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## ► To cite this version:

Esther U. Kadiene, Pei-Jie Meng, Jiang-Shiou Hwang, Sami Souissi. Acute and chronic toxicity of cadmium on the copepod *Pseudodiaptomus annandalei*: A life history traits approach. *Chemosphere*, 2019, 233, pp.396 - 404. 10.1016/j.chemosphere.2019.05.220 . hal-03486983

**HAL Id: hal-03486983**

**<https://hal.science/hal-03486983>**

Submitted on 20 Dec 2021

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1 CHEMOSPHERE

2 Manuscript Number: CHEM62185

3 **Acute and chronic toxicity of cadmium on the copepod *Pseudodiaptomus annandalei*: A life**  
4 **history traits approach**

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15

## 16 Abstract

17 **We** determined the effect of acute and chronic toxicity of cadmium (Cd) on  
18 *Pseudodiaptomus annandalei*, according to their developmental and reproductive stages. **Firstly,**  
19 **to estimate the 50% lethal concentration (LC<sub>50</sub>),** acute exposure of nauplii and copepodids to 20,  
20 40, 80, 150, and 300 µg/L of Cd **was tested**, and the effects of 5 µg/L and 40 µg/L of Cd on  
21 copepod developmental rate was done. Female lifespan and number of nauplii produced were  
22 compared. Secondly, one generation of copepod was exposed to dissolved (WCd) and dietary  
23 (DCd) Cd, and sex-specific Cd uptake and population density were estimated.

24 96 h LC<sub>50</sub> was 40 µg/L Cd for nauplii and 120 µg/L Cd for copepodids. Duration of copepod  
25 development was 3.5 days and 5.5 days longer than the control when exposed to 5 µg/L and 40  
26 µg/L of Cd, respectively. Female lifespan in both treatments were 9 and 8 days shorter than in  
27 the control, respectively. Total number of nauplii produced per female lifespan was 440  
28 (control), 450 (5 µg/L Cd), and 365 (40 µg/L Cd). Cd uptake in copepods increased from nauplii  
29 to adults when exposed to dietary Cd and decreased when exposed to dissolved Cd. Dietary  
30 uptake of Cd was **significantly** higher in males than in females and Cd uptake from water was  
31 higher in males than in females, **but not statistically significant**. The **total population** of copepods  
32 **were significantly affected by Cd. The toxic effects of Cd** in copepods appear to depend on  
33 developmental stage, sex, duration and uptake route.

34

35 **Keywords:** LC<sub>50</sub>, developmental stages, **sublethal effects, life history traits**, bio-accumulation,  
36 sex.

37

## 38 1. Introduction

39 Trace metals such as copper, cadmium, lead, or zinc are increasingly contaminating marine,  
40 brackish, and freshwater environments. **The flow of contaminants into estuaries mostly comes**  
41 **from intra-estuarine tributaries, remobilization of stored contaminants in the environments,**  
42 **including direct discharges from industries (Fisson, 2015; Moshenberg, 2013). The Danshui**  
43 **estuary in northeastern Taiwan experiences high influx of untreated domestic discharge and both**  
44 **treated and untreated industrial discharges from its tributaries and it is heavily polluted with trace**  
45 **metals and their concentrations vary with location and seasons (Fang and Lin, 2002; Fang et al.,**  
46 **2006; 2014; Jeng and Han, 1994; Jiann et al., 2014). Metals, such as copper and zinc, can be**  
47 **functionally essential at low concentrations. However, these metals can become toxic when they**  
48 **exceed a particular threshold concentration in the water.** The rate of **metal** concentrations in the  
49 environment and the level of toxicity to organisms are among other factors affected by the  
50 geochemical behavior of metals, chemical speciation, the presence of other toxicants or  
51 environmental conditions and **the physiology and condition of the organism** (Ansari et al., 2004).  
52 **Metals** cannot be broken down in the environment, but their transfer, bioavailability, or toxicity

53 are affected by conditions such as low pH, low hardness, low suspended matter level, high redox  
54 potential, low salinity (Cole et al., 1999), and temperature (Boeckman and Bidwell, 2006; Khan  
55 et al., 2006; 2007). Cadmium is widely distributed in the Earth's crust at an average  
56 concentration of approximately 0.1 mg/kg, and it enters the aquatic environment through  
57 geological or anthropogenic activities (Sciortino and Ravikumar, 1999; Tchounwou et al., 2012).  
58 Although, cadmium has no vital or biological benefits on organisms (Chang et al., 1996; Singh et  
59 al., 2011), however, for certain organisms or under certain conditions, it can be beneficial (Lane  
60 et al., 2005). Acute or chronic exposure can result in toxic effects such as lethal (acute) and  
61 sublethal (chronic) effects on organism's behavior, immune and endocrine systems,  
62 development, growth, and reproduction (Chen, 2011; Kadiene et al., 2017; Michalec et al., 2013;  
63 Sullivan et al., 1983).

