*Title Page

Mercury accumulation from food decreases collembolans' growth

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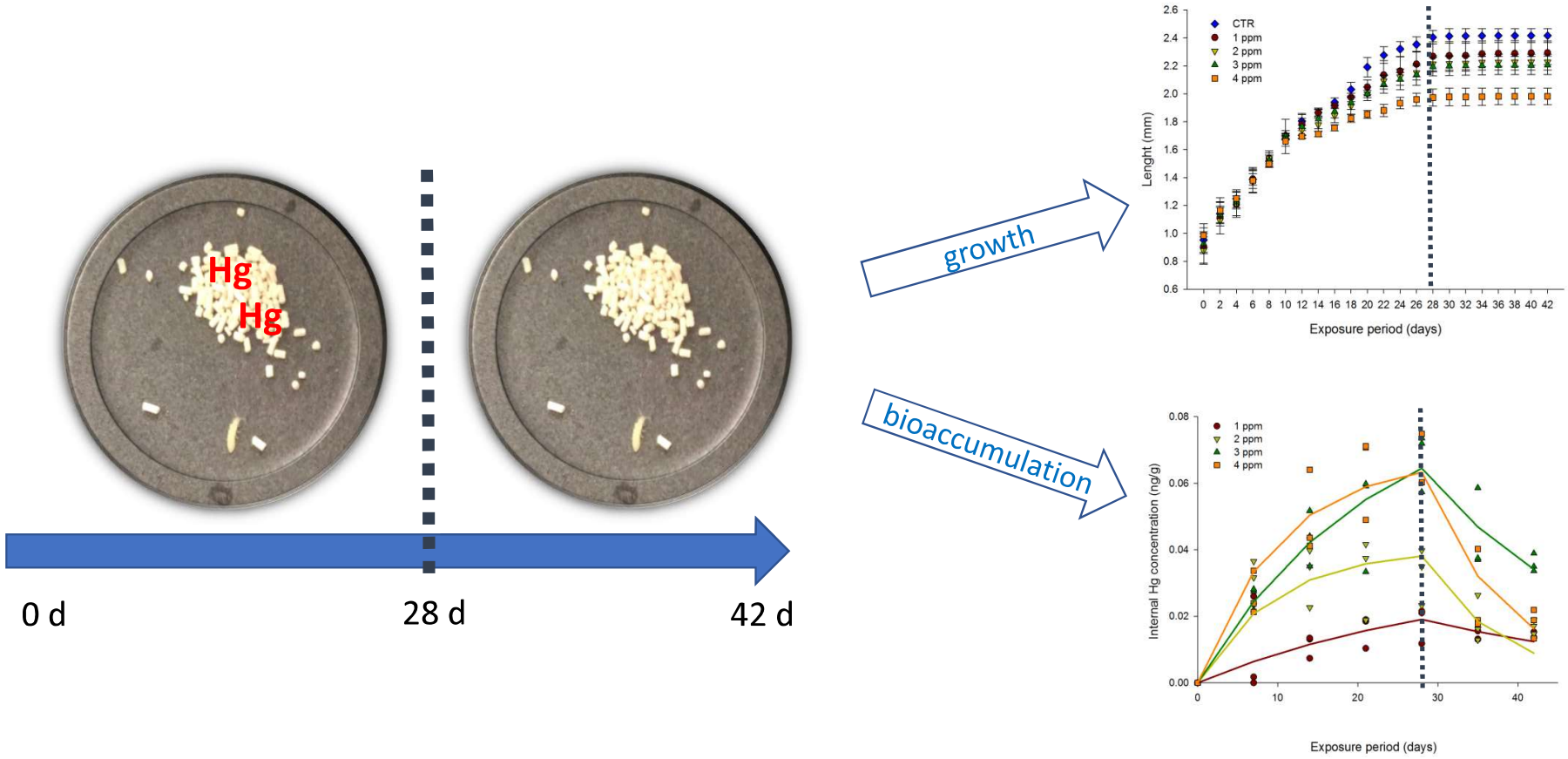
*Graphical Abstract



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

Highlights

- Exposure to mercury through food reduces the growth of collembolans;
- Mercury uptake in the organisms through a dietary exposure increases Hg inside organisms;
- A possible maximum sub lethal concentration of 0.07 ng Hg/g for collembolans was found;
- Food avoidance may occur in the presence of contaminated food at high Hg levels;
- At higher mercury levels, collembolans presented a higher elimination rate.

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Abstract

In the terrestrial environment mercury (Hg) is redistributed and transformed into different inorganic and metal - organic species that are deposited into soils. In the present study, the effects of contaminated food with environmentally relevant concentrations of Hg in the form of $HgCl_2$ on the soil-dwelling collembolan *Folsomia candida* were assessed. Changes in growth rate and Hg bioaccumulation levels were observed at different concentrations of Hg in food complementing data on the effects of Hg on reproduction and survival using standardized protocols. Collembolan growth was recorded every two days, and a Von Bertalanffy's growth curve was derived along with the growth rate. Collembolan growth was dependent on the Hg food concentration. Also, the final length of animals was affected by the presence of Hg in food, with differences in all treatments comparing to non-exposed organisms. Toxicokinetic patterns from Hg exposure in food at different concentrations were not significantly different from each other in the uptake, but differences were found in the depuration phase. Combining the two approaches, collembolans seems to invest their effort in the depuration process, neglecting their efforts in other vital processes, such as growth. Also, metal contaminated food avoidance possibly occurred, a behavior already reported in the literature, thus decreasing their feeding and contaminant intake. Therefore, growth tests can act an important asset to fill the gaps of bioaccumulation tests and reproductive assays, towards a mechanistic understanding. Changes in growth rate, even at low and environmentally relevant concentrations, could be a warning signal when occurring in species with key roles in ecosystems. Also, this study highlights the importance of these complementary tests for a better and complete approach to risk assessment studies.

Key-words: Mercury, contaminated food, bioaccumulation, Von Bertalanffy's growth curve

Capsule

This study is a step forward on the understanding of mercury exposure effects to soil organisms, using growth and bioaccumulation in time as endpoints.

