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1 **Combined effects of metal mixtures and predator stress on the freshwater**
2 **isopod *Asellus aquaticus***

3 M. Van Ginneken*, R. Blust and L. Bervoets

4 Department of Biology, Systemic Physiological and Ecotoxicological Research (SPHERE), University
5 of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium.

6 *Corresponding author. Tel: +32 3 265 3532. E-mail address: marjolein.vanginneken@uantwerpen.be

7 **Abstract**

8 Biotic stressors have been demonstrated to change the toxicity of pollutants. While the
9 combined effects of predator cues and pesticides are well documented, the interaction of
10 predator stress with metals is a topic that has remained largely unexplored. In this laboratory
11 experiment, the freshwater isopod *Asellus aquaticus* is exposed to predator cues and metal
12 mixtures of Cd, Cu and Pb. We examined the effects on growth, respiration and, as behavioral
13 parameters, feeding rate and activity. These were linked to the free ion activities (FIAs) in the
14 water and the metal body concentrations. The findings reveal that Cu accumulation
15 significantly influenced the growth rate, the feeding rate and the activity of isopods exposed to
16 predator stress. Furthermore, we found a concentration-dependent interaction of the Cd + Pb
17 mixtures on the feeding rate and a lower feeding rate for Cd and Pb predator exposed asellids.
18 As several interactions were found between metals and predator stress, it demonstrates the
19 importance of investigating how organisms and whole ecosystems respond to multiple
20 stressors. A better understanding of these interactions will undoubtedly improve risk
21 assessment and management.

22 **Keywords:** bioaccumulation; free ion activity; behavior; feeding rate; growth

23 **1. Introduction**

24 Classic toxicity tests study the impact of a single chemical on test organisms. Generally, the
25 organisms are exposed without additional stressors and while the studied endpoints depend on
26 the test organism, the focus still often lies on mortality. However, in contaminated ecosystems,
27 multiple chemicals are often simultaneously present, resulting in synergistic, additive or
28 antagonistic interactions (Cedergreen, 2014). Sublethal (behavioral) effects may be induced,
29 which could have an important influence on the ecosystem, altering species abundances and

30 diversity (Fleeger et al., 2003). Lastly, organisms rarely live in optimal conditions in natural
31 ecosystems. Coping with predators, fluctuations in food availability or other natural stressors
32 could affect the uptake or toxicity of chemicals as well (Folt et al., 1999; Heugens et al., 2001).
33 Considerable effort is necessary to shed light on the possible effects and interactions of multiple
34 stressors.

35 The present study examined the combined effects of predator stress and metal mixtures of Cd,
36 Cu and Pb on *Asellus aquaticus* L. This freshwater isopod is an important detritivore and can
37 be found throughout Europe and North America (Bloor, 2010). For the mixtures, metals were
38 chosen with dissimilar modes of actions. While copper is known to interfere with the uptake
39 pathway of Na⁺ (De Schamphelaere and Janssen, 2002), cadmium can directly compete with
40 Ca²⁺ for uptake. Lead, on the other hand, can disrupt both Na⁺ and Ca²⁺ homeostasis
41 (Komjarova and Blust, 2008; Rogers et al., 2003). Van Ginneken et al. (2015) already reported
42 synergistic interactions on the mortality and growth rate of *A. aquaticus* exposed to Cd and Pb
43 mixtures.

44 Previous research has shown that predator cues can alter the uptake and toxicity of chemicals,
45 due to costly behavioral and physiological stress responses. When the pesticide carbaryl was
46 combined with predator cues, Relyea (2003) found an increased lethality for two out of six
47 amphibian species. Schulz and Dabrowski (2001) observed a synergistic interaction of fish
48 cues with the organophosphate insecticide azinphos-methyl (AZP) and the pyrethroid
49 insecticide fenvalerate (FV) for mayfly nymphs, resulting in an increased mortality rate. For
50 *Ceriodaphnia dubia*, Qin et al. (2011) demonstrated that predator stress influenced the toxicity
51 of several pesticides differently. While predator cues interacted antagonistically with bifenthrin
52 and thiacloprid, it acted synergistically with fipronil. Thus, interaction patterns differ among
53 pollutants with different modes of action.

54 Although some research has been done on pesticides, less attention has been paid to the
55 interaction of predator cues with metals. Qin et al. (2015) reported an antagonistic effect of
56 predator stress on the acute toxicity of silver nanoparticles to daphnids. Additionally, there are
57 a few articles concerning the effects of metals on predator-prey interactions (Clements, 1999;
58 Kiffney, 1996). Lefcort et al. (2000), for example, reported that snails from heavy metal-
59 polluted environments failed to exhibit antipredator behaviors in contrast to snails from
60 reference lakes. These non-lethal effects of metals and predator cues could significantly change
61 species interactions and community structure (Trussell et al., 2003). Still, knowledge is lacking.

62 For fish, there are indications that certain metals such as Cu and Cd can affect the responses to
63 olfactory cues, leading to possible disturbances in communication, growth and reproduction
64 (Lürling and Scheffer, 2007). Yet, little is known about these effects on Crustacea nor on the
65 effects of various metals combined.

66 As metal pollution is a widespread problem, it is important to comprehend how metals interact
67 with other environmental stressors to estimate the actual effects of these pollutants in nature.
68 Using environmentally realistic metal concentrations, we determined the following sublethal
69 (fitness-related) endpoints of *A. aquaticus*: growth, respiration and, as behavioral parameters,
70 feeding rate and activity. These endpoints were linked to the free ion activities (FIAs) in the
71 water and the metal body concentrations. Additionally, we investigated the interactions of these
72 metals with predator stress for each endpoint. We hypothesized that exposure of *A. aquaticus*
73 to predator cues would cause an increased respiration rate and therefore an increased metal
74 accumulation, leading to a lower growth rate compared to the isopods without this additional
75 stressor. Although the presence of predator cues generally leads to a lower activity (Stoks et
76 al., 2005), defense mechanisms against metals are energetically costly and could be fuelled by
77 **an enhanced food uptake**. Moreover, metals could disrupt the transfer of chemical information,
78 such as predator cues (Lürling and Scheffer, 2007). Therefore, we expected to find an increased
79 slope for feeding rate and activity for isopods exposed to metals both with and without predator
80 cues.

81 **2. Material and Methods**

82 *2.1. Experimental set-up*

83 A ten-day lab experiment was conducted on the aquatic sowbug *Asellus aquaticus*. The isopods
84 were collected in the autumn of 2015 from **a stream**, the Laakbeek, in Lille, Belgium. In the
85 laboratory, they were kept for minimally two weeks in a climate chamber type WT15'+5DU-
86 WB (Weiss Technik, Reiskirchen-Lindenstruth, Germany) at constant temperature (15 ± 1 °C)
87 and light conditions (16:8 h light:dark photoperiod) in 20 L glass aquaria filled with
88 reconstituted medium-hard freshwater (US EPA, 2002). Alder leaves were conditioned by
89 drying them for 72 h and rehydrating them in water from the Laakbeek for a week. These leaves
90 were provided ad libitum as food (Bloor, 2010). After minimally two weeks of acclimation,
91 the experiment was performed under the same conditions.

92 *Asellus aquaticus* was exposed to a combination of predator cues and metal mixtures of Cd, Cu
93 and Pb. Three concentrations for each metal were used (“L” = low, “M” = medium and “H” =
94 high concentrations). We exposed the isopods to the single metals as well as to their binary and
95 tertiary mixtures (Table 1). Four control treatments were added as well. The stable isotopes
96 ⁶⁵Cu, ¹¹⁶Cd and ²⁰⁴Pb (CortecNet, Voisins-Le-Bretonneux, France) were added to moderately
97 hard water (Table 1; US EPA, 2002) to prepare the metal concentrations (the nominal
98 concentrations for Cd were: L = $1.29 \cdot 10^{-3}$ μmol/L, M = 0.013 μmol/L and H = 0.129 μmol/L;
99 for Cu: L = 0.108 μmol/L, M = 1.08 μmol/L and H = 10.8 μmol/L; for Pb: L = 0.035 μmol/L,
100 M = 0.353 μmol/L and H = 3.53 μmol/L). Sublethal metal concentrations were chosen based
101 on the mortality data presented in Van Ginneken et al. (2017). When necessary, the pH was
102 adjusted to 7.8 with 1 N NaOH. Next, 100 mL of this medium was added to acid-washed (1%
103 HCl) polypropylene containers (125 mL). Ten replicates per treatment were made, after which
104 the solutions were left for 24 h to equilibrate. In each container, we placed one individual of *A.*
105 *aquaticus* together with two alder leaf discs (*Alnus glutinosa*, d = 16 mm, 22.0 ± 0.1 mg) that
106 had been dried, weighed and ‘conditioned’ for six days in water from the Laakbeek in Lille
107 (Bloor and Banks, 2006). Each isopod was photographed at the start of the experiment after
108 which the length was determined using ImageJ 1.48v. (U.S. National Institutes of Health,
109 Maryland, USA). Using the following formula determined by Graça et al. (1993), the dry
110 weights (dw) of the isopods were calculated: $\ln(dw) = 2.71\ln(\text{length}) - 4.58$.