64 Copepods are essential trophic links between primary producers and consumers in aquatic  
65 systems. As grazers of phytoplankton and prey to fish and shrimps they mediate in the bio-  
66 accumulation and bio-magnification processes of toxic pollutants in the aquatic food webs  
67 (Fisher et al., 2000; Watras et al., 1998). One of the prevalent subtropical, estuarine copepod  
68 zooplankter is *Pseudodiaptomus annandalei* Sewell, 1919, a dominant euryhaline species found  
69 perennially in coastal and estuarine ecosystems in the tropical and subtropical Indo-Pacific  
70 region. *P. annandalei* is a relatively abundant and dominant brackish water species found in the  
71 Danshui estuary in Northern Taiwan (Chen et al., 2006; Doi et al., 1997; Golez et al., 2004;  
72 Hwang et al., 2010; Liao et al., 2001; Sarkar et al., 1985). The productivity and abundance of  
73 copepods face threats from environmental toxic pollutants. However, toxicity of metals (which is  
74 the degree to which a metal can induce harm) to copepods vary because the rate of uptake,  
75 excretion, and detoxification are inter- and intra-species as well as metal specific (Fang et al.,  
76 2014; Hsiao et al., 2010; Luoma and Rainbow, 2005; Ritterhoff and Zauke, 1997).

77 The natural features of copepods make them suitable candidates as reference species in  
78 aquatic toxicology (Cutts, 2003; Devreker et al., 2004; Kwok et al., 2009, 2015; Souissi et al.,  
79 1997; Wang et al., 2014; Zhang and Uhlig, 1993). Calanoid copepods have distinct  
80 developmental stages and their development time varies and usually depends on environmental  
81 conditions such as temperature and salinity (Beyrend-Dur et al., 2011; Devreker et al., 2007;  
82 Golez et al., 2004). In addition, calanoid copepods including *P. annandalei* exhibits sexual

83 dimorphism (Dur et al., 2010; Katona, 1975). These factors enable easy investigation of stage-  
84 and sex-specific toxicity to chemicals. Their fast growth and short life cycle (8 to 19 days,  
85 depending on their culture conditions) (Beyrend-Dur et al., 2011; Dhanker et al., 2015; Golez et  
86 al., 2004) makes them suitable for toxicity testing of life cycle traits.

87 Copepods accumulate metals by assimilating them from their food or by absorbing them  
88 from water (Wang and Fisher, 1998) and the uptake pathway can determine its internal  
89 distribution (Hare et al., 1991; Hook and Fisher, 2001; Wang and Fisher, 1998). Dietary toxicity  
90 of metal is dependent upon the organism and the life stage that is exposed, as well as the diet  
91 type, form of metal, and the daily dose received (Carpene and George, 1981; Clausen et al.,  
92 1993; Sidoumou et al., 1997). Studies have shown that cadmium uptake is higher from dissolved  
93 phase than through dietary route. For example, *Acartia tonsa* was reported to assimilate 40  
94 percent of the cadmium through their phytoplankton food and more than 50 percent in their  
95 tissue is from the dissolved phase in ambient waters (Fisher et al., 2000). Other studies have  
96 shown similar results of cadmium accumulation from water to be higher than from food  
97 (Borchardt, 1983; Riisgard et al., 1987). Biological factors like age, size, and sex modify uptake  
98 and elimination of metals (Wang and Fisher, 1997). Several studies have highlighted the critical  
99 ecological effect of lethal and sublethal concentrations of toxic metals. However, very few  
100 studies have attempted to elucidate the responses of copepods to toxic metals based on their life  
101 stage or reproductive state. Age and sex are commonly important factors for copepod stress  
102 response (Dipinto et al., 1993; Forget et al., 1998; Hsiao and Fang, 2013; Kadiene et al., 2017;  
103 Sornom et al., 2010; Zidour et al., 2019). Sex-specific sensitivity to metals in *P. annandalei* were  
104 reported in a previous study (Kadiene et al., 2017). In the present study, we further showed toxic  
105 effects of acute exposure of the developmental stages of *P. annandalei* to cadmium (Cd) as well  
106 as the effects of chronic exposure to Cd on growth, development and reproductive performance  
107 of the copepod. In addition, we investigated the sex-specific bio-accumulation of cadmium both  
108 from water and from diet in *P. annandalei*. We followed the hypothesis that life history traits  
109 provide critical indications for sublethal effects of toxicants as a comprehensive approach in  
110 ecotoxicology.

## 111 2. Materials and Methods

### 112 2.1. Copepod culture

113 *P. annandalei* Sewell, 1919, used in this study were cultured in the laboratory of the Institute  
114 of Marine Biology, National Taiwan Ocean University, Taiwan. The strain was initially collected  
115 from coastal brackish ponds in Tungkang, Southern Taiwan, and maintained in the laboratory.  
116 The *P. annandalei* strains were maintained in 2-L beakers in a laboratory incubator at 26 °C,  
117 under a light regime of 12 h light and 12 h dark. We used the protocol described by Souissi et al.  
118 (2010, 2016a), Beyrend-Dur et al. (2013) and Devreker et al. (2009) to maintain the copepods  
119 throughout generations. They were fed one or a combination of these algae; *Diacronema*  
120 (=Pavlova) *lutheri* (Droop) Bendif et al. 2011, *Tisochrysis lutea* Bendif et al. 2013 (Tiso) and  
121 *Nannochloropsis oculata* (Droop) Hibberd 1981. The copepods used in the experiment were  
122 provided from large-scale culture systems. The seawater used for the *P. annandalei* culture was  
123 pumped from the ocean near the National Taiwan Ocean University (Northern Taiwan) and  
124 filtered up to 1-µm. *P. annandalei* copepods were fed *Diacronema lutheri* cultivated in Conway  
125 medium, following the method described by Sadovskaya et al. (2014). *D. lutheri* was used  
126 because it showed a suitable biochemical profile (Fernández-Reiriz et al., 1989; Persoone and  
127 Claus, 1980), and according to preliminary tests, we observed that it was adequate to cultivate *P.*  
128 *annandalei*. The water used for both the large-stock copepod species culture and the experiment  
129 was diluted with distilled water to obtain a salinity of 15. This salinity provides an optimum  
130 growth condition (Beyrend-Dur et al., 2011; Chen et al., 2006).