1 - Introduction

Mercury (Hg) pollution is a worldwide problem, posing a serious threat to ecosystems and consequently to humans, due to its ability to biomagnify along food chains (Boening, 2000). Being a class B metal (Nieboer and Richardson, 1980), Hg has high affinity for reduced sulphur atoms such as those from proteins and peptides containing thiol, which could lead to a disruption of the tertiary structure of proteins, necessary for optimal function and condition of organisms (Valko et al., 2005). Furthermore, Hg can also affect organisms at a cellular level, depleting cellular antioxidation systems, which can lead to the production of reactive oxygen species inducing oxidative stress damage - (e.g., peroxidation of membrane lipids) - (Stohs and Bagchi, 1995). Since Hg is a widely-distributed contaminant, it is transported by air and deposited in areas far from emission sources (Pacyna et al., 2009), on the top-soil layer, and a consequent redistribution and further uptake by vegetation may occur (Miller et al., 2005). Besides that, Hg can be retained in soils for a period of 500 to 1000 years (Pendias et al., 2011), and suffer transformations into organic forms (most commonly methylmercury), through microbial activity, thus becoming more toxic to organisms (Wang et al., 2003). Organic and inorganic forms of Hg will eventually enter food webs and bioaccumulate and biomagnify in soil invertebrates (Pedrini-Martha et al., 2012; Wiener et al., 2006).

Despite the growing concern about the potential adverse effects of elevated mercury concentrations in the environment, the toxicity data available for soil invertebrates is scarce. Some examples are studies in earthworms (Abbasi and Soni, 1983; Beyer et al., 1985; Buch et al., 2017; Fischer and Koszorus, 1992; Mahbub, et al., 2017.), millipedes (Buch et al., 2018),

collembolans and enchytraeids (Buch et al., 2016; Lock and Janssen, 2001). Since those studies were conducted in different substrates, some toxicity differences were observed, as soil characteristics have a significant influence on the bioavailability and toxicity of metals. High pH values and organic matter content could lead to an increase in chemical sorption and consequently a decrease in bioavailability and toxicity (Lock and Janssen, 2001; Sandifer and Hopkin, 1996). The interactions of Hg with temperature were also reported, reducing the cold tolerance of earthworms and collembolans in the presence of Hg, with a dominant synergistic pattern between these two stressors (Bindesbøl et al., 2009; Holmstrup et al., 2008)

Current recommended ecotoxicological tests with the springtail *Folsomia candida* provide useful data regarding their reproduction output and survival, upon a 28-day exposure (ISO, 1999; ISO, 2014). Xenobiotics exposure to collembola is mainly carried out and assessed through the interstitial soil pore water (Fountain and Hopkin, 2005), but the evaluation of other routes of exposure are essential in risk assessment studies. Contaminated food exposure can, therefore, complement the information provided by the already existing standardized tests, facilitating, for example, the observation of growth, and providing additional information on xenobiotics' effects on organisms (Roex et al., 2003). It is widely known that the primary dietary component of most springtails is fungi (Van Straalen and Van Meerendonk, 1987), and most fungal species accumulate metals (e.g., mercury) in their hyphae (Bengtsson et al., 1983), being food a pertinent source of contaminants. Also, using growth as an endpoint is of utmost importance for the equilibrium and sustainability of any species, as a feature closely related to the organism reproduction (Fountain and Hopkin, 2001). In addition, the exposure of xenobiotics through food can also provide new insight on toxicity effects to soil invertebrates as their central role in the ecosystem is related to decomposition processes (Crouau and Moïa, 2006). In an organism, growth is dependent upon food quality availability and the metabolism efficiency, which can be affected by the presence of xenobiotics/contaminants, being,

100 therefore, a relevant endpoint to assess environmental hazard (Crommentuijn et al., 1993;
101 Folker-Hansen et al., 1996).

102 Considering the above mentioned, this study aimed at assessing the effects of Hg on *Folsomia*
103 *candida* growth and to infer if Hg bioaccumulation patterns were related to the observed
104 growth patterns. For that, organisms were exposed to Hg-contaminated food at different
105 relevant concentrations (1 to 4 ppm) for a 28 days' exposure period. During this period
106 collembolan growth was recorded every two days, and a Von Bertalanffy growth curve derived
107 along with the correspondent growth rate. At the same time, a similar experimental setup was
108 conducted to derive bioaccumulation patterns and toxicokinetic parameters (uptake and
109 elimination rates) also at different exposure concentrations. These two experiments
110 complemented each other to understand the mechanisms of toxicity that Hg can induce in *F.*
111 *candida*.

112

113 **2 – Material and methods**

114 **2.1 - Test species and test chemical**

115 Collembolans from the species *F. candida* were obtained from synchronized laboratory
116 cultures maintained at the University of Aveiro, Portugal. Cultures are kept in plastic boxes
117 lined with a mixture of plaster of Paris and activated charcoal in a ratio of 9:1 (ISO, 2014), in
118 the dark, under a constant temperature regime (20 ± 2 °C). Once a week, granulated dry yeast
119 was added as a food source (Fermipan, Setúbal, Portugal).

120 The same yeast provided in cultures was contaminated with mercury (II) chloride ($HgCl_2$ - CAS
121 no: 7487-94-7) purchased from Merck Millipore (99.5 % purity).

122

123 **2.2 – Growth test with *Folsomia candida***

124 The growth test was run in two phases: 1) an exposure phase, where organisms were exposed
125 to contaminated yeast for 28 days and 2) a post-exposure phase, where organisms were
126 moved to clean vials with clean food for 14 days. The test started with 10 days old
127 collembolans that were exposed to Hg via food, provided as contaminated yeast with 1, 2, 3
128 and 4 ppm of Hg. The chosen concentrations are environmental relevant since concentrations
129 ranging from 0.003 to 4.6 mg/kg of Hg have been reported in soils worldwide (E. Steinnes,
130 1997). Also, the mean content of Hg in soils was estimated to be 1.1 mg/kg, with an average
131 background content in different soil types ranging from 0.58 to 1.8 mg/kg (Xu et al., 2015).
132 Each replicate included one organism in a cylindrical plastic pot (30 cm^3), on a moist substrate
133 of 9:1 (w/w) plaster of Paris:charcoal mixture. Granulated dry yeast was previously spiked with
134 the respective Hg solutions and dried by a lyophilization process. Afterward, it was supplied *ad*
135 *libitum* during the exposure phase and being replaced every week or in the presence of fungi.
136 A total of 10 replicates were used per control, Hg treatments and sampling time. Digital
137 photographs were taken every two days for 42 days, and the collembolan's length was
138 recorded from the end of the posterior abdominal segment to the anterior margin of the head,
139 using the Image J analysis software program (Schneider et al. 2012). Calibration run by using a
140 small millimeter paper placed near the measured organism.

141 Considering that collembolan finished the test with 52 days old, the appearance and number
142 of new-born juveniles were also recorded.

143

144 **2.3 – Bioaccumulation test with *Folsomia candida***

145 Running at the same time as the growth test, 18 replicates with 20 organisms each per Hg
146 concentration were also exposed to the same Hg concentrations following similarly the
147 procedures described in section 2.2. Briefly, organisms were exposed to contaminated food (1,
148 2, 3 and 4ppm Hg) for 28 days, followed by a non-contaminated yeast exposure for an extra 14

149 days' period. Organisms were sampled after 7, 14, 21, 28 days during the exposure period and
150 at day 35 and 42 (belonging to the post-exposure test), sacrificing 3 replicates in each sampling
151 time for bioaccumulation analysis (3 replicates used in each one of the 6 sampling times, a
152 total of 18 replicates initially performed). Samples were immediately frozen at -80 °C for
153 following chemical analysis. Before the measurements of total Hg inside the organisms were
154 dried by a lyophilization process.

155

156 **2.4 - Mercury analysis**

157 Total mercury (THg) concentration in yeast (food source) and collembolans was quantified by
158 atomic absorption spectrophotometry with thermal decomposition using an Advanced
159 Mercury Analyser (AMA) LECO 254 (Costley et al., 2000). The analytical procedure was adapted
160 as described by Cabecinhas et al. (2015) and Vieira et al. (2014): drying time of 60 s,
161 decomposition time of 150 s and waiting time of 45 s. The detection limit of the procedure was
162 0.02 ng Hg, and first and second working range of analysis (automatically switched) varied
163 between 0.05 – 40 ng Hg and 40-600 ng Hg, respectively.

164 Mercury determinations were all done in triplicate and blank run in parallel before and after
165 each sample to ensure the equipment was clean from any internal Hg contamination. All blank
166 measurements were run, at least, in triplicate until values obtained were consistently inferior
167 to the equipment detection limit (0.02 ng of Hg); additional blanks were, always performed at
168 the beginning and end of the day.

169 Accuracy and precision of the analytical method were assessed by the analysis of certified
170 reference materials (NRC TORT-2 Lobster hepatopancreas, and NRC DOLT-3 Dogfish liver) in
171 parallel with samples (Pereira et al., 2008). The average recovery of TORT-2 and DOLT-3 were
172 101 and 104%, respectively.

173

174 **2.5 - Data analysis**

175 Growth was described by the Von Bertalanffy expression:

176 $$L_t = L_\infty \left(1 - e^{-k(t-t_0)}\right) \quad (1)$$

177 Where L_t = length at time t (mm), L∞ = the asymptotic length (mm), k = growth rate and t_0 =
178 the hypothetical negative time estimated from hatching date for an individual with length 0
179 (days) (Bertalanffy, 1960). The theoretical growth curve was fitted to the average length of
180 individuals per replicate and the parameters L∞ and k were estimated. For comparisons
181 between the effects of Hg treatments and the negative control, slopes of the regression and
182 growth rate values (k) were compared by a generalized likelihood ratio test. Differences in
183 collembolans' length exposed to different Hg treatments, in different days of exposure were
184 analyzed using a one-way ANOVA, followed by post hoc Dunnett's test ($p < 0.05$), comparing to
185 the correspondent negative control (in time). To infer on the possible effects of Hg exposure
186 in collembolan's reproduction output, the day at which newborn juveniles were first observed
187 in each test box were compared between treatments and the control by using a Kruskal-Wallis
188 test followed by Dunn´s *post hoc* test. Differences in the weight of collembolans at different
189 sampling times were derived by a one-way ANOVA, followed by post hoc Dunnett´s test
190 (α=0.05). A two-way ANOVA followed by a Tukey Test (α=0.05) was also used to observe
191 statistical differences in the weight of collembolans in the different concentrations of Hg, at
192 different sampling times, also looking at the interaction between these two factors. The R-
193 squared (r^2) was obtained by dividing the sum of squares of each factor and of their interaction
194 by the total sums of squares of the two-way ANOVA assessing the percentage of variance
195 accounted for each factor in the ANOVAs.

196 For the bioaccumulation test, the toxicokinetics of Hg was described by applying a one-
197 compartment model. Uptake and elimination equations were fitted simultaneously. In the

model, body concentration of Hg was used, and the initial Hg concentration in the organisms ($Q_{(0)}$) was considered to be zero.

For the uptake phase, the following equation was used:

$$Q_{(t)} = \frac{k1}{k2} \cdot C_e \cdot \left(1 - e^{-k2.t}\right) \quad (2)$$

Where $Q_{(t)}$ - Hg body concentrations at t days (ng/mg); k_1 – uptake rate constant (kg_{food}/kg_{org} .day^{-1}); k_2 – elimination rate constant (day^{-1}); C_e – Hg exposure concentration (ng/mg); and t - time (days).

For the elimination phase, the metal body burden was described as:

$$Q_{(t)} = \frac{k1}{k2} \cdot C_e \cdot \left(e^{-k2.(t-t_c)} - e^{-k2.t}\right) \quad (3)$$

where t_c - time the animals are transferred to clean medium (days).

Differences in k_1 and k_2 values between concentrations were tested by a Generalized Likelihood Ratio test (GLR test). The assimilation rate (a) was calculated as $k1*C_e$, and the bioaccumulation factor (BAF) was calculated as k_1/k_2. Also, $BAF_{org/food}$ was also calculated as the Concentration of Hg in organisms/concentration of Hg in food although no equilibrium was reached (plateau) during the uptake phase. Hg half-lives (DT_{50}) in the collembolans were calculated as $ln2/k_2$. All calculations were performed using SPSS (version 20).

3 – Results and discussion

220 **3.1 Hg measurements in food**

221 The measured concentrations in yeast were 0.918, 2.03, 2.87 and 3.9 ppm of Hg for the
222 nominal concentrations chosen of 1, 2, 3, and 4 ppm, respectively. Since differences between
223 nominal and measured values were lower than 10%, nominal concentrations were used
224 throughout the manuscript. However, measured concentrations were used in the data
225 analysis.

226

227 **3.2 - Growth test with *Folsomia candida***

228 Collembolan growth was dependent on Hg concentration in food as observed in Figure 1. A
229 Von Bertalanffy growth curve was fitted to the data in all cases, and the values of L_∞ and K
230 derived (Table 1). For each Hg treatment and control, two curves were settled, and data are
231 presented separately in Table 1-A for data for the exposure phase only (first 28 days of the
232 experiment), and in Table 1-B for the 42-day period, including both exposure and post-
233 exposure phases (28 days of exposure to Hg-contaminated food and 14 days of recovery with
234 non-contaminated food).

235

236 Table 1 - Parameters in von Bertalanffy growth curves. Estimates of mean maximum length (L∞) and growth rate (k)
237 with asymptotic confidence intervals (C.I.) A- using growth measurements for 28 days of exposure (uptake); B –
238 using growth measurements for 42 days (28 days of exposure followed by 14 days of recovery).

239 A

[Hg] in food (ppm)	L_∞	C.I. 95%	K	C.I. 95%	r^2
0	3.619	3.460 – 3.778	0.029	0.027 – 0.031	0.981
1	3.05	2.906 – 3.194	0.036	0.033 – 0.039	0.958
2	2.917	2.795 – 3.038	0.037	0.035 – 0.040	0.965
3	2.776	2.673 – 2.880	0.041	0.038 – 0.044	0.960
4	2.238	2.191 – 2.285	0.057	0.055 – 0.060	0.960

240

241 B

[Hg] in food (ppm)	L_∞	C.I. 95%	K	C.I. 95%	r^2
0	2.862	2.799 – 2.924	0.042	0.04 – 0.044	0.961
1	2.614	2.551– 2.669	0.047	0.045 – 0.049	0.942
2	2.529	2.482 – 2.576	0.048	0.046 – 0.050	0.955
3	2.2458	2.416 – 2.500	0.051	0.049 – 0.054	0.949
4	2.106	2.083 – 2.130	0.065	0.063 – 0.068	0.949

242

243 As expected, collembolans reached their highest values of L∞ (maximum length) when
244 exposed to uncontaminated food decreasing its value with the increase of Hg food
245 concentration for the 28 days and 42 days fitting curves, as observed in Figure 1. Also, the
246 pattern was inverse for collembolans' growth rate (k) with values increasing with the increase
247 of Hg in food (28 and 42 days fitting curves). Curves fitted with the 28 days' dataset always
248 showed higher values for L_∞ and lower values for k when compared to curves fitted with the
249 42 days' data set. In addition, the statistical analysis showed significant differences in the
250 growth rate within the same treatment when comparing the 28 and 42 days fitting curves
251 (Table SD1; $p<0.05$). Using the same approach for collembolans exposed to for both 28 or 42
252 days curves, the growth rate was statistically different for all treatments comparing to the
253 control curve (Table SD1; $p<0.05$). Due to those differences, an analysis per day of exposure of
254 collembolans' length was performed for the 28 days of exposure. As expected, statistical
255 differences were found regarding growth for the 4-ppm treatment already at day 12 and
256 onward, comparing to the control (Dunnett's test, $p<0.05$). For collembolans exposed to
257 contaminated food with 3 and 2 ppm, statistical differences were found at day 14 and onward,
258 while for the 1ppm treatment only at day 20 and onward differences were attained, always
259 comparing with the control animals supplied with non-contaminated yeast (Dunnett's test,
260 $p<0.05$). These significant differences were maintained during the recovery period thus

showing that organisms were not completely recovering from Hg exposure even when clean food was provided (Dunnett's test, $p<0.05$).

The time of hatched eggs was also evaluated in our study. Analyzing the hatching day of juveniles, a significant delay was observed for organisms exposed to 4 ppm Hg in food (H_4= 15.747; p=0.003). The mean number of days for juveniles to hatch (±SD) in control, 1, 2, 3 and 4 ppm was respectively 22.1 (±0.78); 21.6 (±0.70); 22.3 (±0.71), 22.2 (±0.73) and 23.78 (±1.30) days. These results are in accordance with the ones found by Fountain and Hopkins (2011) where reproduction (eggs laid in time) was delayed, due to retarded growth at high metal concentrations. This species of collembolan has an unpigmented body and a gut that crosses the organisms' body, and when they avoid food and start eating the black mixture of plaster of Paris and activated charcoal, their gut acquires a visible black color. With the increase of Hg in food the number of organisms showing a black ribbon along the body also increased (personal observation/data not shown) thus supporting a possible avoiding food behavior. Avoidance behavior is well explained by Fountain and Hopkin (2001), extrapolating that the reduced growth of collembolans, when exposed to higher concentrations of metals, could be explained to presumably use of the substrate as an alternative food source to springtails on an attempt to acquire nutrition from the graphite.

In the study of Marigomez et al. (1986), the terrestrial slug *Arion ater* was exposed to concentrations of mercury chloride ranging 10 to 100 ppm, and significant food consumption and growth reductions were observed for concentrations higher than 10 ppm in a dose-related manner. These results are in accordance with ours since an increase in Hg concentrations leads to lower growth. Another study performed by Abassi and Soni (1983) kept adult earthworms (*Octochaetus pattoni*) in cement tanks for 60 days, at the same density as the one found in the wild, in a mixture of soil and animal dung contaminated with mercuric chloride ranging from 0.5 - 5.0 ppm Hg. The calculated LC_{50} was 2.39 ppm after 10 days´ decreasing to 0.79 ppm at

286 the end of the 60 days' exposure period. Differences in the Hg-induced toxicity could be
287 explained by different exposure routes, since earthworms would ingest the contaminated
288 food, but would also be exposed to the contaminated soil by dermal contact and soil ingestion.

289 Lock and Janssen (2001) studied the effects of Hg in three different representative soil
290 invertebrates: the enchytraeid *Enchytraeus albidus,* the collembolan *Folsomia candida* and the
291 earthworm *Eisenia fetida*. An EC_{50} of 9.16 ppm was calculated for *E. fetida* (21 days of
292 exposure), while an EC_{50} of 22 ppm for *E. albidus* (42 days of exposure) and an EC_{50} of 3.26
293 ppm for *F. candida* (28 days of exposure) were derived, based on data from reproduction
294 output for all organisms. A similar study conducted by Liu et al. (2010), using only the species
295 *F. candida* found a higher EC_{50} for reproduction of 9.29 ppm and an EC_{50} value for the
296 avoidance of 3.88 ppm in the presence of an agricultural fluvoaquic sandy loam soil (Cambisol,
297 9.0% clay, 21.8% silt, 69.2% sand) spiked with $HgCl_2$. Also, a more recent study from Buch et al.
298 (2016) assessed the effects of natural and artificial soils contaminated with Hg in two
299 collembolan species (*Folsomia candida* and *Proisotoma minuta*). For *F. candida* exposed to
300 natural soil, an AC_{50} (avoidance behavior) of 5.44 (CI 4.13–6.75) ppm, an EC_{50} for reproduction
301 of 3.40 (3.18–3.62) ppm and an LC_{50} of 6.12 (3.74–8.50) ppm were attained. Despite the
302 apparent differences between both studies (different exposure routes and different endpoints
303 analyzed), the range of concentrations used in our study is lower than the LC_{50} and $AC_{50,}$ and
304 the highest concentration used similarly to the EC_{50} derived by Buch et al. (2016). It should also
305 be noticed that in that study *F. candida* was more sensitive to mercury contamination than *P.*
306 *minuta*. Even knowing that different exposure routes could lead to differences regarding
307 toxicity, our results reveal that ingestion of contaminated food could be a very sensitive
308 endpoint and growth could be a relevant sensitive endpoint to look at.

309

310 **3.3 - Bioaccumulation test with *Folsomia candida***

311 Before Hg analysis, 20 collembolans per replicate from each treatment and control were
312 sampled and weighted at each sampling time. The results of the one-way ANOVA in each
313 sampling time revealed that until day 28 (complete uptake period), no statistical differences
314 were found in the weight of collembolans exposed to different Hg concentrations (ANOVA,
315 $p>0.05$). Collembolans sampled at day 35 (first sampling time in the recovery period) from the
316 2 and 3 ppm food exposure presented statistically higher weights than those from the control
317 (Dunnett´s test, $p<0.05$). Surprisingly, no statistical differences were observed for the ones
318 exposed to 4 ppm Hg in food compared to the control. Regarding the collembolans used in day
319 42, statistical differences in their weight were also observed, with collembola from the 4-ppm
320 exposure showing higher weights (Dunnett´s test, $p<0.05$), on a possible attempt to ingest as
321 much food as possible to compensate the lack of quality feeding when previously
322 contaminated food was supplied, as suggested above. Analyzing the results with a two-way
323 ANOVA, as expected, the time of exposure was the factor that explained the majority of the
324 collembola weight differences (two-way ANOVA, $F_{5,88} = 106.007$, $p<0.05$) with an R^2 of 0.82.
325 Regarding the factor for Hg concentration no statistical effect in collembolans' weight was
326 derived (two-way ANOVA, $F_{4,88} = 1.062$, $p>0.05$) with an R^2 of 0.014 with a slight interaction
327 with time of exposure (two-way ANOVA, $F_{20,88} = 2.23$, $p<0.05$; R^2 of 0.07).

328 The fit of the one-compartment kinetics model and the corresponding uptake and elimination
329 rate constants are shown in Figure 2 and Table 2. For 1 and 3 ppm treatments, Hg body
330 concentrations did not reach equilibrium after the 28 days of exposure. Mean Hg body
331 concentration (n=3) at the end of the uptake phase (28 days) was 0.02, 0.03, 0.07 and 0.07
332 ppm, corresponding to a total body burden of 0.07, 0.13, 0.25 and 0.26 ng of Hg at exposure
333 concentrations of 1, 2, 3 and 4 ppm, respectively. Within this, Hg values in collembola at day
334 28 of exposure increased with the increase in Hg concentrations in food, with the two highest
335 concentrations of exposure (3 and 4 ppm) producing similar body burden values.

Considering the size of collembolans decreased with increasing Hg concentrations, and their weight did not follow the same pattern, the hypotheses that an extra source of food occurred can be raised. This was somehow supported by observations of the presence of black material (possibly charcoal) in the digestive system of collembolans during the exposure to the highest Hg concentration. The hypothesis of extra ingestion of plaster of Paris and charcoal to compensate for the lack or low quality of the provided food source can be raised and corroborated with these findings. By looking at the kinetics curves patterns, collembolans exposed to 2 and 4 ppm were those deriving data that formed a (close to) plateau in the uptake phase, showing a potential equilibrium between uptake and elimination. This was also shown by the DT_{50} values at 2 and 4 ppm where Hg half-life in collembola was much lower than those from 1 and 3 ppm, revealing a faster elimination of Hg. The same pattern was also seen when comparing BAF values calculated with the kinetic parameters or through the concentrations measured.

Table 2 - Hg uptake and elimination rate constants (k_1 and k_2), bioaccumulation factor $(BAF)_{kinetics}$ and $BAF_{org/food}$ and time that takes for Hg to be reduced by half inside the organisms (DT_{50}) for the accumulation of Hg in *Folsomia candida* exposed for 28 days to Hg spiked food, followed by an elimination phase of 14 days. The 95 % confidence intervals are presented in brackets.

[Hg] in food (ppm)	K_1 ($kg_{food}/kg_{org}/day$)	K_2 (day^{-1})	$BAF_{kinetics}$ (kg_{food}/kg_{org})	$BAF_{[hg]org/[Hg]food}$ (kg_{food}/kg_{org})	DT_{50} (days)
1	0.0011 (0.001 - 0.002)	0.0307 (-0.001 - 0.062)	0.0359	0.018	22.6
2	0.0020 (0.001 - 0.003)	0.1038 (0.057 - 0.150)	0.0198	0.0165	6.7
3	0.0014 (0.001 - 0.002)	0.0456 (0.031 - 0.061)	0.0312	0.022	15.2
4	0.0016 (0.001 - 0.002)	0.0972 (0.068 - 0.126)	0.0173	0.017	7.1

Uptake rate constants (k_1) were compared using the likelihood-ratio test. The statistical analysis did not show any significant difference between treatments, thus showing that Hg

uptake and accumulation was concentration independent. The same procedure was conducted for the elimination rate constant (k_2) and statistical differences were observed between concentrations of 1 and 2 ppm ($X^2_{(1)} = 5.766$ $p<0.05$), 1 and 4 ppm ($X^2_{(1)} = 5.34$ $p<0.05$), 2 and 3 pp ($X^2_{(1)} = 8.10$ $p<0.05$), and between 3 and 4 ppm ($X^2_{(1)} = 13.04$ $p<0.05$). These differences in k_2 revealed that collembolans are eliminating Hg faster as Hg exposure increases.

Collembolans exposed to the lowest concentration of Hg (1 ppm) in food showed a low Hg uptake (<0.02 ng/g), which is in accordance with the no effects observed in their growth during the test. At the highest concentrations (3 and 4 ppm) a maximum of approximately 0.07 ng/g was attained, which could be close to the maximum sub-lethal concentrations that collembolans can tolerate. Organisms exposed to 4 ppm had to compensate food intake with extra food available (as mentioned above) as possibly they could not tolerate any further increase of Hg in their body. Although this avoidance behavior towards the contaminated food along with the ingestion of plaster led to an increase in their weight, no energy could be allocated from the plaster towards their growth (expressed as body size).

The comparison of these data with literature is difficult as studies on mercury bioaccumulation in soil-dwelling organisms are scarce, and toxicity studies are based on soil exposure using reproduction as a preferred endpoint. However, as stated before, Fountain and Hopkin found an avoidance behavior to Cd, Cu, Pb, and Zn exposures in food, at high concentrations. Although they did not assess metal bioaccumulation, graphite was found in the digestive tract which supports our data regarding food compensation. In the study of Zhang et al. (2009) earthworms showed to accumulate high levels of Hg from a polluted soil with total and methylmercury concentrations differing between earthworms' species. The comparison between earthworms (or other species) and collembolans should be analyzed carefully since the routes of exposure may be different. Also, the exposure route may be constant or avoidable, like is the case of food, which can be avoided, or soil under a reproduction test

where avoidance is not an available option. Fountain and Hopkin (2001) also discussed the potential higher tolerance of Collembolans regarding metal exposure through diet since they can avoid contaminated food. When in contact with contaminated media they excrete metals by molting, exfoliating the midgut epithelium where the elements are retained as part of a storage detoxification system, in order to decrease metal accumulation (Köhler, 2002).

In soil ecotoxicology, the presence of soil as exposure matrix or as substrate can provide some difficulties when assessing some crucial endpoints, like growth. In the present case study, the dietary exposure was assessed in plaster of Paris/charcoal, providing continuous monitoring during the exposure period, and assessing the effects in time. This approach would not have been possible in the soil, due to the difficulty of spotting each collembola and following its growth (in time).

4 - Conclusions

This study provides a new approach to understand Hg-induced effects in *F. candida*, by combining an ecotoxicological endpoint (growth) and with Hg bioaccumulation following a timeline. For the first time, it is reported the Hg-induced effects on growth and bioaccumulation from a dietary source to the springtail *F. candida*. Even at low and environmental relevant Hg concentrations, collembola growth rate was impaired. Despite a clear difference in the growth of collembolans with increasing Hg concentrations, collembolans did not reach a complete steady state in all concentrations of the uptake phase, being independent of the Hg exposure concentration.

Regarding depuration, after exposure to the higher Hg concentration, collembolans showed an increase in the k_2, with a faster elimination and decreasing Hg residence time. Also, by comparing collembolans' length and weight at different Hg concentration, along with their bioaccumulation patterns, it could be highlighted a potential food avoidance and exploring a

different food source available (plaster with charcoal). This was perceived by the dark color of collembola exposed to the highest concentrations. It was also highlighted a possible maximum sub-lethal concentration of 0.07 ng Hg/g of collembolan. The present study can be considered as a step forward in the assessment of the potential risks of mercury or other metals in terrestrial environments, also providing a mechanistic approach looking simultaneously at toxicity and bioaccumulation.

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6- Bibliography

Abbasi, S. A., Soni, R., 1983. Stress-induced enhancement of reproduction in earthworm *Octochaetus pattoni* exposed to chromium (vi) and mercury (ii)— implications in environmental management. International Journal of Environmental Studies. 22**,** 43-47.

Bengtsson, G., et al., 1983. Growth Changes Caused by Metal Uptake in a Population of *Onychiurus armatus* (Collembola) Feeding on Metal Polluted Fungi. Oikos. 40, 216-225.

Bertalanffy, L., Principles and theory of growth. Fundamental Aspects of Normal and Malignant Growth, ed. WW Nowinski. Amsterdam: Elsevier publ. co.(Abstr.), 1960.

Beyer, W. N., et al., 1985. Accumulation of methylmercury in the earthworm, *Eisenia foetida*, and its effect on regeneration. Bulletin of Environmental Contamination and Toxicology. 35**,** 157-162.

Bindesbøl, A.-M., et al., 2009. Impacts of heavy metals, polyaromatic hydrocarbons, and pesticides on freeze tolerance of the earthworm *Dendrobaena octaedra*. Environmental Toxicology and Chemistry. 28**,** 2341-2347.

Boening, D. W., (2000). Ecological effects, transport, and fate of mercury: a general review. Chemosphere. 40**,** 1335-1351.

Buch, A. C., et al., (2016). Ecotoxicity of mercury to *Folsomia candida* and *Proisotoma minuta* (Collembola: Isotomidae) in tropical soils: Baseline for ecological risk assessment . Ecotoxicology and Environmental Safety. 127, 22-29.

Buch, A. C., et al. (2017). "Ecotoxicology of mercury in tropical forest soils: Impact on earthworms." Science of The Total Environment 589: 222-231.

Buch, A. C., et al. (2018). "Food preference and ecotoxicological tests with millipedes in litter contaminated with mercury." Science of The Total Environment 633: 1173-1182.

Cabecinhas, A. S., et al., 2015. Sensitivity of the sea snail *Gibbula umbilicalis* to mercury exposure – Linking endpoints from different biological organization levels. Chemosphere. 119**,** 490-497.

Coelho, J. P., et al., (2005). Macroalgae response to a mercury contamination gradient in a temperate coastal lagoon-Ria de Aveiro. Estuarine, Coastal and Shelf Science, 65, 492–500.

Costley, C. T., et al., 2000. Determination of mercury in environmental and biological samples using pyrolysis atomic absorption spectrometry with gold amalgamation. Analytica Chimica Acta. 405**,** 179-183.

Crommentuijn, T., et al., 1993. Influence of Cadmium on Life-History Characteristics of *Folsomia candida* (Willem) in an Artificial Soil Substrate. Ecotoxicology and Environmental Safety. 26**,** 216-227.

Crouau, Y., Moïa, C., 2006. The relative sensitivity of growth and reproduction in the springtail, *Folsomia candida*, exposed to xenobiotics in the laboratory: An indicator of soil toxicity. Ecotoxicology and Environmental Safety. 64**,** 115-121.

Diogo, J. B., et al., 2007. Tolerance of genetically characterized *Folsomia candida* strains to phenmedipham exposure. Journal of Soils and Sediments. 7**,** 388-392.

E. Steinnes, "Mercury," in Heavy Metals in Soils, B. J. Alloway, Ed., pp. 245–259, Blackie Academics and Professional Press, London, UK, 2nd edition, 1997.

Fischer, E., Koszorus, L., 1992. Sublethal effects, accumulation capacities and elimination rates of As, Hg and Se in the manure worm, *Eisenia fetida* (Oligochaeta, Lumbricidae). Pedobiologia. 36**,** 172-178.

Folker-Hansen, P., et al., 1996. Effect of Dimethoate on Body Growth of Representatives of the Soil Living Mesofauna. Ecotoxicology and Environmental Safety. 33**,** 207-216.

Fountain, M. T., Hopkin, S. P., 2001. Continuous Monitoring of *Folsomia candida* (Insecta: Collembola) in a Metal Exposure Test. Ecotoxicology and Environmental Safety. 48**,** 275-286.

Fountain, M. T., Hopkin, S. P., 2005. *FOLSOMIA CANDIDA* (COLLEMBOLA): A "Standard" Soil Arthropod*. Annu. Rev. Entomol. 50**,** 201-222.

Holmstrup, M., et al., 2008. Exposure to mercury reduces cold tolerance in the springtail *Folsomia candida*. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology. 148**,** 172-177.

ISO, 1999. Soil quality – inhibition of reproduction of Collembola (Folsomia *candida*) by soil pollutants., ISO/DIS 22030, Geneva, Switzerland., 1999.

ISO, 2014. ISO 11267, Soil quality -- Inhibition of reproduction of Collembola (Folsomia candida) by soil contaminants. International Organization for Standardization. Geneve.

Kabata-Pendias, A., 2011. Trace elements in soils and plants, 4th ed. CRC Press, Taylor and Francis Group, USA, p. 534

Köhler, H.-R., 2002. Localization of metals in cells of saprophagous soil arthropods (Isopoda, Diplopoda, Collembola). Microscopy Research and Technique. 56**,** 393-401.

Liu, Y.-R., et al., 2010. Effects of mercury on reproduction, avoidance, and heat shock protein gene expression of the soil springtail *Folsomia candida*. Environmental Toxicology and Chemistry. 29**,** 654-659.

Lock, K., Janssen, C., 2001. Ecotoxicity of mercury to *Eisenia fetida*, *Enchytraeus albidus* and *Folsomia candida*. Biology and Fertility of Soils. 34, 219-221.

Mahbub, K. R., et al. (2017). "Mercury toxicity to Eisenia fetida in three different soils." Environmental Science and Pollution Research 24(2): 1261-1269.

Marigomez J. A., et al., 1986. Feeding and growth responses to copper, zinc, mercury and lead in the terrestrial gastropod arionater (Linne) Journal of Molluscan Studies. 52, 68-78.

Miller, E., et al., 2005. Estimation and Mapping of Wet and Dry Mercury Deposition Across Northeastern North America. Ecotoxicology. 14, 53-70.

Nieboer, E., Richardson, D. H. S., 1980. The replacement of the nondescript term 'heavy metals' by a biologically and chemically significant classification of metal ions. Environmental Pollution Series B, Chemical and Physical. 1, 3-26.

Pacyna, J. M., et al., 2009. Changes of emissions and atmospheric deposition of mercury, lead, and cadmium. Atmospheric Environment. 43, 117-127.

Pedrini-Martha, V., et al., 2012. Patterns of Urban Mercury Contamination Detected by Bioindication With Terrestrial Isopods. Archives of Environmental Contamination and Toxicology. 63, 209-219.

Pereira, E., et al., 2008. Evaluation of an interlaboratory proficiency-testing exercise for total mercury in environmental samples of soils, sediments and fish tissue. TrAC Trends in Analytical Chemistry. 27, 959-970.

Roex, E. W. M., et al., 2003. Acetylcholinesterase inhibition and increased food consumption rate in the zebrafish, *Danio rerio*, after chronic exposure to parathion. Aquatic Toxicology. 64, 451-460.

Sandifer, R.D., Hopkin, S.P., 1996. Effects of pH on the toxicity of cadmium, copper, lead and zinc to *Folsomia candida* Willen, 1902 (Collembola) in a standard laboratory test system. Chemosphere 33, 2475–2486

Schneider, C.A., Rasband, W.S., Eliceiri, K.W. "NIH Image to ImageJ: 25 years of image analysis". Nature Methods 9, 671-675, 2012.

SPSS, 1997. SigmaScan 5. SPSS Inc., Chicago.

Stohs, S. J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biology and Medicine. 18, 321-336.

Tipping, E., Poskitt, J.M., Lawlor, A.J., Wadsworth, R.A., Norris, D.A., Hall, J.R., 2011. Mercury in United Kingdom topsoils; concentrations, pools, and critical limit exceedances. Environ. Pollut. 159, 3721–3729.

Valko, M., et al., 2005. Metals, Toxicity and Oxidative Stress. Current Medicinal Chemistry. 12, 1161-1208.

Van Straalen, N. M., Van Meerendonk, J. H., Biological half-lives of lead in *Orchesella cincta* (L.) (Collembola). Bulletin of Environmental Contamination and Toxicology. 38, 213-219.

Vieira, H. C., et al., 2014. Real and Potential Mercury Accumulation in Human Scalp of Adolescents: A Case Study. Biological Trace Element Research. 163, 19-27.

Wang, D., et al., 2003. Accumulation and transformation of atmospheric mercury in soil. Science of The Total Environment. 304, 209-214.

Wiener, J. G., et al., 2006. Mercury in Soils, Lakes, and Fish in Voyageurs National Park (Minnesota): Importance of Atmospheric Deposition and Ecosystem Factors. Environmental Science & Technology. 40, 6261-6268.

Xu, J., et al., 2015. Sources and remediation techniques for mercury contaminated soil. Environment International. 74, 42-53.

Zhang, Z., et al., 2009. Bioaccumulation of Total and Methyl Mercury in Three Earthworm Species (*Drawida sp.*, *Allolobophora sp.*, and *Limnodrilus sp.*). Bulletin of Environmental Contamination and Toxicology. 83, 937-942.

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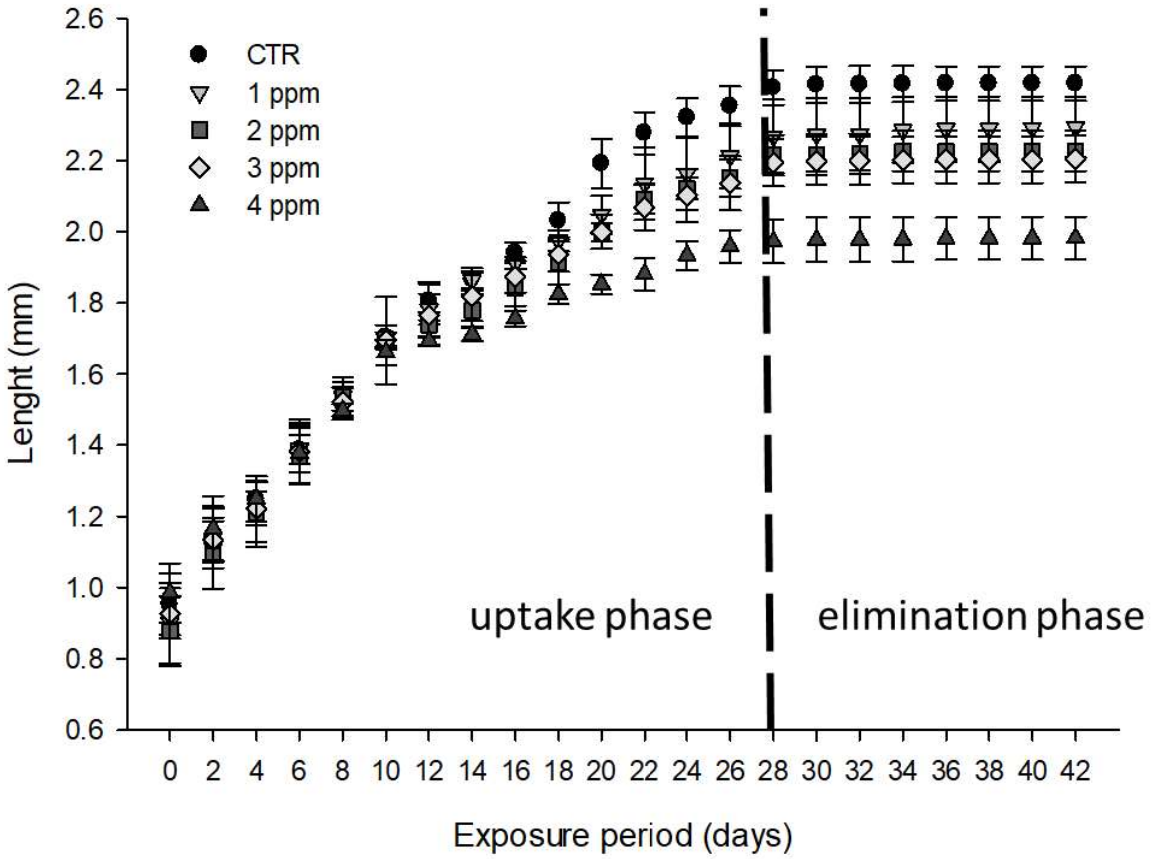

Figure 1 – Body length of *Folsomia candida* recorded during a 28 day exposure to Hg contaminated yeast (uptake phase), followed by a 14 day exposure to clean yeast (elimination phase). The negative control was clean yeast (diamond) and the vertical dashed line separates the uptake phase from the elimination phase. Data is expressed as average ± standard error.

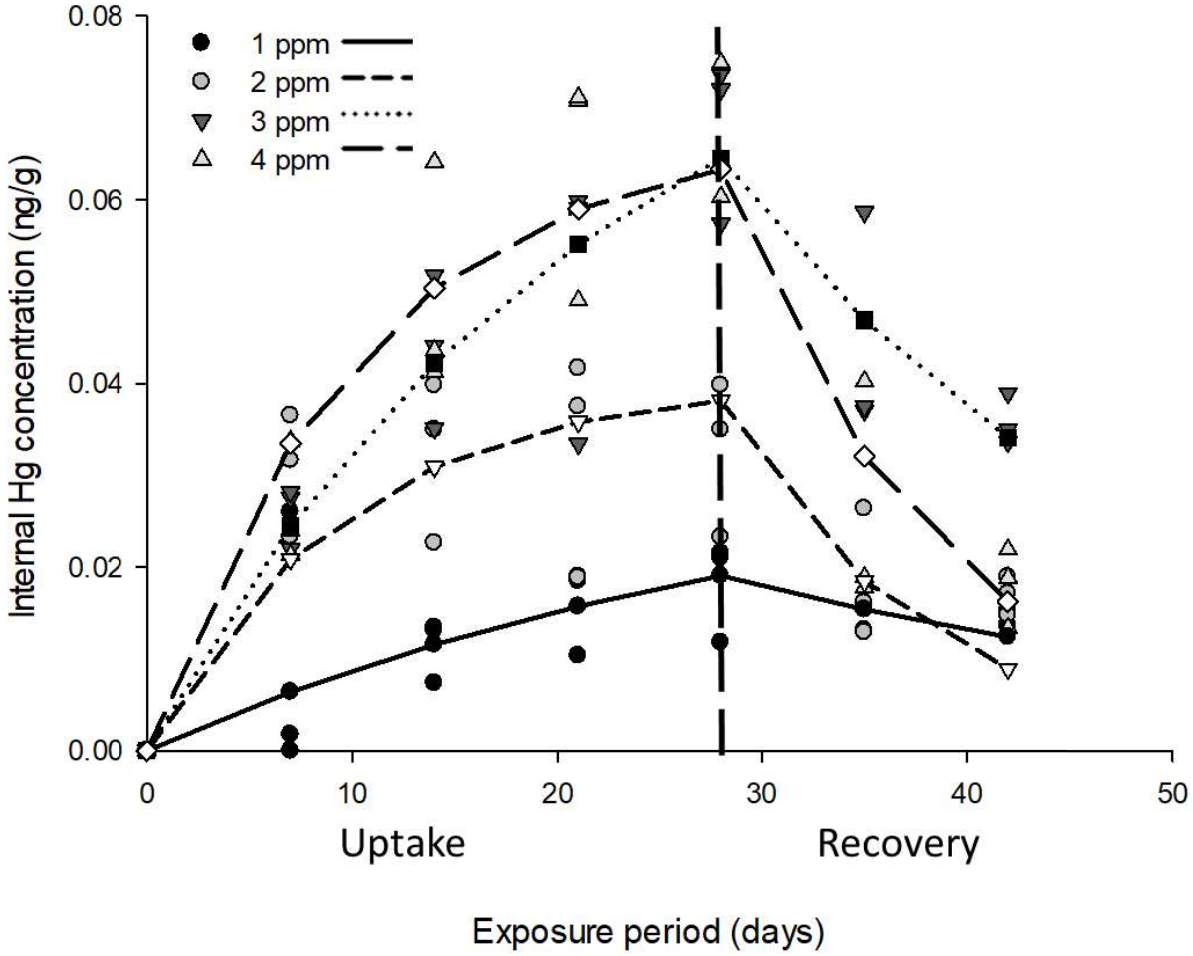

Figure 2 - Uptake and elimination kinetics of Hg in *Folsomia candida* exposed to nominal concentrations ranging from 1 to 4 ppm in contaminated dry yeast. Uptake and elimination phases lasted for 28 and 14 days, respectively. The vertical dashed line separates the uptake phase from the elimination phase. Lines represent the modeled Hg body concentration, using the one compartment model.

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