111 All metal treatments were combined with two predator treatments in which cues of both
112 invertebrate and vertebrate predators were absent or present. Predators were caught in the same
113 river as *A. aquaticus* to ensure predator recognition (Harris et al., 2013). The damselfly larvae
114 *Calopteryx splendens* was used as the invertebrate predator. One larva was put in each
115 container filled with 300 mL medium-hard water (US EPA, 2002) for 72 h (Janssens and Stoks,
116 2013). Additionally, one adult individual of the three-spined stickleback *Gasterosteus*
117 *aculeatus* and the ninespine stickleback *Pungitius pungitius* were used for fish cues and were
118 placed in a bucket with 8 L water for 24 h (based on Harris et al., 2013). After removing these
119 animals, equal parts of these cues were mixed ((0.125 fish + 1.67 damselfly larvae)/L). This
120 stimulus water was frozen at -80 °C until needed. It retains its activity for at least two months
121 (Pettersson et al., 2000). To add conspecific alarm cues, prior to use one *A. aquaticus* per 20
122 mL cue medium was homogenized. One mL of this predator cue mixture was added daily to
123 all predator treatments. To compensate for this additional volume, 1 mL of medium-hard water
124 was added to the non-predator stress treatments. Additionally, non-predator stress containers

125 were taped, so that only predator treated isopods could see each other. Because *A. aquaticus* is
126 cannibalistic, this ensured additional visual cues. General water characteristics (pH, dissolved
127 oxygen and electrical conductivity) and mortality were monitored daily (Table 2). Immobile
128 animals were considered dead and were removed from the solution.

129 At the end of the experiment, the animals were placed in medium-hard water without stressors.
130 After acclimating for 15 min, they were filmed for 30 minutes. Videos were processed with the
131 tracking program Lolitrack v.4 (Loligo Systems, Tjele, Denmark) that calculated the active
132 time (%). Next, they were placed in glass chambers with oxygen mini sensors in which oxygen
133 concentrations (mg/L) were measured for 4 h using the programs WitroxView v.1.0.2 and
134 Fibsoft v.1.0 (Loligo Systems, Tjele, Denmark). Respiration rates (RR) were calculated as
135 follows: $RR = ((O_1 - O_2) * (V - V_A)) / (m_A * t)$, where O_1 is the oxygen concentration in the
136 respiration chamber at the start of the experiment (mg/L), O_2 is the oxygen concentration in the
137 respiration chamber at the end of the experiment (mg/L), V is the volume of the empty
138 respiration chamber (mL), V_A is the volume of the isopod (assuming 1 mg isopod = 0.001 mL)
139 (mL), m_A is the mass of the isopod (mg wet weight) and t is the duration of the respiration
140 experiment (h). Additionally, the growth rate (GR) and feeding rate (FR; Bloor and Banks,
141 2006) of each animal were determined as follows: $GR = (m_2 - m_1) / 10$, where m_1 is the dry
142 weight of the animal at day 0 (mg) and m_2 is the dry weight of the animal at day 10 (mg); and
143 $FR = (L_1 * C_L) - L_2 / m_2 * 10$, where L_1 is the dry weight of the *Alnus* discs initially supplied and
144 L_2 is the dry weight of leaf material remaining after 10 days (mg), m_2 is the dry weight of the
145 animal at day 10 (mg) and C_L is the leaf weight change correction factor given by: $C_L =$
146 $\Sigma(C_2/C_1) / N$, where C_1 is the initial dry weight of control leaves (mg), C_2 is the dry weight of
147 the control leaves after 10 days (mg) and N is the number of control leaves. There were six
148 control leaves per metal treatment. Finally, the animals were rinsed with Milli-Q water, wiped
149 dry and placed in a -20 °C freezer awaiting further analysis.

150 2.2. Metal and DOC analysis

151 At day 0 and 10, water samples (50 mL) were taken from the containers with a syringe and
152 filtered through a 0.20 µm filter (Chromafil, Macherey-Nagel, Düren, Germany). Next, one
153 part was acidified to 1% HNO₃ to determine the major ions (¹¹⁶Cd, ⁶⁵Cu, ²⁰⁴Pb, Ca, K, Mg, Na)
154 with an inductively coupled plasma optic emission spectrometer (ICP-OES; Thermo scientific,
155 ICAP 6300 Duo, Waltham, MA, USA) and the trace metals with a high resolution inductively
156 coupled plasma mass spectrometer (HR-ICP-MS, Element XR, Thermo Scientific, Finnigan

157 element 2, Bremen, Germany). The remaining water sample was acidified to pH 2 with 2N HCl
158 and was used to quantify dissolved organic carbon with a TOC-analyzer (TOC-VCPH,
159 Shimadzu Corporation, Kyoto, Japan).

160 To measure the metal body concentrations of the isopods, three process blank samples and
161 three samples of certified reference mussel material (SRM2796) of the Community Bureau of
162 Reference (European Union, Brussels, Belgium) were included. The isopods were dried for 72
163 h at 60 °C in a laboratory furnace. They were cooled in a desiccator after which they were
164 weighed on a Sartorius SE2 ultra microbalance (accuracy of 1 µg). Next, they were digested in
165 a solution of trace-metal-grade HNO₃ (69%) and high-purity H₂O₂ (29%) (3:1 for the isopods)
166 for 1 h at 125 °C in a hot block (Environmental Express, Charleston, SC, USA). Digested
167 isopod samples were diluted to 3% HNO₃ and metal concentrations were measured using the
168 HR-ICP-MS (Element XR, Thermo Scientific, Finnigan element 2, Bremen, Germany). Isopod
169 body concentrations were expressed as µmol/g dw. Metal concentrations in the blank samples
170 were all below quantification limits (<0.075 µg/L for ¹¹⁶Cd, <0.001 µg/L for ⁶⁵Cu and ²⁰⁴Pb)
171 and the recoveries from the reference samples were consistently within 10 % of the certified
172 values for all three metals. The Windermere Humic Aqueous Model 6.0.13 (Natural
173 Environment Research Council) was used to compute the free ion activities (FIAs) for the
174 different metals. Dissolved metal concentrations below the detection limit were entered as the
175 detection limit itself and 100% of the DOC was entered as fulvic acids.

176 2.3. Statistical analyses

177 Statistical analyses (ANCOVAs) were performed in R version 3.4.2, with metal concentrations
178 as continuous variable and metal and predator treatment as categorical variables. Controls were
179 added for each metal treatment. All models were first made with metal body concentrations
180 and later compared to the model with free ion activities. All metal concentrations were log-
181 transformed (log₁₀) to normalize distributions. Diagnostic plots were used to test the data for
182 normality and homoscedasticity. We assessed the effects and interactions of the stressors by
183 interpreting the interaction term and differences between slopes. If a significant difference was
184 found (p < 0.05), a Tukey HSD test was conducted. Possible differences between the control
185 treatments with and without predator stress were tested with a two-sample t-test or the
186 Wilcoxon rank sum test. Graphs were made in Sigmaplot (Version 11.0; Systat, Chicago, IL,
187 USA). **Given the substantial amount of data, only graphs with significant trends or differences**
188 **are shown.** The animals that died before the end of the experiment were not included in the
189 analyses.

