FC water). We found slight differences in the controls: survival was 80% in control FC water while no mortality was observed in the control DMW. However, when the media were spiked with Hg, the toxicity response relative to the respective control media was similar between DMW and FC water. These findings suggest that our conclusions based on DMW medium may be environmentally relevant.

Environmental concentrations for Hg are generally much lower than the Hg concentrations used in the present study (0 - 22 ng L^{-1} in average based on a worldwide survey of published measurements collected in rivers flowing into estuaries; Amos et al., 2014). However, at industrially contaminated sites (e.g. mining etc.) concentrations can be up to thousand times greater than maximum baseline concentrations for total Hg (Wentz et al., 2014; Brooks and Southworth, 2011). We experimentally used $SnCl_2$ treatment to remove Hg from DMW media.

Previous field pilot studies have demonstrated the efficiency of $SnCl_2$ for Hg stripping from water with abatement of ~90% (Mathews et al., 2015; Southworth, 1996; Southworth et al., 2009). In the present study, $SnCl_2$ used at a Sn:Hg stoichiometric ratio of 5:1 (Jackson et al., 2013; Looney et al., 2003) and coupled with air bubbling air was effective for Hg stripping with 100% of Hg removed after a 16-h treatment regardless of the initial concentrations of Hg used (i.e. 888 and 2019 ng L^{-1}). While $SnCl_2$ appeared to be effective in reducing concentrations of toxic heavy metals in field trials, the effects of the added Sn to aquatic ecosystems had not been previously evaluated. The present study demonstrates the absence of toxicological effects of Sn at concentrations of 2.1 ± 0.4 and 10.9 ± 0.6 µg L^{-1} in the model invertebrate *C. dubia* that is consistent with Kungolos et al. (2004) and Ziksari et al. (2014) who found EC_{50} and LC_{50} values ranging from 10 to 2000 µg L^{-1} (i.e. concentrations 10 to 200-fold concentrations used in the present study) for tin nanoparticles and Sn^{4+} respectively in *D. magna*. As a comparison, Mathews et al. (2015) found that pilot $SnCl_2$-based treatment system increased aqueous Sn concentrations from <0.5 to ~12 µg L^{-1} in treated stream. Interestingly, in addition to the absence of toxicological effects of Sn on growth, survival and reproduction, we also demonstrated that the stripping of Hg using $SnCl_2$ allowed a full recovery of the three investigated endpoints, including predicted population-level effects. Nevertheless, in the present study, the bioaccumulation of Sn was not been measured in *C. dubia* and the potential long-term impacts of an Sn enrichment at low trophic levels in freshwater ecosystems remain unknown (Mathews et al., 2015) and need to be investigated before $SnCl_2$ can be considered a treatment to remediate Hg in natural environments.

5. Conclusion

Our experimental results indicate that inorganic Hg causes chronic effects on *C. dubia*. Increasing aqueous concentrations of Hg induced mortality, reproduction inhibition, reduced growth, and decreased long term population growth rates. Toxicity of Hg has been confirmed both in artificial media and stream water. We demonstrated that $SnCl_2$ treatment is efficient to remove Hg from the water column and also removes the toxicological effects of Hg on survival, reproduction, growth, and predicted population growth. Our findings indicate that at concentrations relevant for Hg removal, the addition of Sn does not cause any toxicological effects on *C. dubia*. The effectiveness of $SnCl_2$-based remediation of Hg has already been demonstrated in the field but the toxicological risks of this remediation treatment were previously unknown.