## 131 2.2. Experiment 1: Acute exposure of *P. annandalei* to cadmium

### 132 2.2.1. Lethal concentrations (LC<sub>50</sub>) of cadmium in nauplii and copepodids

133 The evaluation of LC<sub>50</sub> of Cd in *P. annandalei* nauplii and copepodids was carried out  
134 following the protocol of Kadiene et al. (2017). Nauplii and copepodids (n=25) were placed in  
135 100 ml beakers containing 90 ml media of different concentrations of cadmium (0, 20, 40, 80,  
136 150 and 300 µg/L) in triplicate. The beakers were covered with aluminum foil, without aeration,  
137 unfed and kept in the incubator at 26 °C, for a duration of 96 hours and mortality was estimated  
138 daily.

### 139 2.2.2. Effect of cadmium on developmental duration, female lifespan, and total number of 140 nauplii produced per female lifespan

141 A selected number of ovigerous females were incubated. Nauplii hatched within 24 hours  
142 were placed individually in 12-well plates containing 5 mL of medium spiked with 0, 5  
143 (corresponding to No Observed Effect Concentration (NOEC) estimated from the results of  
144 2.2.1.), and 40 µg/L Cd (corresponding to 50% lethal concentration for nauplii (refer to results  
145 from 2.2.1.)). They were monitored every 12 to 24 hours and fed with an uncontaminated diet  
146 once in 2 days sufficiently then transferred to a new medium of their respective treatments to  
147 permit exposure only from the contaminated water. Presence of molted exoskeletons and the  
148 time until they reached the adult stage were recorded.

149 C5 female and C6 male copepods were selected from the respective treatments in  
150 developmental duration tests. A single C5 female was paired with 2 male partners in a new  
151 medium with the same treatments (control, 5, and 40 µg/L Cd) in 15mL beakers and 10  
152 replicates. They were fed as described above and frequently observed (every 12 or 24 hours).  
153 After spawning (formation of egg sacs) and hatching, the adults were transferred to a new  
154 medium with the same treatment and the number of produced nauplii were counted. Dead males  
155 were replaced and the reproduction process was continued until the females died. The total  
156 number of nauplii produced by individual females from first clutch to death was enumerated, and  
157 the lifespan of the females were recorded.

## 158 2.3. Experiment 2: Chronic exposure of *P. annandalei* to cadmium

### 159 2.3.1. Effect of Cd on cohort populations and sex-specific bio-accumulation of Cd

160 The experiment consisted of dissolved metal exposure from water only (represented as  
161 WCd), a dietary metal exposure (represented as DCd) and control, following Hook and Fisher  
162 (2001) and Arias et al. (2016) with modifications. Copepod nauplii were collected by filtering  
163 the copepod stock cultures through a 120 µm mesh filter and were set to acclimate at the  
164 experimental salinity and temperature conditions. 5 L culture media were spiked with 40 µg/L of  
165 Cd (WCd), corresponding to the LC<sub>50</sub> of *P. annandalei* nauplii for 12 hours before stocking the  
166 copepods to allow a sufficient time for Cd to react with ligands which are naturally present in the  
167 water (Hook and Fisher, 2001). On the other hand, other sets of 5 L culture media were prepared  
168 without Cd contamination (labelled as DCd and control). The copepods were transferred  
169 randomly into each of the experimental beakers. The algae (*Diacronema lutheri*) used to feed the  
170 copepods in the control and WCd were not contaminated with Cd. *D. lutheri* was inoculated and