190 3. Results

191 3.1. General

192 Mortality for the control treatments varied between 0 and 10 % for the isopods that were not
193 exposed to predator stress, mortality for the predator exposed isopods ranged between 10 and
194 40 %. Other mortality rates did not exceed 20 %, except for the Cd + Pb L treatment (30 %)
195 exposed to predator stress. No significant difference in mortality rate was found between the
196 predator treatments. For growth rate, feeding rate, activity and respiration rate, substantial
197 variations were observed between isopods from the same treatment, resulting in relatively low
198 R^2 . No significant three-way interactions were found.

199 3.2. Metal accumulation

200 Cadmium FIAs varied between $0.155 \cdot 10^{-3} \pm 0.009 \cdot 10^{-3} \mu\text{mol/L}$ (Cd + Cu L without predator
201 stress) and $78.0 \cdot 10^{-3} \pm 4.9 \cdot 10^{-3} \mu\text{mol/L}$ (Cd + Cu + Pb H without predator stress; Table S1).
202 Cadmium body burdens ranged from $1.75 \cdot 10^{-3} \pm 0.20 \cdot 10^{-3} \mu\text{mol/g dw}$ (Cd + Pb L with predator
203 stress) to $0.432 \pm 0.057 \mu\text{mol/g dw}$ (Cd H with predator stress; Table S2). In general, we found
204 a positive correlation between body concentration and $\text{FIA}_{\text{water}}$ ($F = 394, p < 0.001$).
205 Additionally, there was a significant interaction between body burden and metal treatment (F
206 $= 4.64, p = 0.003$), indicating significant differences in slopes between the mixture treatments
207 (Table 3, Figure 1A). The single Cd treatment had a significantly larger slope than Cd + Cu (t
208 $= -3.20, p = 0.001$) and Cd + Cu + Pb ($t = -2.73, p = 0.007$), suggesting an antagonistic
209 interaction between those metals on Cd uptake. Also Cd + Cu and Cd + Pb had a significantly
210 different slope ($t = 2.45, p = 0.015$). No interaction was found between body burden and
211 predator treatment. Although the ANCOVA model indicated significant differences between
212 the isopods with and without predator exposure, a Tukey HSD test revealed no significance.

213 Copper FIAs varied between $0.188 \cdot 10^{-6} \pm 0.082 \cdot 10^{-6} \mu\text{mol/L}$ (Cu + Cd + Pb L without predator
214 stress) and $0.169 \pm 0.008 \mu\text{mol/L}$ (Cu + Cd + Pb H with predator stress; Table S3), while body
215 concentrations varied between $0.753 \pm 0.074 \mu\text{mol/g dw}$ (Cu L without predator stress) to 8.26
216 $\pm 1.32 \mu\text{mol/g dw}$ (Cu H without predator stress; Table S4). A positive correlation between
217 $\text{FIA}_{\text{water}}$ and the Cu body burden was found ($F = 897, p < 0.001$), but no significant differences
218 in slopes were found between the metal treatments. However, there was an interaction between
219 Cu exposure and predator treatment ($F = 8.19, p = 0.004$), resulting in a significantly larger

220 slope of Cu accumulation for *Asellus* exposed to predator stress (Figure 1B). However, the
221 difference in slopes between the treatments with and without predator cues is small.

222 For Pb, FIAs ranged from $24.8 \cdot 10^{-9} \pm 6.6 \cdot 10^{-9}$ $\mu\text{mol/L}$ (Pb L without predator stress) to 10.5
223 $10^{-3} \pm 1.1 \cdot 10^{-3}$ $\mu\text{mol/L}$ (Pb + Cd + Cu H without predator stress; Table S5) and body
224 concentrations from $27.8 \cdot 10^{-3} \pm 8.7 \cdot 10^{-3}$ $\mu\text{mol/g dw}$ (Pb + Cu L without predator stress) to 6.33
225 ± 1.51 $\mu\text{mol/g dw}$ (Pb + Cd H without predator stress; Table S6). Again, body burden increased
226 when $\text{FIA}_{\text{water}}$ increased ($F = 1750$, $p < 0.001$). While there was no interaction of body
227 concentrations with predator stress, we did find an interaction with metal treatment ($F = 3.82$,
228 $p = 0.01$) (Table 3). Significantly larger slopes for the single Pb and Pb + Cd treatment were
229 found than for Pb + Cu ($t = -2.39$, $p = 0.017$ and $t = -2.16$, $p = 0.03$, respectively) and the
230 tertiary mixture ($t = -2.60$, $p = 0.009$ and $t = -2.38$, $p = 0.018$, respectively), which indicates an
231 antagonistic interaction for the latter metal mixtures (Figure 1C). The ANCOVA model
232 indicated a significant difference in Pb body burden between the treatments with and without
233 predator stress, but this was not confirmed by a Tukey HSD test.

234 3.3. Respiration rate

235 Respiration rates varied from 0.106 ± 0.022 (Cu M without predator stress) to 0.311 ± 0.056
236 $\mu\text{g O}_2/\text{mg ww/h}$ (Cd L without predator stress) (Table S7). The ANCOVA model comparing
237 respiration rates to metal body concentrations revealed no significant differences between the
238 Cd treatments. For Cu and Pb, only a significant difference in respiration was found between
239 the treatments with and without predator stress ($F = 4.07$, $p = 0.044$ and $F = 4.04$, $p = 0.045$,
240 respectively). Copper isopods exposed to predation stress respired $0.018 \mu\text{g O}_2/\text{mg ww/h}$ or 12
241 % more (Table 4, Figure 2A). For Pb, the respiration rate was $0.017 \text{ O}_2/\text{mg ww/h}$ or 11% higher
242 (Figure 2B). We investigated whether there was a stronger link with the metal FIAs in the
243 water, but no significances were found for the three metals. Furthermore, no significant
244 differences were observed between the control treatments with and without predator cues.

245 3.4. Growth rate

246 The highest average growth rate found for the three metals was 0.106 ± 0.028 mg/day (Cd +
247 Cu + Pb L with predator stress), the lowest was $62.5 \cdot 10^{-3} \pm 19.2 \cdot 10^{-3}$ mg/day (Cu H without
248 predator stress) (Table S8). For the control treatments, no significant difference in growth rate
249 was found between isopods with and without predator stress. A negative correlation ($F = 7.02$,
250 $p = 0.008$) was found between Cd body burden and growth, but no interactions with metal or

251 predator treatment were detected (Table 5, Figure 3A). Growth significantly decreased with
252 increasing Cu body burden as well ($F = 43.2$, $p < 0.001$). There were no interactions of Cu
253 body burden with metal treatment, but there was an interaction with predator treatment (Figure
254 3B), resulting in a significantly lower slope for the growth rate of isopods exposed to predator
255 stress ($t = -2.08$, $p = 0.038$). For Pb, no significant correlation or interactions with metal or
256 predator treatment were found between body burden and growth rate.

257 We found similar results for the Cd and Cu FIA_{water} model. For Pb, however, there was a
258 significant negative correlation between growth rate and Pb FIA_{water} ($F = 12.0$, $p < 0.001$).

259 3.5. Feeding rate

260 We found a significant difference between the feeding rates for the control treatments with and
261 without predator cues: isopods that were not exposed to predator cues fed 0.035 mg/mg/day or
262 84% more than predator exposed isopods ($W = 843$, $p = 0.005$). Feeding rates ranged from $0 \pm$
263 0 (Cu + Pb H) to 0.358 ± 0.088 mg/mg/day (Cd + Pb M without predator stress) (Table S9).

264 While for Cd no significant relation was found for body concentrations and feeding rate, there
265 was a significant interaction between Cd body burden and metal treatment ($F = 4.48$, $p = 0.004$)
266 (Table 6, Figure 4A). The slope of feeding rate for Cd + Pb was significantly higher than for
267 the other treatments, caused by a significant increase in feeding rate for *Asellus* exposed to the
268 Cd + Pb M concentrations (for Cd: $t = 2.78$, $p = 0.006$; for Cd + Cu: $t = 3.34$, $p < 0.001$; for Cd
269 + Cu + Pb: $t = 2.50$, $p = 0.013$). There was no significant difference in slopes between the
270 predator treatments, but isopods that were not exposed to predator stress generally fed 0.028
271 mg/mg/day or 53% more than those with predator stress ($F = 16.3$, $p < 0.001$) (Figure 4B). We
272 found similar significant effects for the Cd FIA_{water} model. However, here we did find a
273 significant negative correlation between Cd FIA_{water} concentrations and feeding rate ($F = 35.5$,
274 $p < 0.001$). Furthermore, we also observed other significant differences in slopes between metal
275 treatments. The slope of Cd + Cu + Pb was less negative (0.001 ± 0.007) than the slopes for
276 the other metal treatments (for Cd: -0.037 ± 0.009 , $t = -3.26$, $p = 0.001$; for Cd + Cu: $-0.024 \pm$
277 0.004 , $t = -2.53$, $p = 0.012$; for Cd + Pb: -0.032 ± 0.008 , $t = -3.13$, $p = 0.002$).

278 For Cu, we found a significant negative correlation between body burden and feeding rate (F
279 $= 44.3$, $p < 0.001$). Only Cu + Cd + Pb had a significantly larger slope than the other treatments
280 (for Cu: $t = -2.54$, $p = 0.011$; for Cu + Cd: $t = -3.07$, $p = 0.002$; for Cu + Pb: $t = -2.39$, $p =$
281 0.017), suggesting an antagonistic interaction between the three metals (Figure 4C). Isopods

282 that were not exposed to predator stress fed 0.023 mg/g/day or 49% more (Figure 4D) and the
283 slope of their feeding rate decreased more with increasing body burden ($t = 2.00$, $p = 0.046$)
284 compared to *Asellus* treatments with predator cues. Again, the same significances were
285 observed for the Cu FIA_{water} model. Only the significant difference in slopes between Cu + Cd
286 and Cu + Cd + Pb was not found.

287 Lead feeding rates showed no significant decrease in feeding rate with increasing body
288 concentration, but we found a significantly larger slope for Pb + Cd than for the other
289 treatments (for Pb: $t = -2.08$, $p = 0.038$; for Pb + Cu: $t = -3.19$, $p = 0.002$; for Pb + Cd + Cu: t
290 $= -2.91$, $p = 0.004$) (Figure 4E). Furthermore, asellids that were not exposed to predator stress
291 again had a significantly higher feeding rate (Figure 4F): they fed 0.032 mg/mg/day or 59 %
292 more ($F = 20.4$, $p < 0.001$). The FIA_{water} model only showed the significant difference in
293 feeding rates between isopods with and without predator stress ($F = 19.7$, $p < 0.001$).

294 3.6. Activity

295 No significant difference in activity was found between the controls with and without predator
296 exposure. Activity ranged from 15.7 ± 2.8 (Cd + Pb H without predator stress) to 53.1 ± 2.6 %
297 (Cd M without predator stress) (Table S10). For the three metals, activity decreased with
298 increasing body burden (for Cd: $F = 12.9$, $p < 0.001$; for Cu: $F = 37.8$, $p < 0.001$; and for Pb: F
299 $= 17.8$, $p < 0.001$) (Table 7, Figure 5). Also for Cu, the slope of activity for isopods with
300 predator stress decreased less than for the isopods without predator exposure ($t = 3.16$, $p =$
301 0.002). No other significant interactions were observed. For the FIA_{water} models, only
302 significant negative correlations between FIA_{water} and activity were found (for Cd: $F = 32.7$, p
303 < 0.001 ; for Cu: $F = 22.9$, $p < 0.001$; and for Pb: $F = 17.9$, $p < 0.001$).

304 4. Discussion

305 We observed changes caused by either metal or predator stress for all endpoints. In several
306 cases, the effects of predator stress were affected by the accumulated metals, thus providing
307 strong evidence for stressor interactions. For the control treatments we only found a significant
308 difference in feeding rate between asellids with and without predator exposure. We also
309 expected a significantly lower activity for the predator exposed isopods, but this was not
310 observed. The animals were transferred to water without predator cues for filming. Therefore,
311 this is most likely caused **due to the absence of the stressor**.

312 Comparisons of metal body burdens to FIAs resulted in relatively high R^2 . Part of the variability
313 can be explained by binding of the metals to the leaf discs, resulting in a secondary uptake
314 route through ingestion. Consistent with the finding of our previous study (Van Ginneken et
315 al., 2015), the present study observed for the Cd and Pb treatments significantly higher body
316 concentrations for the single metals and Pb + Cd than for the other mixtures. Their slopes
317 decreased approximately by a factor of 1.3. Synergistic interactions of Cd and Pb have already
318 been reported and are most likely caused by combined disturbances of Ca^{2+} and Na^+
319 homeostasis (Birceanu et al., 2008). Though the slopes of the Cd and Pb mixture were similar
320 to the ones for the single metals. Therefore, it is more logical that Cd + Cu, Pb + Cu and Cd +
321 Cu + Pb interacted antagonistically. An et al. (2004) also found a decreased toxicity of a ternary
322 mixture of Cd, Cu and Pb for *Cucumis sativus*. Both Cu and Pb are known to compete for the
323 same uptake sites, namely via Na^+ channels, so an antagonistic interaction is expected.
324 Cadmium, however, has a different mode of action and enters via Ca^{2+} channels. Barata et al.
325 (2006) demonstrated a less than additive toxicity of mixtures of Cd and Cu for *Daphnia magna*
326 and interpreted their findings as the result of both metals being metallothionein inducers.
327 Another explanation could be that these metals share a common uptake site and that this
328 inhibited their uptake. Komjarova and Blust (2008) suggested Divalent Metal Transporter 1
329 (DMT1), an Fe^{2+} transporter.

330 Although no effects of predator stress were seen for Cd and Pb accumulation, we did see a
331 significantly higher slope for Cu accumulation. The difference is small, but could be explained
332 by the increased respiration rate we observed for isopods exposed to predator cues. An
333 increased respiration rate was also found for Pb, but this did not result in an altered slope for
334 Pb accumulation. An increase in oxygen consumption when exposed to predator cues is often
335 observed (Beckerman et al., 2007; Slos and Stoks, 2008). However, we did not see an increase
336 for Cd. A possible explanation could be that costs associated with repair processes were masked
337 by reducing other metabolic costs, e.g. associated with locomotion (Knops et al., 2001).
338 Furthermore, no studies could be found that investigated the effect of predator cues on
339 accumulation, but e.g., Qin et al. (2011) found significantly smaller LC values for
340 *Ceriodaphnia dubia* when exposed to fipronil and predator cues, which could be the result of
341 an increased body concentration. On the other hand, Qin et al. (2015) found that predator stress
342 and silver nanoparticles interacted antagonistically for the LC_{50} s of *Daphnia magna*, indicating
343 that effects can vary depending on factors such as the predator-prey model and the chemical
344 bioavailability (Qin et al., 2011).

345 For Cd, a negative correlation was found between body burden and growth rate, but feeding
346 rate did not decrease significantly (when linked to the body concentrations). This lower food
347 conversion efficiency has been reported before. After exposing *Enallagma cyathigerum* to
348 predator stress, Janssens and Stoks (2013) reported significantly lower growth rates, while food
349 intake for pesticide treatments even increased. Again, this can be caused by a trade-off: by
350 converting less food into biomass and investing more in defense mechanisms and strategies
351 (Preisser et al., 2005).

352 In the literature, a reduced feeding rate is most often reported after exposure to pollutants (Bloor
353 and Banks, 2006; Maltby et al., 2002). Yet, for the medium mixture of Cd and Pb, a very steep
354 increase was observed for feeding rate, resulting in a slope that was increased by a factor of 6
355 minimally. For the highest concentrations of this mixture, feeding rates again decreased
356 drastically. This mixture of cadmium and lead did result in a high accumulation for both metals,
357 but no concentration-dependence was observed nor were any other effects on endpoints. Van
358 Ginneken et al. (2015) found a synergistic interaction resulting in a higher mortality rate and a
359 lower growth rate. However, the concentrations of Cd used in that study were in general much
360 higher (0.120 – 1.09 $\mu\text{mol/L}$) than the ones used in the present study. So, the interactions of
361 these two metals do not only depend on the endpoint, but also seem to depend on the exposure
362 concentrations. Concentration-dependent interactions have been reported before for metals
363 (Jonker et al., 2005). Sharma et al. (1999) studied the effects of Cd, Cu and Zn on the root
364 growth of the plant *Silene vulgaris* and found non-additive or antagonistic responses for low
365 concentrations. However, if one of the components in the mixture exceeded a certain level of
366 toxicity, synergistic interactions were observed. It is possible that the medium concentrations
367 we used triggered a range of energetically costly defense mechanisms, which had to be fuelled
368 by a higher feeding rate, but that after some critical point these mechanisms (metallothioneins,
369 antioxidant enzymes) became too costly to maintain. Further investigations are required to
370 evaluate the effects on biochemical markers. When Cu was added to a mixture of Cd and Pb,
371 there was longer a significant increase in feeding rate. This is reflected in the accumulation
372 data, where the tertiary mixture produced an antagonistic effect on the uptake of Cd and Pb.
373 This antagonistic interaction was also observed for the feeding rates of Cu, where negative
374 slopes for the single Cu treatment and the binary mixtures were decreased by at least a factor
375 of 14 compared to the feeding rate of the tertiary mixture.

376 A lower foraging rate reduces the risk of being detected by a predator. This is evidenced by the
377 Cd and Pb feeding rates of the predator exposed asellids. Yet, no significant differences in

378 growth rate between the predator treatments for Cd and Pb were found, which could be an
379 indication of a higher predator-induced growth efficiency. Normally, as explained above, lower
380 growth efficiencies are reported. However, this higher growth efficiency for *Asellus* could be
381 advantageous for two reasons. Firstly, their reproductive success is positively correlated with
382 size (Bloor, 2010). The presence of predator cues could have triggered the isopods to invest
383 more in reproduction. However, *A. aquaticus* forms precopula pairs, which swim more slowly
384 and are more visible. This makes them far more likely to be consumed by a predator. Dunn et
385 al. (2008), for example, observed that pair formation for *Gammarus duebeni* declined when
386 predator cues were present. Thus, it is more likely that growth efficiency increased due to
387 another reason. An alternative explanation is that larger isopods move faster and have more
388 chance to escape than smaller individuals as swimming speed could be positively correlated to
389 length or body weight (Eroukmanoff and Svensson, 2009; Takeuchi and Watanabe, 1998).

390 Although we observed a negative correlation of activity and Cu body burden, we found that
391 the slope for predator exposed isopods decreased less (by a factor of 3.4), meaning that Cu and
392 predator stress acted antagonistically. The same can be seen for feeding rate, where the negative
393 slope was also increased by a factor of 2 in the presence of a predator. As (foraging) activity
394 was increased for isopods exposed to predator stress, they were most likely more susceptible
395 to predation when Cu body burdens were increased. Clements et al. (1989) also observed that
396 two species of caddisflies were significantly predated more in streams dosed with 0.094 $\mu\text{mol/L}$
397 copper. Also for crustaceans, copper can impair responses to female odors or food (Krång and
398 Ekerholm, 2006; Sherba et al., 2000). McIntyre et al. (2012) found that juvenile coho salmon
399 (*Oncorhynchus kisutch*) exposed to low levels of copper ($< 0.315 \mu\text{mol/L}$) were unresponsive
400 to conspecific alarm cues, therefore being more vulnerable to predatory attacks. For fish, Cu is
401 known to inhibit physiological responsiveness, and even cause cell death, of olfactory receptor
402 neurons (Baldwin et al., 2003; Hansen et al., 1999). The less sensitive chemo-receptor cells of
403 Crustacea are possibly similarly affected (Olsén, 2010). Scott et al. (2003) found that Cd could
404 also disrupt predator-avoidance behaviors in juvenile rainbow trout, but this was not found in
405 the present study. While the mechanisms of toxicity of these metals remain largely unclear,
406 they are believed to interfere predominantly with the internal signaling pathways (Lürding and
407 Scheffer, 2007).

408 A limitation of our study that needs to be acknowledged is the high variation between
409 individuals, resulting in relatively low R^2 . Despite these low R^2 , a remarkable number of clear
410 trends and significant differences between treatments became apparent. We found a lower

411 growth rate for Cd, a decreased feeding rate for Cd and Pb predator stress treatments and a
412 higher respiration rate for Cu and Pb predator stress treatments. Additionally, we also
413 demonstrated that predator cues affected the slopes for the accumulation, growth rate, feeding
414 rate and activity of the Cu treatments. It remains an open question whether these, sometimes
415 small, differences could cause severe changes to the ecosystem. Yet, we must consider that
416 *Asellus aquaticus* is an important decomposer. There is evidence that high accumulated
417 concentrations of Cu cause a disruption of olfactory responses. As a result, they seem less able
418 to detect predator cues and most likely will also struggle with finding food and mating partners.
419 Additionally, it was demonstrated that cadmium and lead can interact synergistically for
420 mortality and growth rate (Van Ginneken et al., 2015) and in the present study we found that
421 they interact in a concentration-dependent manner for feeding rate as well. Consequently, these
422 metals and their interactions with predator cues could cause serious alterations to the food web.
423 Together these findings show that it is essential to consider the interactions of abiotic and biotic
424 stressors in environmental risk assessment.

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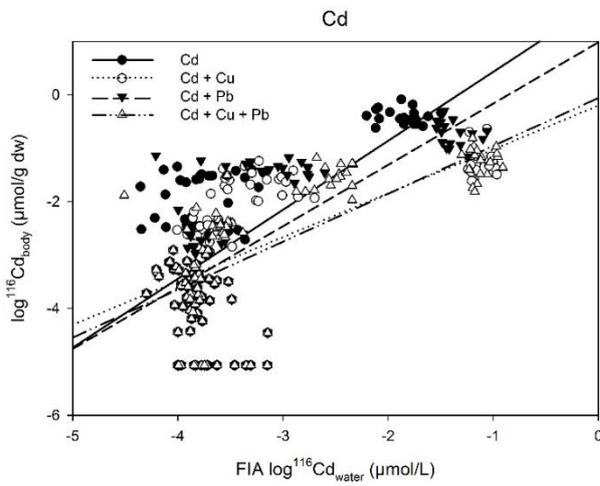
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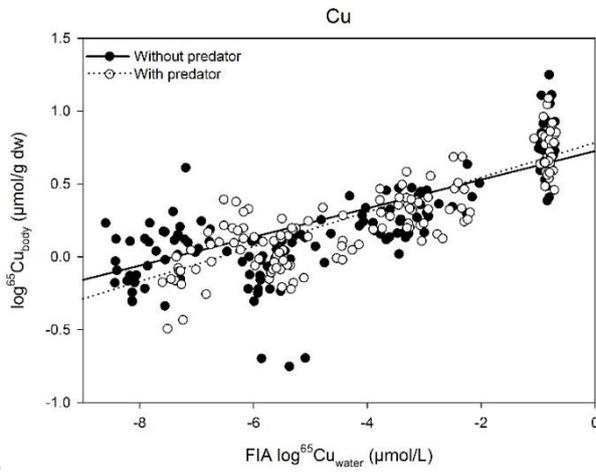
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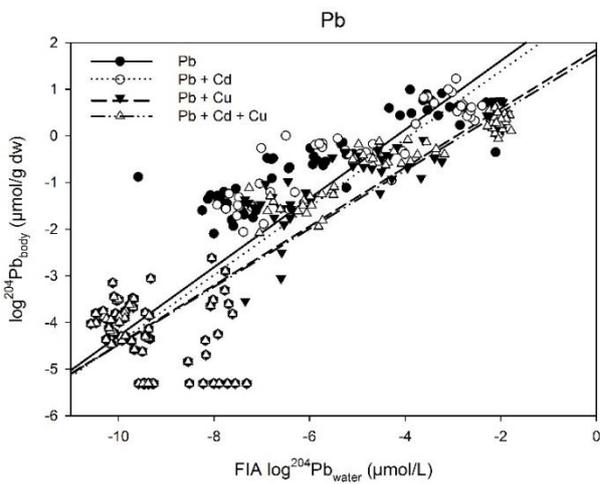
569 **Figures**



570 **A**

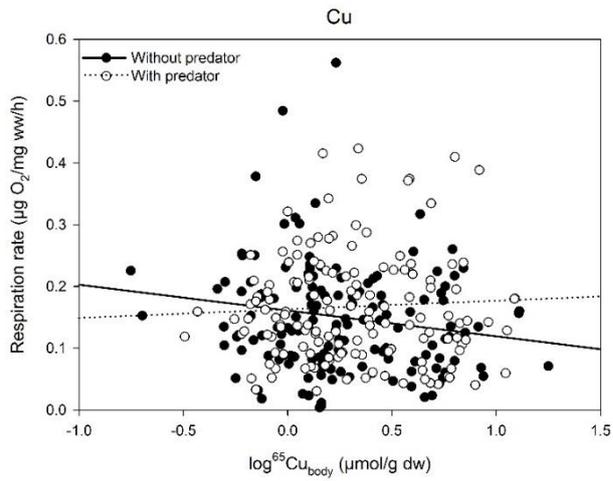


571 **B**

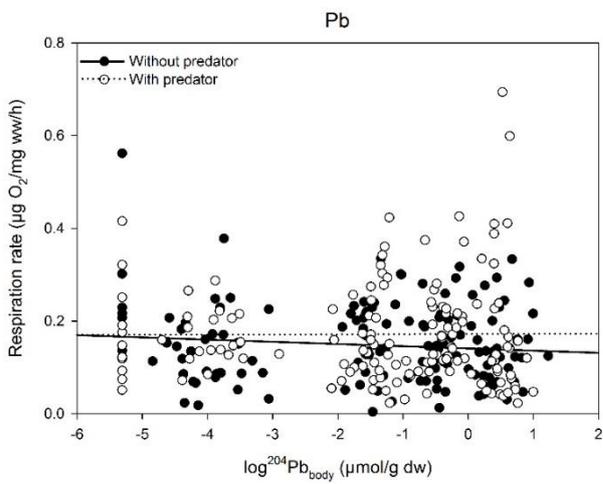


572 **C**

573 **Figure 1: Metal body concentrations after 10 days in function of the free ion activities (FIAs):**
574 **A) Cd metal treatments (N = 287); B) Cu predator treatments (N = 293); and C) Pb metal**
575 **treatments (N = 292).**



576 A



577 B

578 **Figure 2: Respiration rates after 10 days in function of the metal body concentrations: A) Cu**
 579 **predator treatments (N = 287); and B) Pb predator treatments (N = 285).**

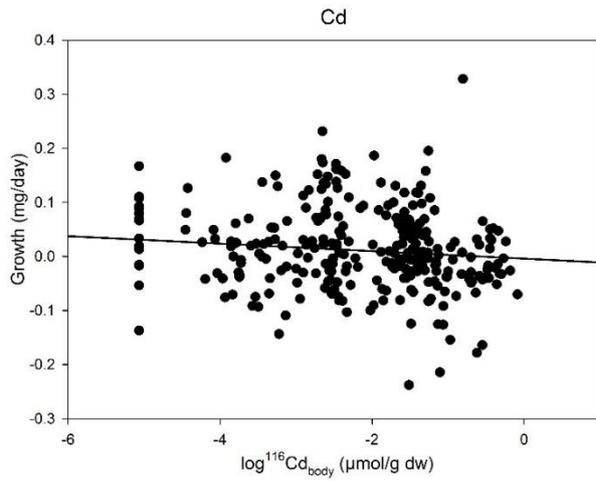
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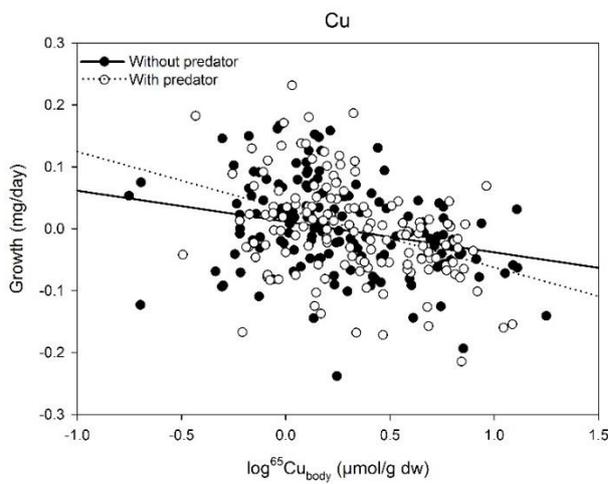
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586 B

587 **Figure 3: Growth rates after 10 days in function of the metal body concentrations: A) Cd (N =**
 588 **287); and B) Cu predator treatments (N = 293).**

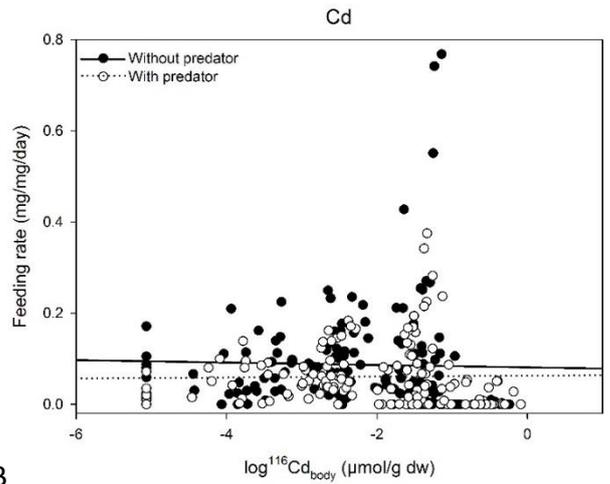
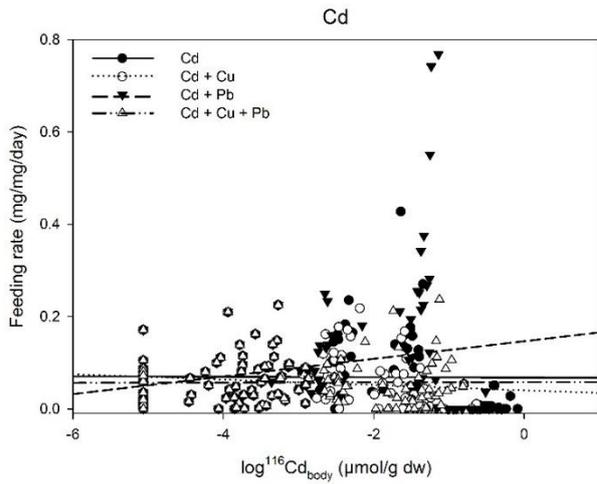
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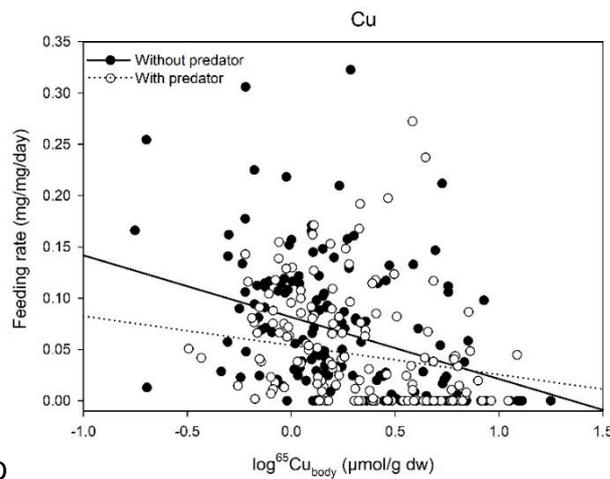
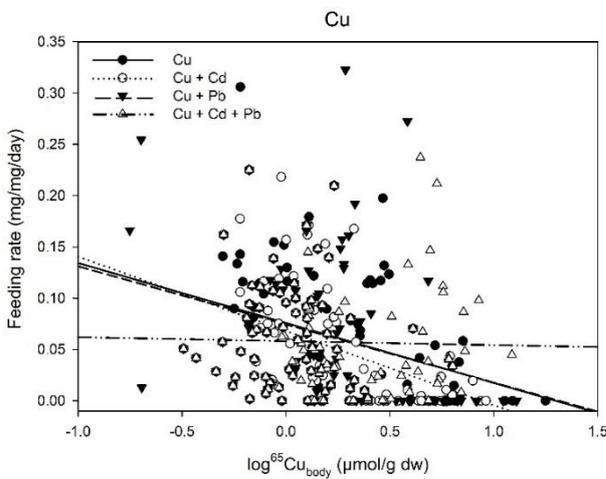
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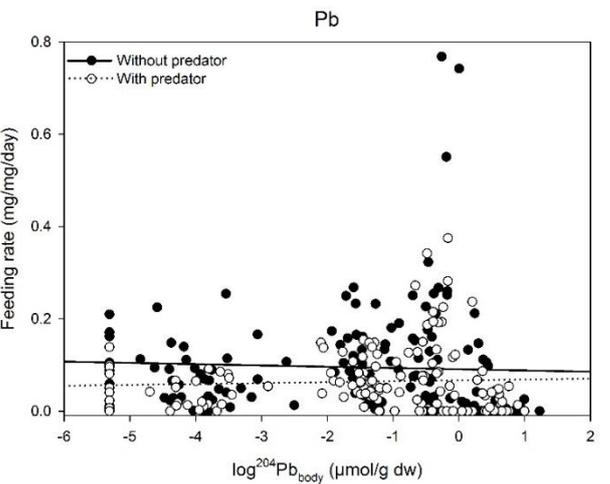
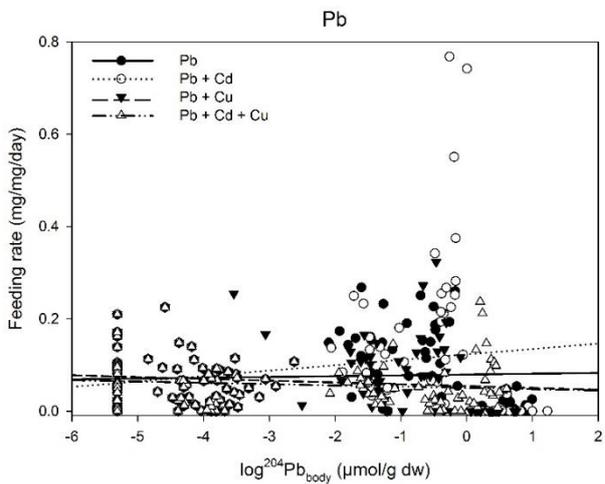
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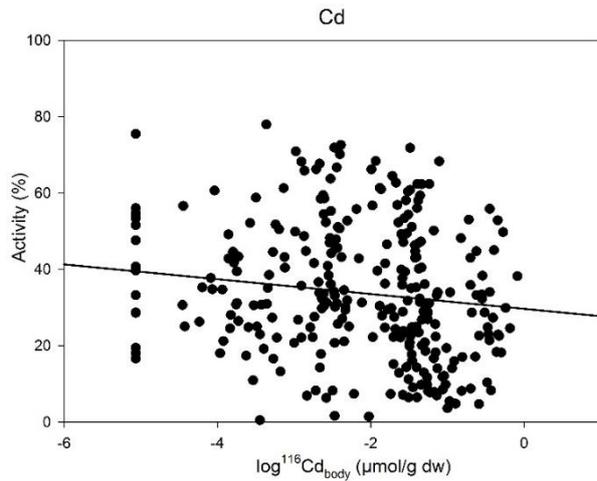


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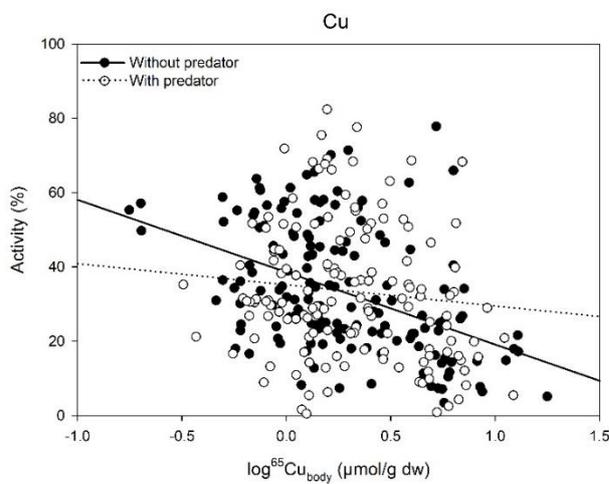
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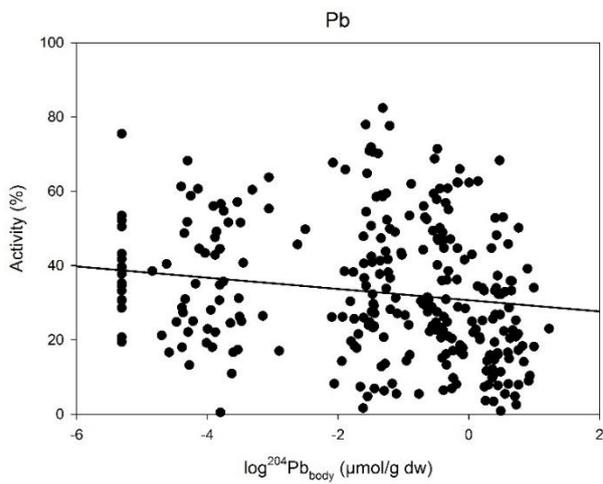
598 **Figure 4: Feeding rates after 10 days in function of the metal body concentrations: A-F) metal**
 599 **and predator treatments for the three metals (for Cd: N = 287; for Cu: N = 293; and for Pb: N**
 600 **= 292).**



601 A



602 B



603 C

604 **Figure 5: Activities after 10 days in function of the metal body concentrations: A) Cd (N = 287);**
 605 **B) Cu predator treatments (N = 293); and C) Pb (N = 292).**

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607 **Tables**

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609 **Table 1: Overview of the dissolved metal concentrations at day 0. BMQL = Below method**
 610 **quantification limit. BMQL of $^{116}\text{Cd} = 0.647 \cdot 10^{-3} \mu\text{mol/L}$; BMQL of $^{65}\text{Cu} = 0.015 \cdot 10^{-3}$**
 611 **$\mu\text{mol/L}$; and $^{204}\text{Pb} = 0.005 \cdot 10^{-3} \mu\text{mol/L}$.**

	$^{116}\text{Cd} (\mu\text{mol/L})$	$^{65}\text{Cu} (\mu\text{mol/L})$	$^{204}\text{Pb} (\mu\text{mol/L})$
Controls	BMQL	$6.61 \cdot 10^{-3} \pm 2.08 \cdot 10^{-3}$	$0.427 \cdot 10^{-3} \pm 0.078 \cdot 10^{-3}$
Cd L	$1.78 \cdot 10^{-3} \pm 0.34 \cdot 10^{-3}$	$1.57 \cdot 10^{-3} \pm 0.19 \cdot 10^{-3}$	$0.628 \cdot 10^{-3} \pm 0.020 \cdot 10^{-3}$
Cd M	$12.1 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	$2.50 \cdot 10^{-3} \pm 0.32 \cdot 10^{-3}$	$0.711 \cdot 10^{-3} \pm 0.034 \cdot 10^{-3}$
Cd H	0.179 ± 0.022	$5.04 \cdot 10^{-3} \pm 0.03 \cdot 10^{-3}$	BMQL
Cu L	BMQL	0.110 ± 0.002	$0.417 \cdot 10^{-3} \pm 0.088 \cdot 10^{-3}$
Cu M	$1.25 \cdot 10^{-3} \pm 0.22 \cdot 10^{-3}$	1.22 ± 0.13	$0.721 \cdot 10^{-3} \pm 0.059 \cdot 10^{-3}$
Cu H	BMQL	23.3 ± 2.0	BMQL
Pb L	BMQL	$1.97 \cdot 10^{-3} \pm 0.12 \cdot 10^{-3}$	$23.2 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$
Pb M	$1.54 \cdot 10^{-3} \pm 0.18 \cdot 10^{-3}$	$1.68 \cdot 10^{-3} \pm 0.25 \cdot 10^{-3}$	0.276 ± 0.002
Pb H	BMQL	$8.35 \cdot 10^{-3} \pm 1.20 \cdot 10^{-3}$	3.08 ± 0.66
Cd + Cu L	$1.54 \cdot 10^{-3} \pm 0.06 \cdot 10^{-3}$	$0.133 \pm 0.092 \cdot 10^{-3}$	$0.476 \cdot 10^{-3} \pm 0.034 \cdot 10^{-3}$
Cd + Cu M	$13.2 \cdot 10^{-3} \pm 0.5 \cdot 10^{-3}$	1.23 ± 0.09	$0.515 \cdot 10^{-3} \pm 0.069 \cdot 10^{-3}$
Cd + Cu H	0.259 ± 0.022	17.2 ± 1.0	$0.059 \cdot 10^{-3} \pm 0.015 \cdot 10^{-3}$
Cd + Pb L	$1.38 \cdot 10^{-3} \pm 0.66 \cdot 10^{-3}$	$0.847 \cdot 10^{-3} \pm 0.308 \cdot 10^{-3}$	$5.25 \cdot 10^{-3} \pm 0.040 \cdot 10^{-3}$
Cd + Pb M	$10.6 \cdot 10^{-3} \pm 0.2 \cdot 10^{-3}$	$0.462 \cdot 10^{-3} \pm 0.231 \cdot 10^{-3}$	$80.9 \cdot 10^{-3} \pm 1.0 \cdot 10^{-3}$
Cd + Pb H	0.286 ± 0.002	$13.6 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$	4.08 ± 0.16
Cu + Pb L	BMQL	0.118 ± 0.004	$23.1 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$
Cu + Pb M	$1.41 \cdot 10^{-3} \pm 0.29 \cdot 10^{-3}$	0.995 ± 0.011	0.119 ± 0.008
Cu + Pb H	BMQL	18.8 ± 0.9	4.34 ± 0.17
Cd + Cu + Pb L	BMQL	$74.1 \cdot 10^{-3} \pm 3.1 \cdot 10^{-3}$	$4.85 \cdot 10^{-3} \pm 0.42 \cdot 10^{-3}$
Cd + Cu + Pb M	$11.5 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$	0.935 ± 0.014	$88.7 \cdot 10^{-3} \pm 1.6 \cdot 10^{-3}$
Cd + Cu + Pb H	0.294 ± 0.019	18.4 ± 1.8	3.87 ± 0.66

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613 **Table 2: Water chemistry measurements, presented as means (\pm SE) of 72 water samples at day 0. Water hardness was calculated**
 614 **according to the formula: $2.5 \text{ Ca} + 4.1 \text{ Mg}$.**

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T (°C)	pH	EC ($\mu\text{S/cm}$)	O ₂ (mg/L)	DOC (mg/L)	Ca (mg/L)	K (mg/L)	Mg (mg/L)	Na (mg/L)	Water hardness (mg/L CaCO ₃)
15 \pm 1	7.89 \pm 0.02	404 \pm 16	9.34 \pm 0.07	1.25 \pm 0.07	15.1 \pm 0.3	2.16 \pm 0.04	13.6 \pm 0.3	37.5 \pm 2.3	93.6 \pm 2.2

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628 **Table 3: Slopes for metal accumulation, calculated based on FIA_{water} , after 10 days per metal or predator treatment (for Cd: N = 287; for Cu: N**
 629 **= 293; for Pb: N = 292). Average slopes and standard errors are presented, together with R^2 values for the ANCOVA model. **Superscript lower****

630 **case letters** indicate statistical differences between treatments.

Metal	Mixture with/Predator stress	Slope _{accumulation} ($\log(\mu\text{mol/g dw}) \cdot \log(\mu\text{mol/L})^{-1}$)	R^2
Cd	-	1.29 ± 0.13^a	47.0%
	Cu	0.821 ± 0.087^b	
	Pb	1.15 ± 0.10^a	
	Cu + Pb	0.898 ± 0.076^b	
Cu	Without	$98.1 \cdot 10^{-3} \pm 5.0 \cdot 10^{-3}^a$	65.3%
	With	0.118 ± 0.005^b	
Pb	-	0.738 ± 0.043^a	78.6%
	Cd	0.729 ± 0.036^a	
	Cu	0.632 ± 0.027^b	
	Cd + Cu	0.623 ± 0.026^b	

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632 **Table 4: Slopes for respiration rates, calculated based on body concentrations, after 10 days (N = 293). Average slopes and standard errors are**
 633 **presented, together with R^2 values for the ANCOVA model.**

Metal	Predator stress	Respiration rate ($\mu\text{g O}_2 \cdot \text{mg ww}^{-1} \cdot \text{h}^{-1} \cdot \log(\mu\text{mol/g dw})^{-1}$)	R^2
Cu	/	$-8.45 \cdot 10^{-3} \pm 12.11 \cdot 10^{-3}$	2.21%
Pb	/	$-2.12 \cdot 10^{-3} \pm 2.11 \cdot 10^{-3}$	2.25%

634 **Table 5: Slopes for growth rates, calculated based on body concentrations, after 10 days per predator treatment (N = 293). Average slopes and**
 635 **standard errors are presented, together with R² values for the ANCOVA model. **Superscript lower case letters** indicate statistical differences**
 636 **between treatments.**

Metal	Predator stress	Growth rate (mg dw.day ⁻¹ .log(μmol/g dw) ⁻¹)	R ²
Cd	/	-7.04 10 ⁻³ ± 2.66 10 ⁻³	4.18%
Cu	Without	-49.8 10 ⁻³ ± 13.8 10 ⁻³ ^a	12.1%
	With	-93.4 10 ⁻³ ± 16.2 10 ⁻³ ^b	

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648 **Table 6: Slopes for feeding rates, calculated based on body concentrations, after 10 days per metal or predator treatment (for Cd: N = 287; for**
 649 **Cu: N = 293; for Pb: N = 292). Average slopes and standard errors are presented, together with R² values for the ANCOVA model. Superscript**
 650 **lower case** letters indicate statistical differences between treatments.

Metal	Mixture with	Feeding rate (mg.mg ⁻¹ .day ⁻¹ .log(μmol/g dw) ⁻¹)	Predator stress	Feeding rate (mg.mg ⁻¹ .day ⁻¹ .log(μmol/g) ⁻¹)	R ²
Cd	-	-0.462 10 ⁻³ ± 4.298 10 ⁻³ a	/	3.70 10 ⁻³ ± 2.70 10 ⁻³	10.2%
	Cu	-5.77 10 ⁻³ ± 3.85 10 ⁻³ a			
	Pb	19.1 10 ⁻³ ± 7.8 10 ⁻³ b			
	Cu + Pb	0.202 10 ⁻³ ± 3.617 10 ⁻³ a			
Cu	-	-58.4 10 ⁻³ ± 14.0 10 ⁻³ a	Without	-60.3 10 ⁻³ ± 10.6 10 ⁻³ a	14.9%
	Cd	-72.4 10 ⁻³ ± 15.0 10 ⁻³ a	With		
	Pb	-56.6 10 ⁻³ ± 15.6 10 ⁻³ a			
	Cd + Pb	-3.84 10 ⁻³ ± 14.26 10 ⁻³ b			
Pb	-	1.75 10 ⁻³ ± 2.97 10 ⁻³ a	/	1.88 10 ⁻³ ± 1.81 10 ⁻³	9.93%
	Cd	11.7 10 ⁻³ ± 5.2 10 ⁻³ b			
	Cu	-3.92 10 ⁻³ ± 2.81 10 ⁻³ a			
	Cd + Cu	-2.76 10 ⁻³ ± 2.24 10 ⁻³ a			

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654 **Table 7: Slopes for activities, calculated based on body concentrations, after 10 days per predator treatment (N = 293). Average slopes and**
 655 **standard errors are presented, together with R² values for the ANCOVA model. **Superscript lower case** letters indicate statistical differences**
 656 **between treatments.**

Metal	Predator stress	Activity (%.log($\mu\text{mol/g dw}$) ⁻¹)	R ²
Cd	/	-1.98 ± 0.55	6.23%
Cu	Without	-19.5 ± 2.65 ^a	10.8%
	With	-5.70 ± 3.56 ^b	
Pb	/	-1.54 ± 0.37	4.45%

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