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Captions to Figures

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

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

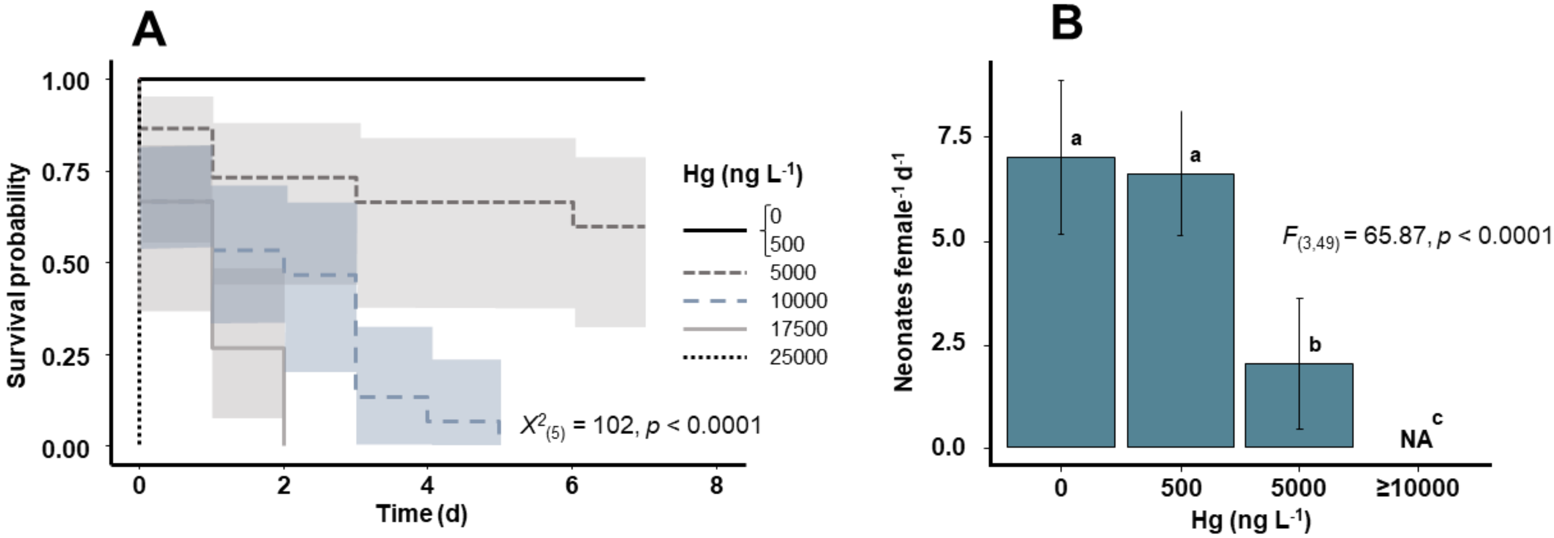
Figure 3. Survival kinetics (A) and reproduction rates (expressed as the number of neonates female^{-1} d^{-1}, Means ± SD, see Section 2.5 for details) (B) of *Ceriodaphnia dubia* (n = 10 per condition) exposed for 7 days to experimental media containing $SnCl_2$ and/or Hg based on DMW or First Creek water. Letters denote significant differences.

Figure 4. Final length (in mm from the top of the eye to the base of the spine; (Agatz et al., 2015) *of Ceriodaphnia dubia* (n = 4-10 per condition) exposed for 7 days to experimental media containing $SnCl_2$ and/or Hg based on free-EDTA DMW or First Creek water. Letters denote significant differences.

Figure 5. Long term population growth rates (*r* values) for *Ceriodaphnia dubia* exposed to experimental media containing $SnCl_2$ (Experiment 1, Panel A) and/or Hg based on DMW or First Creek water (Experiment 2, Panel B). Population growth rates cannot be calculated for treatments in which no individuals survived or reproduced (e.g. Hg concentrations above 10000 ng L^{-1}), however these populations are predicted to decline to extinction, similar to those with negative *r*

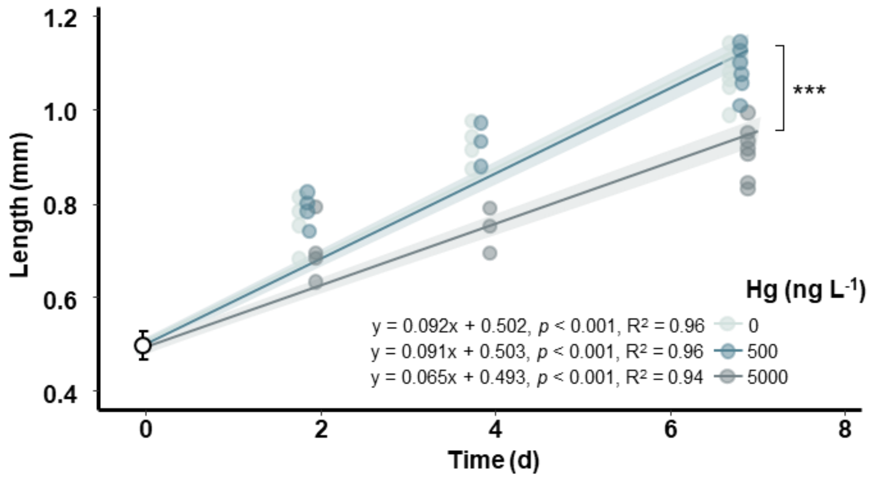
values. The boxplots represent the distribution of 1000 iterations of bootstrapped *r* values with replacement (see text for methods description) and the red dots represent the *r* value of the data set.