171 cultured in salinity 15-20 and spiked with 40 µg/L of Cd for 4 days and used for feeding  
172 copepods in DCd. The algae were centrifuged to remove the culture medium, re-suspended using  
173 the medium from the respective treatments, then fed to the copepods. This was done particularly  
174 in WCd, to prevent dilution of Cd concentration in the medium or to prevent Cd contamination  
175 of DCd medium. The copepods were fed every 2 days, according to a standard feeding protocol  
176 of the laboratory (Beyrend-Dur et al., 2009; Souissi et al., 2016b). Moreover, algae were still  
177 present in observable amounts in their gut when observed under the microscope the next day  
178 after adequately feeding the previous day. Salinity 15 was used to culture *D. lutheri* because a  
179 preliminary test showed that low salinity increased Cd uptake in *D. lutheri*. The amount of Cd  
180 bio-accumulated in *D. lutheri* after exposure to 40 µg/L of Cd for 4 days, ranged from 8 µg/g to  
181 11 µg/g dry weight (DW) throughout the experiment. During inoculation, Conway medium was  
182 used and prepared without the addition of EDTA known to be a ligand that complexes metal  
183 ions, and can decrease the concentration of free Cd<sup>2+</sup> in the solution (Grčman et al., 2001).  
184 Copepods were grown to adults at the above conditions, with periodic sampling at nauplius stage  
185 (day 3), copepodid stage (day 6), and adult stage (day 14). The culture medium was not changed  
186 throughout the experiment. When large numbers of ovigerous females were observed, males and  
187 females were sorted separately in triplicates for bio-accumulation analysis and the remaining  
188 copepods were counted to calculate population densities. The copepods were filtered through  
189 acid washed polycarbonate filter papers, rinsed slightly with Milli-Q water to remove salt and  
190 dried at 60 °C for 72 h and kept in a desiccator until further analysis.

#### 191 2.4. Cadmium analysis

192 Dried filter papers containing experimental samples were digested for 3 h at 120 °C using 1  
193 mL of nitric acid (Merck Suprapur, 65%) and 3 mL of hydrochloric acid (Merck Suprapur, 33%)  
194 in Teflon tubes. The digested solutions were diluted to a final volume of 10 mL with ultrapure  
195 water and stored at 4 °C until analysis. Cadmium concentrations were measured in triplicate  
196 using GAAS (graphite atomic absorption spectrophotometry, Hitachi Z-8000, Japan). The data  
197 quality was assessed and validated using certified reference material samples (NASS-3)  
198 including bovine liver (NBS-SRM-1577) and orchard leaf (NBS-SRM-1571).

#### 199 2.5. Kinetic uptake model



200 The kinetic uptake model, expressing the **concentration** of metal uptake ( $\mu\text{g/g/d}$ ), was  
201 calculated following Tlili et al. (2016) and Zidour et al. (2019):

$$202 \quad C_{cp} = K_u C_w t \text{ and } C_{cp} = K_u C_D t$$

203 where  $C_{cp}$  is the cadmium concentration in copepod ( $\mu\text{g/g}$  dry weight),  $k_u$  is the uptake rate  
204 constant or the slope of the graph for water exposure ( $\text{L.g}^{-1}$  (dry weight).day $^{-1}$ ) and ( $\text{g}$  (dry  
205 weight).g $^{-1}$  (dry weight).day $^{-1}$ ; i.e. day $^{-1}$ ) for dietary exposure,  $C_w$  is Cd concentration in water  
206 ( $\mu\text{g/L}$ ),  $C_D$  is Cd concentration in diet ( $\mu\text{g/g}$  dry weight), and  $t$  is exposure time (days).

## 207 2.6. Statistical analysis

208 Probit analysis was performed using Microsoft Excel 2016, and  $\text{LC}_{50}$  was calculated as  
209 described by Tlili et al. (2016). Mortality was corrected for probit analysis by using Abbot's  
210 formula. Data are expressed as the mean  $\pm$  standard deviation (SD). Comparisons between means  
211 were made by one-way ANOVA, followed by a Tukey's test for identification of the statistically  
212 distinct groups. Significant differences were accepted for  $p < 0.05$ . Statistical analyses were  
213 performed using SPSS, v.18.0 (SPSS Inc., Chicago, IL, USA).

## 214 3. Results

215 Figure 1A and 1B show the 96 h concentration–mortality regression lines for *P. annandalei*  
216 naupliar and copepodid stages. Mortality increased with an increase in cadmium concentration.  
217 Mortality was probit transformed, and lethal concentration (LC) values extrapolated from  
218 regression lines. The lethal concentration that resulted in 50% of naupliar mortality was 40.3  
219  $\mu\text{g/L}$  Cd and for copepodids it was 120.4  $\mu\text{g/L}$  Cd.

220 Delayed development was observed in individual *P. annandalei* exposed to low (5  $\mu\text{g/L}$ ) and  
221 high (40  $\mu\text{g/L}$ ) Cd concentrations. Significant difference ( $p < 0.05$ ) between the treatments were  
222 observed from the early copepodid stages. Naupliar development from N1 to N3 was fast,  
223 however, under Cd treatments at least 1 or 2 days' delay was observed between each molt from  
224 N3 onward (Fig. 2). The duration of copepod development from N1 to C5 was 3.5 days and 5.5  
225 days longer than in the control groups when exposed to 5  $\mu\text{g/L}$  and 40  $\mu\text{g/L}$  of Cd respectively.  
226 In addition, the difference in developmental delay (N1-C5) between high (40  $\mu\text{g/L}$ ) and low (5  
227  $\mu\text{g/L}$ ) Cd concentration was 2 days. Mortality in both control and 5  $\mu\text{g/L}$  Cd groups were  
228 approximately 16% and in those exposed to 40  $\mu\text{g/L}$  Cd was 33%.

229 Lifespan of female copepods under Cd treatment were lower than in the control, although not  
 230 statistically significant ( $p>0.05$ ). Female lifespan in the control treatment was  $61.0\pm 22.1$  days,  
 231 and was  $52.4\pm 17.6$  and  $53\pm 19.9$  days for those under  $5\ \mu\text{g/L}$  and  $40\ \mu\text{g/L}$  of Cd treatments  
 232 respectively. The total number of nauplii produced by individual females per lifespan were  
 233  $440.3\pm 127.2$ ,  $450.7\pm 65.2$  and  $365.7\pm 73.4$  in the control,  $5\ \mu\text{g/L}$  and  $40\ \mu\text{g/L}$  Cd treatments  
 234 respectively, and were not statistically ( $p>0.05$ ) different.

235 The life cycle bio-accumulation of Cd in *P. annandalei* copepods exposed to dissolved Cd  
 236 and dietary Cd are presented in Figure 3A and B. After 2 days of exposure to dissolved Cd, the  
 237 concentration of Cd in *P. annandalei* (late naupliar stages) was  $25.81\pm 2.86\ \mu\text{g/g DW}$ , after 6  
 238 days (copepodid stages) it was  $20.70\pm 0.83\ \mu\text{g/g DW}$  and at the adult stage, it was  $20.17\pm 2.02$   
 239  $\mu\text{g/g DW}$  (Fig. 3A). After 2 days of exposure to dietary Cd, the concentration of Cd in *P.*  
 240 *annandalei* (late naupliar stages) was  $2.15\pm 0.80\ \mu\text{g/g DW}$ ; after 6 days (copepodid stages) it was  
 241  $3.27\pm 0.50\ \mu\text{g/g DW}$ ; and at the adult stage, it was  $9.00\pm 1.41\ \mu\text{g/g DW}$  (Fig. 3B). Experimental  
 242 calculation of the uptake rate constant ( $K_u$ ) from aqueous solution and from diet are presented in  
 243 Table 1.

244 Table 1. Uptake rate constant ( $K_u$ ) calculated from the accumulation kinetics of Cd in *P.*  
 245 *annandalei* exposed to dissolved Cd (WCd) and dietary Cd (DCd).

	WCd	DCd
Uptake rate constant $K_u$	$-0.0836\ (\text{L}\cdot\text{g}^{-1}\cdot\text{day}^{-1})$	$0.3147(\text{day}^{-1})$
$r^2$	0.7636	0.8575

246

247 Sex-specific uptake of Cd from dissolved Cd (WCd) and from dietary Cd (DCd) are shown  
 248 in Figures 3C and D. Cd taken up from water resulted in a concentration of  $22.53\pm 2.05\ \mu\text{g/g DW}$   
 249 in males and  $18.99\pm 2.01\ \mu\text{g/g DW}$  in females. The differences between male and female in WCd  
 250 were not statistically significant ( $p>0.05$ ) (Fig. 3C). Dietary uptake of Cd resulted in a  
 251 concentration of  $11.82\pm 4.35\ \mu\text{g/g DW}$  in males and  $7.59\pm 1.41\ \mu\text{g/g DW}$  in females. The  
 252 differences between male and female in DCd were statistically significant ( $p<0.05$ ) (Fig. 3D).

253 Individual and total population of *P. annandalei* copepods exposed to dietary Cd and  
 254 dissolved Cd (water) are shown in Figure 4. The number of copepodids were significantly

255 (p<0.05) lower in the control than in WCd. However, the number of male, non ovigerous and  
256 ovigerous female copepods were significantly (p<0.05) higher in the control, lower in DCd than  
257 in control but lowest in WCd. The total population of copepods followed a similar trend, where  
258 total population in WCd was significantly (p<0.05) lower than in the control group. In the  
259 control, the total number of males were significantly (p<0.05) higher than the copepodids and the  
260 females. In WCd, copepodids were significantly more abundant (p<0.05) than males and  
261 females.

#### 262 4. Discussion

263 Studies have shown that the response of copepods to different metal toxicity can be specific  
264 to sex (Bao et al., 2008; Lotufo and Fleeger, 1997; Stringer et al., 2012). We supported these  
265 findings in a previous study, where males of *P. annandalei* were shown to be more sensitive to  
266 cadmium toxicity (LC<sub>50</sub> of 121 µg/L) than female copepods (LC<sub>50</sub> of 240 µg/L) (Kadiene et al.,  
267 2017). In the present study, we showed that naupliar stages were more sensitive to cadmium  
268 toxicity (LC<sub>50</sub> of 40 µg/L) than copepodid stages (LC<sub>50</sub> of 120 µg/L). This confirms that toxicity  
269 of cadmium (Cd) is also specific to the stage of development. Similar findings were also  
270 reported, where nauplii were more sensitive (LC<sub>50</sub> of 17 µg/L) to cadmium toxicity than  
271 copepodids (LC<sub>50</sub> of 20 µg/L) for the copepod *Tigriopus brevicornis* (Forget et al., 1998). Dahms  
272 et al. (2014) also showed that the early developmental stages of *Tisbe* sp. are more sensitive than  
273 advanced stages when exposed to effluents from hydrothermal vents, containing high  
274 concentrations of metals (Dahms et al., 2018). Before this, Verriopoulos and Moraitou-  
275 Apostolopoulou (1982) reported that sensitivity of the copepod *Tisbe holothuriae* to cadmium  
276 toxicity depends on the stage of development. Furthermore, we showed that cadmium both at  
277 low and high concentrations delays the development of individual copepods. A similar delay in  
278 development was observed by Gnanamoorthy et al. (2012) in copepods, in sea urchin  
279 (*Paracentrotus lividus*) (Filosto et al., 2008) and in shrimp (Munshi et al., 1998). Exposure of *P.*  
280 *annandalei* nauplii to Cd during their developmental stages and further exposure of the female  
281 copepods till death negatively affected total number of nauplii produced and reduced female  
282 lifespan, which could be a consequence of increasing bio-accumulation of Cd over the exposure  
283 period. Similar findings were reported by Moraitou-Apostolopoulou et al. (1979) where an  
284 increasing concentration of cadmium affects the longevity of the copepod *Acartia clausi*.

285 In the present study, *P. annandalei* bio-accumulation of Cd from the dissolved phase was  
286 high at the earlier **developmental** stages of the copepod (nauplii). However, as they grew from  
287 nauplii to copepodids and to adults in a static system, Cd concentration in the copepod  
288 decreased. In contrast, dietary accumulation of Cd was **low** at the earlier stages of the copepod  
289 (nauplii). However, as they grew from nauplii to copepodids and to adults under periodic feeding  
290 of similar concentration of Cd contaminated algae, the Cd concentration in the copepod  
291 increased. The increasing Cd concentration with time/ developmental stages could be a result of  
292 increased feeding or ingestion rate (Almeda et al., 2010). Bio-accumulation of metals from the  
293 dissolved phase alone has been reported to be higher than from the consumption of metal-  
294 contaminated diets in mussels and copepods (Borchardt, 1983; Riisgård et al., 1987; Wang and  
295 Fisher, 1996; Wang et al., 1996; Wang and Fisher, 1998). The amount or rate of metal uptake  
296 from water can increase over time (Zidour et al., 2019), depending on the copepod stage,  
297 metabolism and rate of excretion. In this study, **the decrease of Cd concentration in copepod**  
298 **exposed to dissolved Cd over time was probably a result of growth dilution (Borgå, 2013), that**  
299 **is, the decrease in concentration of Cd with an increase in body size, hence the negative slope or**  
300 **uptake rate constant ( $K_u$ ).** However, the concentration of Cd in all developmental stages of  
301 **copepod exposed to dissolved Cd were** higher than those **exposed to dietary Cd**. This difference  
302 in accumulation could be due to different exposure routes and times, the concentration they are  
303 exposed to, the rate of uptake from each route, metabolic or excretion rate (Arunakumara et al.,  
304 2008; Berrojalbiz et al., 2009; Gomez et al., 2010; Jennett et al., 1983; Peake et al., 2015). The  
305 rate of Cd excretion is slower when copepods are exposed to Cd in water than to a Cd  
306 contaminated diet (Wang and Fisher, 1998; Williams et al., 2010; Xu and Wang, 2002). It has  
307 been demonstrated that assimilation efficiency of trace elements increases with longer gut  
308 passage time (Chong and Wang, 2000; Wang and Fisher, 1996; Xu et al., 2001). Moreover, the  
309 efflux rate of metals **is** higher following uptake from food than uptake from the dissolved phase  
310 in the copepod *Temora longicornis* (Wang and Fisher, 1998). In an egestion study with the  
311 branchiopoda *Daphnia magna* and the insecta *Chironomus riparius*, Scherer et al. (2017) found  
312 that an exposure to food led to a shorter gut evacuation period of polystyrene spheres. This could  
313 imply that with a shorter gut evacuation time, the possibility of reducing or removing toxic  
314 pollutants is high. Therefore, the longer the organisms are exposed to contaminants especially  
315 from the dissolved phase, the more the contaminants are accumulated (Wallenstein et al., 2009),

316 consequently increasing their **toxic effects** (Javanshir, 2012; Kadiene et al., 2017). Furthermore,  
317 Cervetto et al. (1995) reported that the gut evacuation rate in males were slower than in female  
318 copepods. This could explain why the concentration of Cd in male copepods was higher than in  
319 female copepods under both exposure routes in the present study. Although, **in this study**, the Cd  
320 concentration in males and females of *P. annandalei* exposed to dissolved Cd were not  
321 statistically **different**, the males were reported to be more sensitive to Cd toxicity than the  
322 females in an earlier account (Kadiene et al., 2017). **The expression of metallothioneins, an**  
323 **heme-binding protein, is induced when an organism is exposed to metal, their expression is**  
324 **involved in metal homeostasis and detoxification (Amiard et al., 2006; Liberge and Barthélémy,**  
325 **2007; Wang and Wang, 2010) and could also be expressed in a sex-specific manner. This would**  
326 **mean that the proteins responsible for metal detoxification and elimination maybe more**  
327 **effectively expressed in females than in male copepods. However, this hypothesis needs to be**  
328 **verified in future investigations by using adequate molecular tools.**

329 The high accumulation observed in copepods exposed to dissolved Cd, **more** significantly  
330 affected individuals and the total population of copepods than when fed a Cd contaminated diet  
331 (DCd). **The number of copepodids in WCd was higher than in DCd and both were higher than**  
332 **the number of copepodids in the control. This means a large number of copepods stayed longer**  
333 **in the copepodid stage, which corresponds to the delay observed in the individual copepod**  
334 **development in experiment 1 (see Fig. 2). In addition, the total copepod population at Cd**  
335 **exposure was lower, probably as a result of high mortality in all stages prior to the adult stage,**  
336 **particularly at the naupliar stage, since the concentration of Cd in water was the LC<sub>50</sub> of *P.***  
337 ***annandalei* nauplii.**

338 In conclusion, bio-accumulation of Cd is dependent on the uptake route, and its **toxic effects**  
339 **are dependent on the developmental stages and can be sex-specific.** The long-term implication of  
340 a persistent pollutant like Cd in the aquatic environment is of significant concern to copepod  
341 population structure. A better understanding of the effect of metals to the population structure of  
342 copepods can be demonstrated through long-term or multigenerational approach. Such attempts  
343 will not only provide risk assessment of the largest mesozooplankton group in terms of  
344 biodiversity and abundance, being a critical mediator, if not keystone taxon between primary  
345 producers and secondary consumers. The life history study of copepods as an example can also

346 provide new insights in the use of population issues in the use of model species for  
347 ecotoxicology. Such data are particularly useful when they are modelled to provide generalized  
348 predictions about single species population effects that in turn will affect community structures  
349 in the future.

### 350 **Acknowledgements**

351 This study is part of a co-tutorial PhD thesis between the University of Lille (France) and  
352 National Taiwan Ocean University (Taiwan). This research was funded by a Scholarship from  
353 the President (Ching-Fong Chang) of National Taiwan Ocean University and the Eiffel  
354 Excellence Scholarship by the French Ministry for Europe and Foreign Affairs. This work is a  
355 contribution to the project CPER 2015-2020 MARCO funded by Europe (FEDER), the French  
356 government, the region Hauts-de-France and IFREMER, and the International Associated  
357 Laboratory between the University of Lille and the National Taiwan Ocean University. We are  
358 also grateful to Dr. Li-Chun Tseng, Chih-Ming Lin and Hsin-Yi Lo and the laboratory members  
359 for all their technical assistance.

### 360 **Author contributions statements**

361 Kadiene E. U. carried out the experiment and wrote the main manuscript text and prepared the  
362 figures. Meng P. J. was a collaborator in the chemical analysis of the metals, Hwang J.S. and  
363 Souissi S. jointly provided the experiment resources, supervised the work, reviewed the  
364 manuscript.

### 365 **Competing interests**

366 The authors declare no competing interests.

### 367 **Data availability statement**

368 The authors confirm that the data supporting the findings of this study are available within the  
369 article and further information needed on this study is available from the corresponding author,  
370 S.S., upon reasonable request.

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689 **Fig. 1.** Ninety-six-hour concentration–mortality curves for *P. annandalei* nauplii (A) and  
690 copepodid (B) copepods exposed to cadmium.

691 **Fig. 2.** Developmental duration of *P. annandalei* exposed to 0 (control), 5 and 40 µg/L of  
692 cadmium.

693 **Fig. 3.** Cadmium accumulation kinetics in *P. annandalei* exposed to dissolved Cd (WCd) (A)  
694 and dietary Cd (DCd) (B) measured in copepod populations at different growth stages (A). Cd  
695 concentration in male and female copepods exposed to dissolved Cd (WCd) (C) and dietary Cd  
696 (DCd) (D).

697 **Fig. 4.** Individual and total population of *P. annandalei* copepod cohort exposed to a diet  
698 containing about 8.4 µg/L of Cd (DCd) and water containing 40 µg/L of Cd (WCd). x, y, z  
699 represents significant differences (p<0.05) between treatments (control, DCd, and WCd). a, b  
700 represents significant differences (p<0.05) between individual stages within a treatment  
701 (copepodid, male, non-ovigerous female, and ovigerous females). Asterisks (\*) and hash (#)  
702 represents significant differences (p<0.05) between the total production between treatments.

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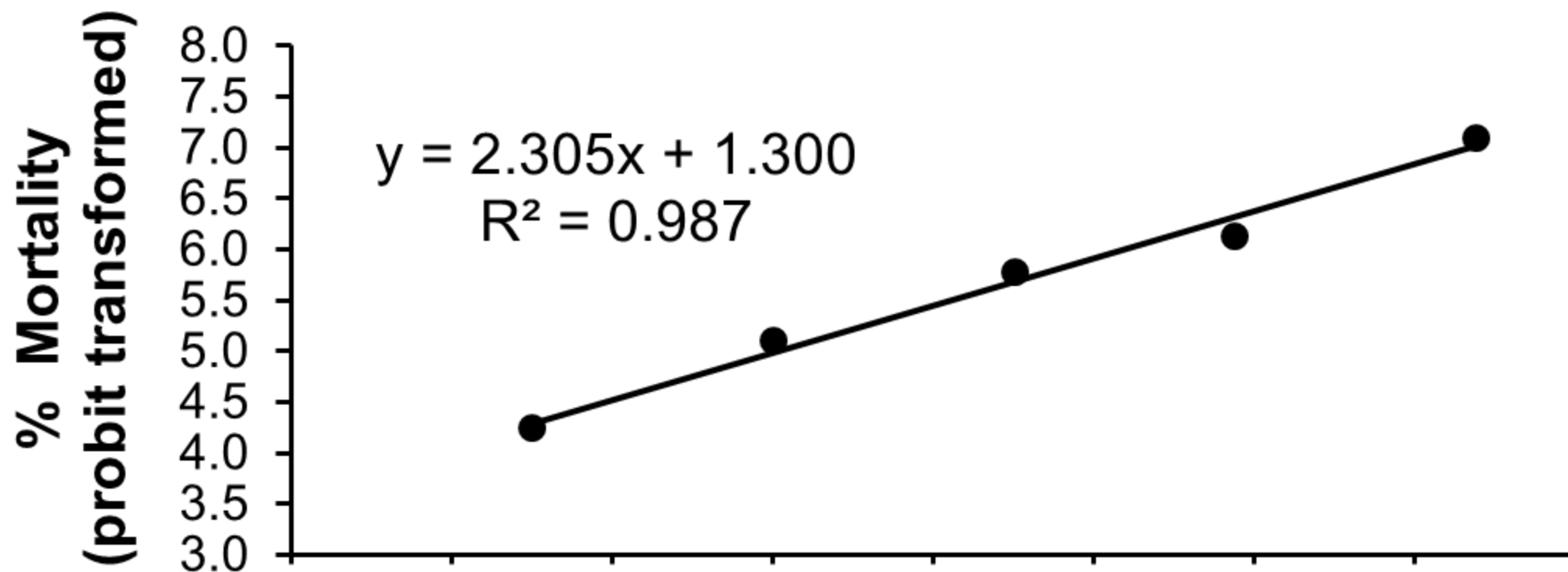
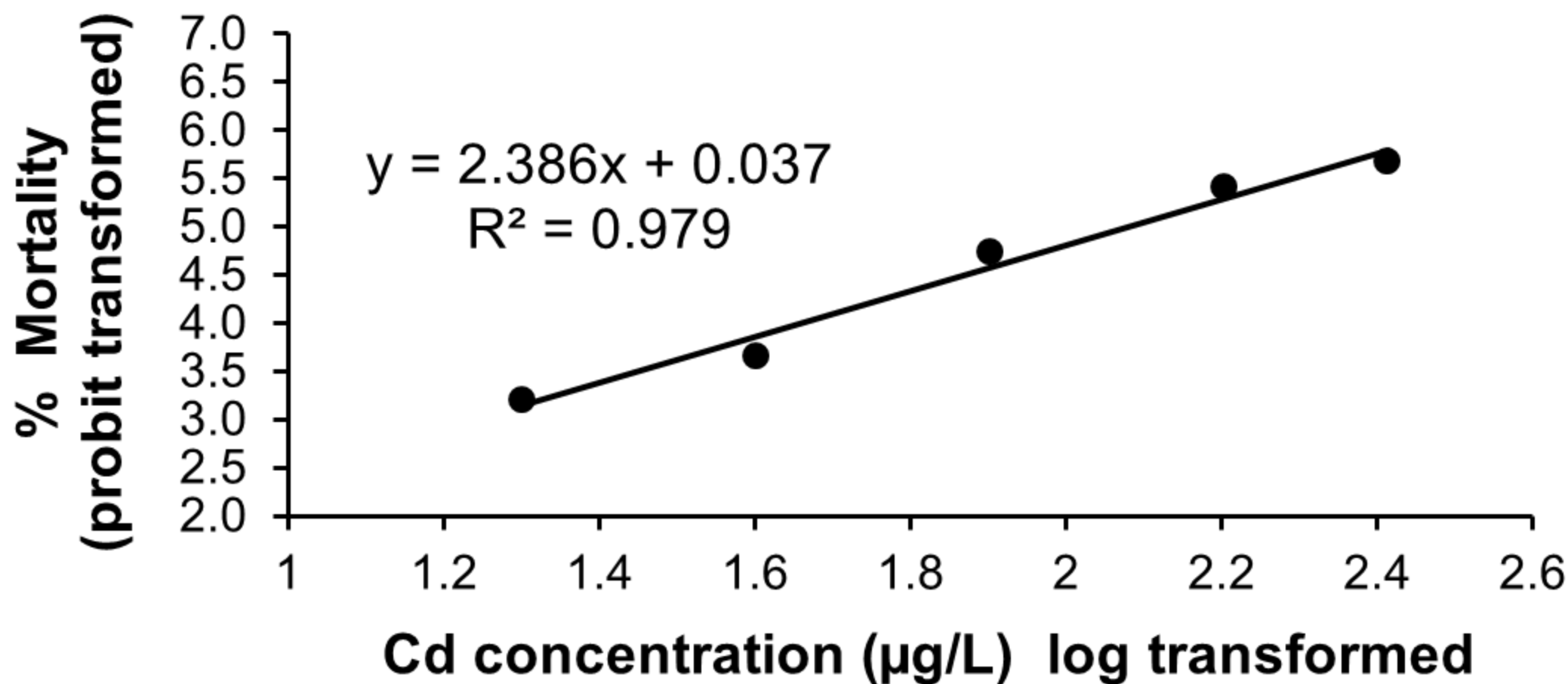
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**A****B**

Cumulative stage duration

(days)