Figure 6. Long term population growth rates (*r* values) for *Ceriodaphnia dubia* exposed to experimental media containing Hg comparing controls using DMW (freshwater media) or First Creek water. The boxplots represent the distribution of 1000 iterations of bootstrapped *r* values with replacement (see text for methods description) and the red dots represent the *r* value of the data set.



A
Survival probability
1.00
0.75
0.50
0.25
0.00
0
2
4
6
8
Time (d)
Hg (ng L-1)
0
500
5000
10000
17500
25000
X2(5) = 102, p < 0.0001
B
Neonates female-1 d-1
7.5
5.0
2.5
0.0
a
a
b
NAc
F(3,49) = 65.87, p < 0.0001
0
500
5000
≥10000
Hg (ng L-1)

Figure 1



1.2
1.0
0.8
0.6
0.4
Length (mm)
0
2
4
6
8
Time (d)
***
Hg (ng L-1)
y = 0.092x + 0.502, p < 0.001, R² = 0.96
y = 0.091x + 0.503, p < 0.001, R² = 0.96
y = 0.065x + 0.493, p < 0.001, R² = 0.94
0
500
5000

666 Figure 2

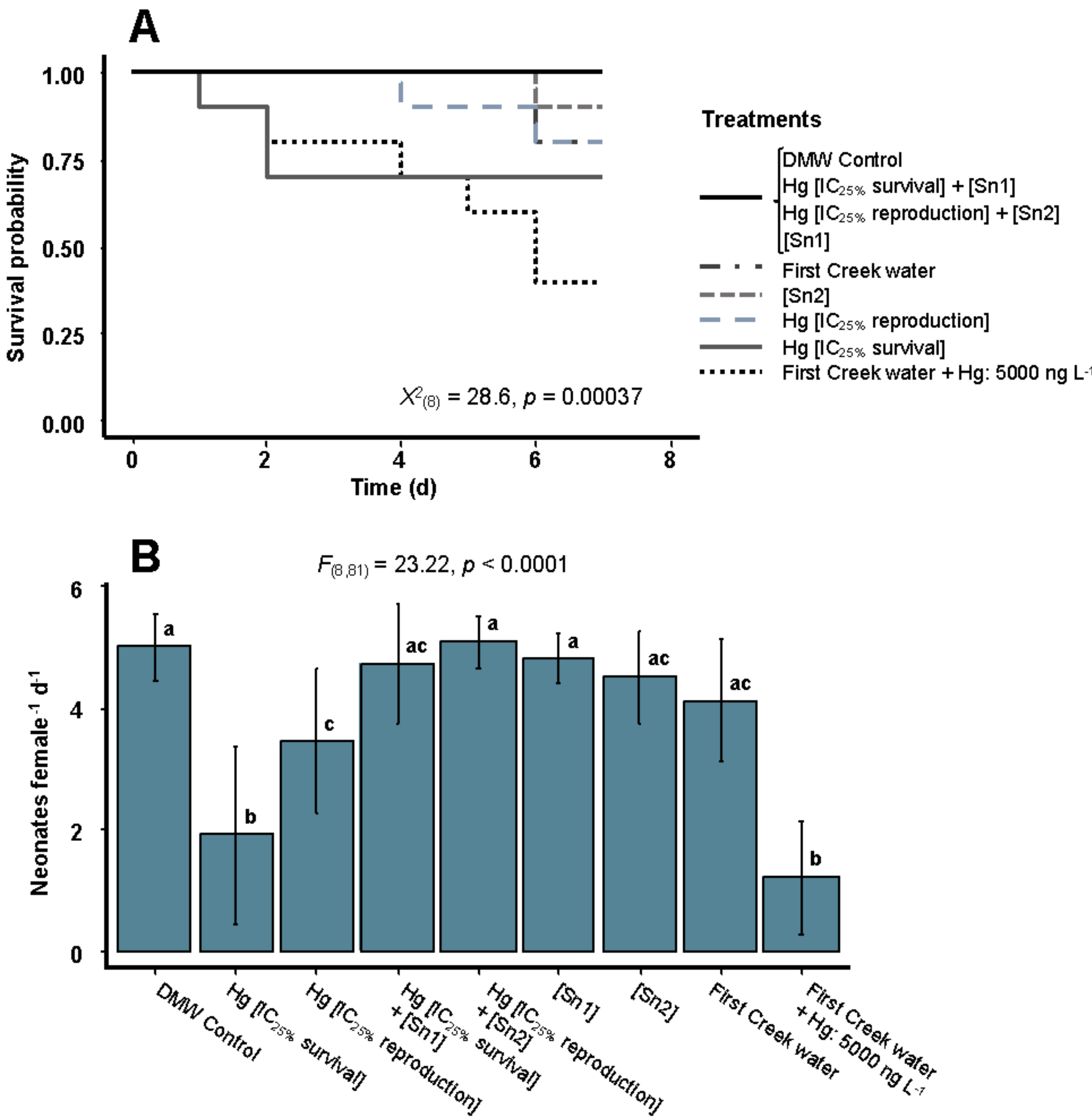
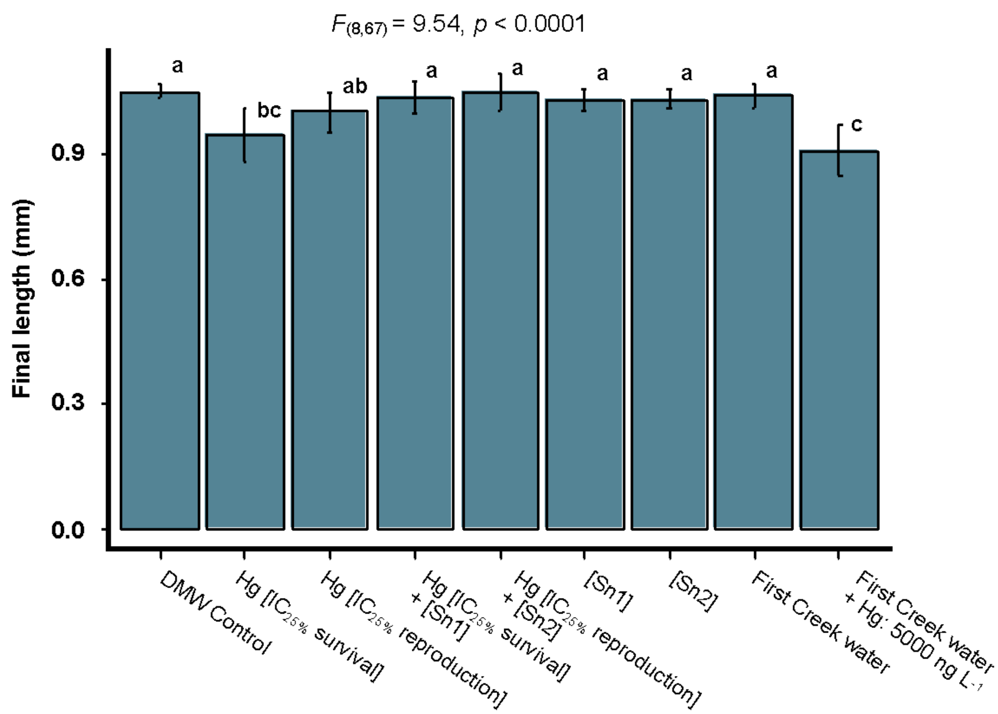

A
Survival probability
1.00
0.75
0.50
0.25
0.00
0
2
4
6
8
Time (d)
$X^2_{(8)}$ = 28.6, p = 0.00037
Treatments
DMW Control
Hg [$IC_{25\%}$ survival] + [Sn1]
Hg [$IC_{25\%}$ reproduction] + [Sn2]
[Sn1]
First Creek water
[Sn2]
Hg [$IC_{25\%}$ reproduction]
Hg [$IC_{25\%}$ survival]
First Creek water + Hg: 5000 ng L^{-1}
B
$F_{(8,81)}$ = 23.22, p < 0.0001
Neonates female^{-1} d^{-1}
6
4
2
0
a
b
c
ac
a
a
ac
ac
b
DMW Control
Hg [$IC_{25\%}$ survival]
Hg [$IC_{25\%}$ reproduction]
Hg [$IC_{25\%}$ survival] + [Sn1]
Hg [$IC_{25\%}$ reproduction] + [Sn2]
[Sn1]
[Sn2]
First Creek water
First Creek water + Hg: 5000 ng L^{-1}

Figure 3



$F_{(8,67)}$ = 9.54, p < 0.0001
Final length (mm)
0.0
0.3
0.6
0.9
a
bc
ab
a
a
a
a
a
c
DMW Control
Hg [IC25% survival]
Hg [IC25% reproduction]
Hg [IC25% survival] + [Sn1]
Hg [IC25% reproduction] + [Sn2]
[Sn1]
[Sn2]
First Creek water
First Creek water + Hg: 5000 ng L-1

668 Figure 4

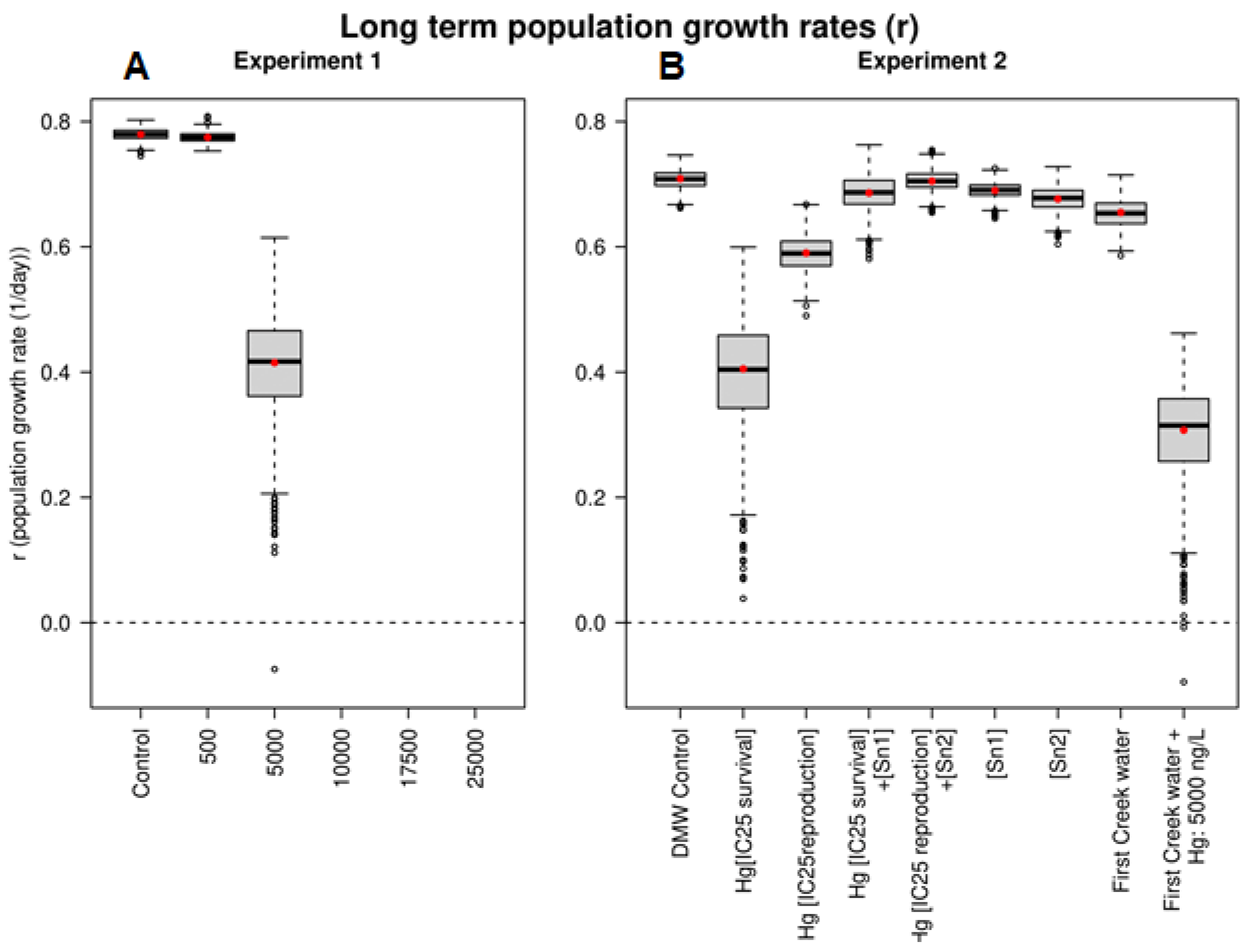
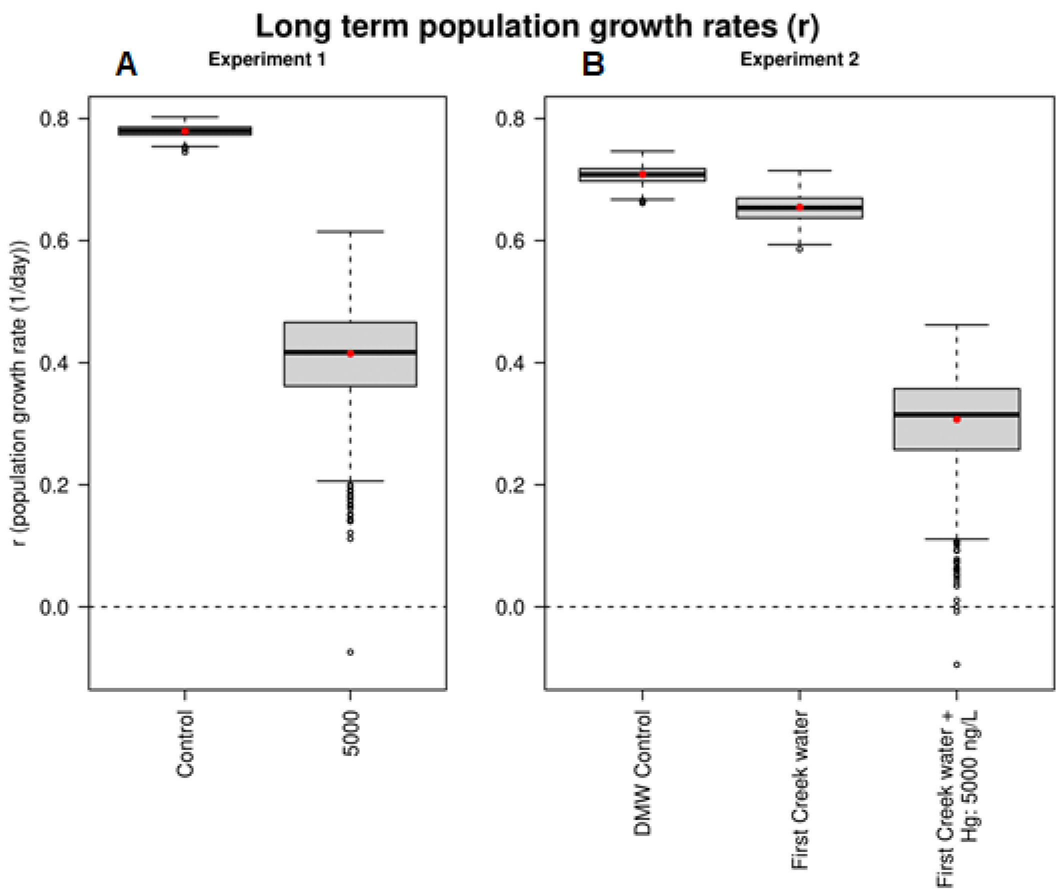

Long term population growth rates (r)
A
Experiment 1
B
Experiment 2
r (population growth rate (1/day))
0.8
0.6
0.4
0.2
0.0
Control
500
5000
10000
17500
25000
DMW Control
Hg[IC25 survival]
Hg [IC25reproduction]
Hg [IC25 survival] +[Sn1]
[IC25 reproduction] +[Sn2]
[Sn1]
[Sn2]
First Creek water
First Creek water + Hg: 5000 ng/L

Figure 5



Long term population growth rates (r)
A
Experiment 1
B
Experiment 2
r (population growth rate (1/day))
0.8
0.6
0.4
0.2
0.0
Control
5000
DMW Control
First Creek water
First Creek water +
Hg: 5000 ng/L

670 Figure 6

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 $SnCl_2$-treated media. Values are Means ± 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 (µg L^{-1})	pH	Conductivity (µS 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 ± 0^a	-	8.37 ± 0.05	150 ± 3	8.41 ± 0.14	55 ± 1	68 ± 1	0.04 ± 0.02	0.004 ± 0.003	0.36 ± 0.11
Hg: 500 ng L^{-1}	501 ± 17^b	-	-	-	-	-	-	-	-	-
Hg: 5000 ng L^{-1}	4791 ± 145^c	-	-	-	-	-	-	-	-	-
Hg: 10000 ng L^{-1}	9825 ± 435^d	-	-	-	-	-	-	-	-	-
Hg: 17500 ng L^{-1}	17119 ± 282^e	-	-	-	-	-	-	-	-	-
Hg: 25000 ng L^{-1}	23279 ± 841^f	-	-	-	-	-	-	-	-	-
Experiment 2										
DMW Control	0 ± 0^a	0.2 ± 0^a	8.33 ± 0.11^a	156 ± 3^a	8.53 ± 0.19^a	56 ± 2^a	70 ± 2^a	0.03 ± 0.03^a	0.004 ± 0.002^a	0.44 ± 0.03^a
Hg [IC_{25} survival]	2023 ± 113^b	-	-	-	-	-	-	-	-	-
Hg [IC_{25} reproduction]	854 ± 71^c	-	-	-	-	-	-	-	-	-
Hg [IC_{25} survival] + [Sn1]	0 ± 0^a	8.6 ± 0.6^b	-	-	-	-	-	-	-	-
Hg [IC_{25} reproduction] + [Sn2]	0 ± 0^a	3.1 ± 0.6^c	-	-	-	-	-	-	-	-
[Sn1]	0 ± 0^a	10.9 ± 0.6^b	8.33 ± 0.05^a	156 ± 3^a	8.51 ± 0.25^a	57 ± 1^a	69 ± 2^a	0.05 ± 0.03^a	0.005 ± 0.003^a	0.36 ± 0.04^a
[Sn2]	0 ± 0^a	4.3 ± 0.8^c	8.34 ± 0.05^a	156 ± 3^a	8.53 ± 0.23^a	58 ± 2^a	69 ± 1^a	0.04 ± 0.05^a	0.005 ± 0.003^a	0.036 ± 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 $SnCl_2$-treated media.

Media	Hg in media (ng L^{-1})	Survival (%)	Reproduction (neonates $female^{-1}$)	Hg in *C. dubia* (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	4812
Hg: 5000 ng L^{-1}	4791 ± 145	60	14 ± 11	10.0	2005
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 [IC_{25} survival]	2023 ± 113	70	15 ± 10	-	-
Hg [IC_{25} reproduction]	854 ± 71	80	23 ± 10	-	-
Hg [IC_{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	31 ± 6	-	-
First Creek water	0 ± 0	80	28 ± 7	-	-
First Creek water + Hg: 5000 ng L^{-1}	4530 ± 1045	40	9 ± 6	-	-

BCF: Bioconcentration factor

Table 3. Standardized mean differences (Cohen's *d* values) and associated confidence intervals (CI) comparing the effect of the various treatments on the long-term population growth rate (*r* value) of *C. dubia*. We compared *r* values within each experiment (comparing the treatment to that experiment's control). CIs that do not include 0 indicate statistical significance ($p < 0.05$, denoted by an asterisk), however refer to text discussion of the relevance of the statistical significance of these calculations.

Media	r value calculated from full data set (d^{-1})	Mean r value of 1000 bootstrapped iterations (d^{-1})	Cohen's d[a]	CI[a]
Experiment 1				
DMW Control	0.78	0.78	0	
Hg: 500 ng L^{-1}	0.77	0.77	-0.52	[-0.61 -0.43]*
Hg: 5000 ng L^{-1}	0.42	0.41	-6.32	[-6.54 -6.11]*
Experiment 2				
DMW Control	0.71	0.71	0	
Hg [IC_{25} survival]	0.41	0.40	-4.89	[-5.07 -4.72]*
Hg [IC_{25} reproduction]	0.59	0.59	-5.19	[-5.37 -5.01]*
Hg [IC_{25} survival] + [Sn1]	0.69	0.69	-1.01	[-1.1 -0.91]*
Hg [IC_{25} reproduction] + [Sn2]	0.70	0.70	-0.2	[-0.29 -0.11]*
[Sn1]	0.69	0.69	-1.26	[-1.36 -1.17]*
[Sn2]	0.68	0.68	-1.76	[-1.86 -1.66]*
First Creek water	0.65	0.65	-2.77	[-2.89 -2.65]*
First Creek water + Hg: 5000 ng L^{-1}	0.31	0.30	-7.12	[-7.35 -6.88]*

[a]Compared to the control for that experiment

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: