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1 **THE TOXICOLOGICAL INTERACTION BETWEEN OCEAN ACIDITY AND**  
2 **METALS IN COASTAL MEIOBENTHIC COPEPODS**

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14

15        **Abstract**

16            Increased atmospheric CO<sub>2</sub> concentrations are causing greater dissolution of CO<sub>2</sub> into  
17 seawater, and are ultimately responsible for today's ongoing ocean acidification. We  
18 manipulated seawater acidity by addition of HCl and by increasing CO<sub>2</sub> concentration and  
19 observed that two coastal harpacticoid copepods, *Amphiascoides atopus* and *Schizopera*  
20 *knabeni* were both more sensitive to increased acidity when generated by CO<sub>2</sub>. The present  
21 study indicates that copepods living in environments more prone to hypercapnia, such as  
22 mudflats where *S. knabeni* lives, may be less sensitive to future acidification. Ocean  
23 acidification is also expected to alter the toxicity of waterborne metals by influencing their  
24 speciation in seawater. CO<sub>2</sub> enrichment did not affect the free-ion concentration of Cd but did  
25 increase the free-ion concentration of Cu. Antagonistic toxicities were observed between CO<sub>2</sub>  
26 with Cd, Cu and Cu free-ion in *A. atopus*. This interaction could be due to a competition for  
27 H<sup>+</sup> and metals for binding sites.

28        **Key Words**

29            Ocean acidification, metal, cadmium, copper, harpacticoid copepod

30

## 31 **1. Introduction**

32 Over the last century, the atmospheric concentration of CO<sub>2</sub> has risen at a rate 100 times  
33 faster than any change observed during the past 650,000 years (Siegenthaler et al., 2005).  
34 There is broad consensus that this ongoing change is a direct result of human activity,  
35 principally by fossil fuel burning, cement production and changing land use (Hansen et al.,  
36 2007). Atmospheric levels of CO<sub>2</sub> have consequently increased from pre-industrial levels of  
37 280 ppm to a concentration of approximately 380 ppm (Feely et al., 2004). Almost 50% of all  
38 anthropogenic CO<sub>2</sub> emitted to the atmosphere has diffused passively into the ocean,  
39 significantly decreasing the rate of global warming (Sabine et al., 2004). Concentrations of  
40 atmospheric CO<sub>2</sub> are rising at a rate of 3.3% per year and will likely continue to rise (Canadell  
41 et al., 2007).

42 Political, social and environmental pressures to reduce atmospheric CO<sub>2</sub> have led  
43 governments to seek new options for CO<sub>2</sub> mitigation. A potential approach is the injection of  
44 CO<sub>2</sub> in underground porous reservoir rocks. Sub-seabed sequestration of CO<sub>2</sub> is considered to  
45 be a practical tool (Gibbins et al., 2006), and is already in use in the Norwegian sector of the  
46 North Sea (Holloway, 2005). Subsurface leakage is possible over time and has the potential to  
47 considerably increase local CO<sub>2</sub> concentration (Hawkins, 2004).

48 The net effect of CO<sub>2</sub> enrichment to seawater is to increase the concentration of  
49 carbonic acid (H<sub>2</sub>CO<sub>3</sub>), bicarbonate ion (HCO<sub>3</sub><sup>-</sup>), and hydrogen ion (H<sup>+</sup>), and to decrease the  
50 concentration of carbonate (CO<sub>3</sub><sup>2-</sup>). The production of H<sup>+</sup> lowers the pH and causes the  
51 phenomenon called “ocean acidification”. Carbonate ions can react with excess H<sup>+</sup> to form  
52 HCO<sub>3</sub><sup>-</sup>, and it was thought that the carbonate buffering system would allow oceans to retain a  
53 stable pH despite rising emissions. However, as the partial pressure of CO<sub>2</sub> increases, the  
54 buffering capacity of seawater decreases and the ocean has been reduced by 0.1 pH unit since

55 start of the industrial revolution, representing a 30% increase in the concentration of  $H^+$  ions  
56 (Caldeira and Wickett, 2003; Key et al., 2004). Recently, an increase in the area exposed to  
57 corrosive seawaters during seasonal upwelling was observed along the North American  
58 Pacific continental shelf (Feely et al., 2008). Hydrological models predict that, based on  
59 proposed future emissions of  $CO_2$ , the average oceanic pH will decline by 0.3-0.5 units by the  
60 year 2100 and by 0.7 units within the next 300 years (Caldeira and Wickett, 2003). Leakage  
61 from  $CO_2$  seabed storage would create locally faster and stronger acidification than that  
62 induced by atmospheric  $CO_2$  (Hawkins, 2004).

63 Reduced availability of carbonate ions, induced by increased  $CO_2$  concentrations,  
64 decreases calcification rates of organisms producing  $CaCO_3$  shells or skeletons (Gattuso et al.,  
65 1998; Kleypas et al., 2006; Riebesell et al., 2000). In addition to calcification, a number of  
66 other physiological processes, including growth, development, metabolism, ionoregulation  
67 and acid-base balance, can be affected directly by increases in  $CO_2$  (hypercapnia) or resulting  
68 acidosis (Fabry et al., 2008; Pörtner, 2008; Pörtner et al., 2004; Widdicombe and Spicer,  
69 2008).

70 It appears inevitable that marine biota will be faced with ocean acidification for decades  
71 or centuries to come. Further, effects of acidification may exacerbate the potential effects of  
72 other anthropogenic stressors such as heavy-metal enrichment, especially in coastal  
73 sediments. Specifically, ocean acidification will change the organic and inorganic speciation  
74 of metals and will modify interactions of metals with marine organisms (Millero et al., 2009).

75 In the general context of ocean acidification, as well as in the context of  $CO_2$  leakage  
76 from seabed storage, data are urgently needed to quantify the potential effects of  $CO_2$  on  
77 organisms from shallow coastal areas (Raven et al., 2005). Meiofauna are the most abundant  
78 metazoans living in soft sediment, and this environment constitutes the majority of seabed  
79 habitats. The first aim of the present study was to compare toxic impacts of acidity induced by

80 HCl and CO<sub>2</sub> on two species of harpacticoid copepods: *Schizopera knabeni* and  
81 *Amphiascoides atopus* in aqueous exposures. A wide range of acidity conditions was tested  
82 (including conditions that caused 100% mortality) to examine the entire H<sup>+</sup> concentration-  
83 response curve. A second aim of the present study was to evaluate the interactive toxicity of  
84 CO<sub>2</sub> with cadmium (Cd) and copper (Cu) for the copepod *A. atopus*. Those heavy metals are  
85 produced by human activity and both are widespread in the marine environment. Cd and Cu  
86 were chosen for study because they have different behaviors in CO<sub>2</sub> acidified water, which  
87 may create differences in toxicity.

88

## 89 **2. Material and method**

### 90 *2.1 Test organism and laboratory culture*

91 A culture of *Schizopera knabeni* was initiated in 1993 using animals from a salt marsh  
92 near Port Fourchon, Louisiana (Lotufo, 1997), and a culture of *A. atopus* was established in  
93 1992 (Lotufo and Fleeger, 1995). Both cultures were kept at 25°C in 1 L sediment-free  
94 Erlenmeyer flasks containing 600 mL of 30 psu artificial seawater (ASW), which was  
95 renewed every 14 days. Copepods were fed weekly with T-*Isochrysis* paste (Brine Shrimp  
96 Direct, Odgen, UT, USA). Continuous reproduction in both species was apparent. Copepod  
97 were harvested by rinsing culture medium through a 125 µm aperture screen and retained  
98 copepods were sorted under a stereo dissection microscope via Pasteur pipette.

### 99 *2.2 96-h toxicity tests*

100 All materials used to conduct bioassays were acid-cleaned prior to use. Fifteen  
101 nonovigerous adult female copepods (*A. atopus* or *S. knabeni*) were placed in 600 mL ASW  
102 plastic flasks, covered with a lid to avoid exchange with CO<sub>2</sub> from the air, and three replicates  
103 were used for each treatment. ASW was previously oxygenated by bubbling air for 24 h.  
104 Incubations at each condition were performed for 96 h at 25°C and 16:8 light:dark  
105 photoperiod in an environmental chamber.

106 Acidity was manipulated by adding mineral acid (HCl) to ASW in flasks. Range-  
107 finding tests were used to determine acidities that resulted in 100% mortality to both copepod  
108 species. *A. atopus* and *S. knabeni* were exposed to 5 (ranging from 5.62 to 6.22) and 8  
109 (ranging from 4.75 to 5.67) different pH conditions, respectively (N = 3 × 15 animals for each  
110 pH level). The pH (NBS scale) was measured, using a pH meter (Oakton) at the beginning  
111 and at the end of the incubation. pH varied by an average of 0.059 ± 0.015 units over the  
112 course of the exposures, representing an average H<sup>+</sup> concentration variation of 0.250 ± 0.145

113  $\mu\text{mol.L}^{-1}$ . Carbon dioxide effects were studied by bubbling pure  $\text{CO}_2$  in ASW. This  $\text{CO}_2$ -rich  
114 water (pH = 4.5) was mixed with air-bubbled ASW in order to achieve desired pH levels.  $\text{CO}_2$   
115 concentrations were tested at increasing levels until 100% mortality was achieved. *A. atopus*  
116 and *S. knabeni* were exposed to 10 (ranging from 5.47 to 6.67) and 9 (ranging from 5.29 to  
117 6.44) different  $\text{CO}_2$ -induced pH conditions, respectively ( $N = 3 \times 15$  specimens). Mean  
118 variations of pH and concentration of  $\text{H}^+$  measured between the end and the beginning of  
119 incubation were  $0.089 \pm 0.053$  and  $0.132 \pm 0.093 \mu\text{mol.L}^{-1}$  respectively. Total alkalinity in the  
120 ASW used under the conditions of our experiments was determined potentiometrically using  
121 a Gran function (Dickson et al., 2007). The  $p\text{CO}_2$  was calculated from pH and total alkalinity  
122 using the R package seacarb (Proyle, 2003).

123 In the present study, the pH values measured at the end of the incubations were used  
124 for the calculation of  $\text{LC}_{50}\text{s}$ .

125 Only *A. atopus* was studied for metal exposure. A small volume (20  $\mu\text{L}$ ) of acidified  
126 solution with the calculated amount of Cd and Cu to achieve a target concentration was  
127 spiked into each 600 mL incubation flask. At pH of 8.20, 6.30, 6.23 and 6.07, respectively 9,  
128 6, 5 and 5 different Cd concentrations were tested ( $N = 3 \times 15$  specimens). At pH of 8.20,  
129 6.36, 6.23, 6.15 and 6.08, respectively 6, 3, 3, 4 and 3 different Cu concentrations were tested  
130 ( $N = 3 \times 15$  specimens).

131 After 96 h, water was sampled for pH and metal concentrations. Contents of each  
132 microcosm were rinsed through a 125  $\mu\text{m}$  mesh sieve and copepods retained were enumerated  
133 as live or dead. Missing copepods were presumed dead and percent mortality was calculated.  
134 For *A. atopus* and *S. knabeni*, mean fractions of missing copepods represented  $7.27 \pm 14.22 \%$   
135 and  $6.42 \pm 14.18 \%$  of the total animals, respectively.

136 *2.3 Metal measurements*



137 Total Cd concentrations were determined by flame r atomic absorption spectroscopy  
138 (Varian AA240FS), and Cu concentrations were analyzed using ICP-OEC (Inductively  
139 Coupled Plasma, Optical Emission Spectrometer). All modeling for Cd and Cu was  
140 performed using the geochemical equilibrium modeling software program MINEQL+,  
141 version 4.6. Modeling simulations were run using components from the existing internal  
142 MINEQL+ database including,  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{SO}_4^{2-}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{HCO}_3^-$ ,  $\text{Br}^-$ ,  $\text{BO}_3$ ,  $\text{Sr}$ , and  $\text{F}^-$ , at  
143 the nominal concentrations typical of 30 psu ASW (Instant Ocean). Two successive iterations  
144 were performed; the first at a fixed pH of 4.5 with an open atmosphere in order to obtain the  
145 dissolved inorganic carbon (DIC) concentration, and the second using this DIC in a closed  
146 system, and using the components at the concentrations as described above, plus the different  
147 measured total Cd and Cu concentrations added to the components. These conditions  
148 mimicked the experimental protocols used in this study, which included bubbling  $\text{CO}_2$  into  
149 ASW to a pH of 4.5 (under open atmosphere conditions), then mixing this water with air-  
150 bubbled ASW to achieve a desired pH (as described above) before sealing the container to  
151 container with copepods inside.

152 Concentrations of  $\text{H}^+$ , metals and free-ion metal concentrations that caused 10%  
153 ( $\text{LC}_{10}$ ) and 50% ( $\text{LC}_{50}$ ) lethality were estimated, as well as their 95% confidence intervals  
154 using log-probit analysis (SPSS Version 17.0.1, Chicago, IL).

155 The sum toxic unit approach (Sprague, 1970) was used to assess the joint effects of  
156 binary mixtures of (i)  $\text{CO}_2/\text{Cd}$ , (ii)  $\text{CO}_2/\text{Cu}$  and (iii)  $\text{CO}_2/\text{Cu}$  free-ion. Toxic units (TU) are a  
157 means of expressing the toxicity of a mixture of compounds as a portion of its threshold effect  
158 concentration (Sprague, 1970). The toxicity of a mixture of compounds is expressed as a sum  
159 of the ratios of the exposure concentration and threshold-effect concentrations ( $\text{LC}_{50}$   
160 expressed as concentration that causes 50% mortality [ $\text{LR}_{50}$ ]) of the individual compounds in  
161 the mixture, where: sum toxic units = (concentration  $x$  /  $\text{LR}_{50} x$ ) + (concentration  $y$  /  $\text{LR}_{50} y$ ).

162 If compounds in a mixture are acting additively, the sum TU required to cause a 50%  
163 mortality of the exposed organisms will equal 1. A TU including 1 within the 95% confident  
164 interval was considered additive, a TU that did not include 1 in its high end 95% confidence  
165 interval (<1) was considered greater than additive (synergistic), while a TU that did not  
166 include 1 in its low end 95% confidence interval was considered less than additive  
167 (antagonistic).

#### 168 *2.4 Statistical analysis*

169 Differences between (i) acidity tolerances of *A. atopus* vs. *S. knabeni* and (ii) acidity  
170 induced by HCl vs. CO<sub>2</sub> were tested with analysis of covariance (ANCOVA) (Green et al.,  
171 1996) using SAS software (SAS-Institute, 1985). The observed survival proportions were  
172 transformed via angular transformation (Anscombe, 1948).

173

## 174 **3. Results**

### 175 *3.1 Acid toxicity*

176 Over the 96-h exposure period, mortality of *A. atopus* and *S. knabeni* ranged,  
177 respectively, from 0 to 2.2 % and from 0 to 4.4% in control experiments.

178 *A. atopus* had an LC<sub>10</sub> of 5.99 (5.86 – 6.19) pH units and the LC<sub>50</sub> was 5.75 (5.63 -  
179 5.84) pH units when acidity was manipulated with HCl. When water was acidified with CO<sub>2</sub>,  
180 LC<sub>10</sub> was 6.41 (6.29 – 6.82) pH units and LC<sub>50</sub> was 6.11 (6.04 - 6.17) pH units, equivalent to  
181 LC<sub>10</sub> of 23.6 (9.1 – 31.1) and LC<sub>50</sub> of 47.2 (41.0 - 55.4) matm CO<sub>2</sub> (Fig. 1).

182 *S. knabeni*, had an LC<sub>10</sub> of 5.51 (5.38 – 5.90) pH units and the LC<sub>50</sub> was 5.00 (4.90 -  
183 5.06) pH units when acidity was manipulated with HCl. When water was acidified with CO<sub>2</sub>,  
184 LC<sub>10</sub> was 6.06 (5.92 - 6.39) pH units and LC<sub>50</sub> was 5.54 (5.49 - 5.59) pH units, equivalent to  
185 LC<sub>10</sub> of 52.9 (24.7 – 73.1) and LC<sub>50</sub> of 175.1 (156.6 – 197.1) matm CO<sub>2</sub> (Fig. 2).

186 ANCOVA of the angular-transformed survival proportions showed that both species  
187 were significantly more sensitive to acidification from CO<sub>2</sub> relative to acidification from HCl  
188 ( $p < 0.001$ ). Furthermore, regardless of the method used to acidify the ASW, *A. atopus* was  
189 significantly more sensitive to acidity than *S. knabeni* ( $p < 0.001$ ).

### 190 *3.2 Metal toxicity*

191 The 96-h LC<sub>50</sub> of *A. atopus* for Cd was 1.38 mg.L<sup>-1</sup> (1.26 - 1.52) at pH 8.20. When  
192 acidity was changed by adding CO<sub>2</sub>, Cd LC<sub>50</sub> was 1.41 mg.L<sup>-1</sup> (1.22 – 1.60) at pH 6.30, 0.98  
193 mg.L<sup>-1</sup> (0.72 – 1.18) at pH =6.23 and 0.05 mg.L<sup>-1</sup> (0 – 0.307) at pH 6.07 (Fig. 3). The Toxic  
194 Unit required to create 50% mortality was 1.47 mg.L<sup>-1</sup> (1.28-1.62) (Fig. 4). As TU did not  
195 include 1 in its low end 95% confidence interval, CO<sub>2</sub> and Cd can be considered to have  
196 antagonistic toxicity. MINEQL+ modeling revealed that the Cd free-ion concentration was  
197 not affected by CO<sub>2</sub> under the conditions of the experiment.

198           The 96-h LC<sub>50</sub> of *A. atopus* for Cu was 0.65 mg.L<sup>-1</sup> (0.40 – 0.99) at pH 8.20. When  
199 acidity was changed by adding CO<sub>2</sub> this Cu LC<sub>50</sub> was 0.32 mg.L<sup>-1</sup> (0.25 – 0.55) at pH 6.37  
200 and 0.26 mg.L<sup>-1</sup> (0.19 – 0.48) at pH = 6.23 (Fig. 5). The Toxic Unit required to create 50%  
201 mortality was 1.17 (1.01 – 1.25). TU was slightly > 1 in its low end 95% confidence interval  
202 (Fig. 6), which indicated that the toxicity of CO<sub>2</sub> and Cu were antagonistic. MINEQL+  
203 modeling revealed that the free-ion concentration of Cu<sup>2+</sup> was affected by CO<sub>2</sub>. The 96h LC<sub>50</sub>  
204 free-ion Cu<sup>2+</sup> concentration was 2.73 μM (2.08-3.32) at pH 8.2. Free-ion concentrations of  
205 Cu<sup>2+</sup> were evaluated for each incubation condition and TU was calculated according to the  
206 individual toxicities of CO<sub>2</sub> and free-ion Cu. The TU that created 50% mortality was 1.53  
207 (1.24 - 1.92) (Fig. 7). Consequently, when calculated with free-ion concentrations,  
208 toxicological interactions between CO<sub>2</sub> and Cu appeared to be more strongly antagonist than  
209 when calculated with total Cu concentrations (TU = 1.17 (1.01 – 1.25)). Interactions between  
210 CO<sub>2</sub> and Cd as well as CO<sub>2</sub> and free-ion Cu displayed a similar range of antagonist effects  
211 with TU LC<sub>50</sub>s of 1.47 (1.28-1.62) and 1.53 (1.24 - 1.918) respectively.

212

## 213 **4. Discussion**

### 214 *4.1 Methodological consideration*

215 To our knowledge, this is the first study of the influence of CO<sub>2</sub> on shallow marine  
216 harpacticoid copepods. However, the present results should be interpreted with caution as  
217 several methodological aspects may underestimate the toxicity of elevated CO<sub>2</sub> concentrations  
218 in seawater. Sublethal effects may produce CO<sub>2</sub> sensitivity affecting many physiological  
219 parameters. Mechanisms that mitigate acidity by acid-base balance comes at a metabolic cost  
220 and, while not life threatening, could decrease growth and reproductive capacity (Langenbuch  
221 and Pörtner, 2004). For instance, egg production in the pelagic copepod *Acartia steueri* was  
222 found to decrease at pH of 6.8 (Kurihara et al., 2004). The fact that only the response of adults  
223 was studied, may also lead to an underestimation of CO<sub>2</sub> toxicity. It is widely accepted that  
224 early life history stages may be more sensitive to high pCO<sub>2</sub> (Pörtner and Farrell, 2008),  
225 especially in invertebrates (Dupont and Thorndyke, 2009a; Kurihara, 2008; Mayor et al.,  
226 2007). Early developmental stages of copepods are especially sensitive to contaminants  
227 (Green et al., 1996; Lotufo, 1997).

228 However, other aspects of the present study may lead to an overestimate of the impact  
229 of ocean acidification. Transfers of animals to each condition were abrupt and consequently  
230 not representative of the gradual changes that animals would experience in natural  
231 environments. Specifically, our acute exposures did not allow for either physiological  
232 acclimation or genetic modification in response to altered environmental conditions. A  
233 multigenerational experiment on the pelagic copepod *Acartia tonsa* showed that only one  
234 generation is needed to cope with impacts of ocean acidification on life cycle dynamics  
235 (Dupont and Thorndyke, 2009b).

236           Such potential biases must be kept in mind when interpreting results dealing with the  
237 toxicity of acidity. However, the impact of those biases would not apply to a (i) comparison  
238 of toxicity between CO<sub>2</sub> and HCl, (ii) comparison of CO<sub>2</sub> tolerance between species or (iii)  
239 comparison of metal and CO<sub>2</sub> interactive toxicity.

#### 240           4.2 CO<sub>2</sub> vs. HCl toxicity

241           The present study suggests that acidification using CO<sub>2</sub> produced an LC<sub>10</sub> of pH 6.41 for  
242 *A. atopus* (Fig. 1) and pH 6.06 for *S. knabeni* (Fig. 2). A model of the Southern North Sea  
243 suggests an average decrease of 0.1 pH unit over the next 50 years (Blackford and Gilbert,  
244 2007), while a global model predicts ocean pH values as low 7.5 within the next 300 years  
245 (Caldeira and Wickett, 2003). Both predictions are, however, well above observed toxicities.  
246 Our results suggest that adult benthic copepods from coastal environment may be relatively  
247 insensitive to CO<sub>2</sub> and may not be highly affected by an increase of ocean acidity. However,  
248 leakage from CO<sub>2</sub> seabed storage can create a faster and stronger local acidification  
249 (Hawkins, 2004) than the one induced by atmospheric CO<sub>2</sub>. The present study suggests that  
250 some harpacticoid species may be sensitive to those strong changes expected for seepage  
251 scenario.

252           The 96-h LC<sub>50</sub> observed for *A. atopus* and *S. knabeni* were, respectively, pH 5.75 (Fig.  
253 1) and 5.00 (Fig. 2) when acidity was manipulated with HCl. In a similar type of experiment,  
254 the sensitivity of pelagic copepods to acidity was shown to be species-specific: among 10  
255 oceanic zooplankton species studied, only two had lower sensitivity to acidification than we  
256 observed: *Eucalanus bungii bungii* and *Themisto japonica* LC<sub>50</sub>'s of pH 5.16 and 5.00  
257 respectively (Yamada and Ikeda, 1999). Thus, adult benthic copepods may be slightly more  
258 tolerant to acidity than pelagic copepods.

259           Unmixed sediments are characterized by strong geochemical gradients. pH changes of 1  
260 unit with depth (Fisher and Matisoff, 1981) and 1.5 units over short time scales (Gnaiger et

261 al., 1978) have been measured. In such variable environments, it is unlikely that benthic  
262 animals would be affected by small changes of pH. However, some meiofauna appear to be  
263 specialists, living at preferred depth locations within the sediment (Fleeger and Gee, 1986;  
264 Steyaert et al., 2003). Depth specialists could be adapted to highly specific conditions from  
265 that sediment stratum and consequently some species may be intolerant to small changes in  
266 pH.

267 CO<sub>2</sub> sensitivity of benthic fauna has been studied in the deep sea in order to evaluate the  
268 potential impact of large-scale sequestration of CO<sub>2</sub>. Deep-sea harpacticoid copepods suffered  
269 70-100% mortality when exposed to a reduction in pH of 0.75 units (Thistle et al., 2005).  
270 Copepods may not be able to burrow deeper to avoid CO<sub>2</sub> (Thistle et al., 2006), but they may  
271 be able to swim into the water column in order to attempt escape from the advancing front of  
272 carbon dioxide-rich seawater CO<sub>2</sub> (Thistle et al., 2007). Other studies have illustrated  
273 sensitivity of deep-sea nematodes, foraminifera and euglenoid flagellates (Barry et al., 2004;  
274 Fleeger et al., 2006; Fleeger et al., In press; Ishida et al., 2005; Ricketts et al., 2009) at pH  
275 decreases of only 0.1-0.2 units (Barry et al., 2005). The deep sea is characterised by highly  
276 stable physico-chemical parameters. In contrast, shallow-dwelling aquatic animals have  
277 evolved acid-base regulatory capabilities to combat seasonal, or even daily fluctuations in  
278 water pH. Deep-sea animals are unlikely to possess well-developed acid-base regulatory  
279 capacity (Pane and Barry, 2007). Consequently, the high CO<sub>2</sub> sensitivity of deep-sea  
280 copepods may not be observed among species from shallow coastal environments.

281 To our knowledge, this is the first study to examine the impact of changing seawater pH  
282 on harpacticoid copepods from a shallow-water environment. Among other members of  
283 shallow meiofauna, nematodes have a higher tolerance to CO<sub>2</sub> than do macrofauna (Dashfield  
284 et al., 2008; Widdicombe et al., 2009) and this apparently short-term tolerance could be due to  
285 their impermeable proteinaceous cuticle. Three species of nematodes from subtidal coastal

286 environments were highly resistant to high concentrations of CO<sub>2</sub> and lethal effects were only  
287 observed after increasing acidity to pH 5.5-6 (Takeuchi et al., 1997). At pH 7.5, a subtidal  
288 nematode community was unaffected by high CO<sub>2</sub> after several days of exposure (Dashfield  
289 et al., 2008), but a pH of 7.3 produced a change in nematode community composition  
290 (Widdicombe et al., 2009). Nematodes and copepods share the same habitat and have  
291 consequently evolved in similar environmental conditions. The present study suggests that, as  
292 with nematodes, shallow-water harpacticoid copepods would be largely unaffected by future  
293 ocean acidification.

294 Carbonate chemistry of seawater can be manipulated in various ways, and the most  
295 commonly used is by bubbling seawater with gases at different *p*CO<sub>2</sub> values. In the present  
296 study, different *p*CO<sub>2</sub> seawaters were prepared by mixing different ratios of water bubbled  
297 with pure CO<sub>2</sub> and water bubbled with air. This technique increases dissolved inorganic  
298 carbon at constant total alkalinity, and provides realistic simulations of past and predicted  
299 changes of ocean chemistry (Gattuso and Lavigne, 2009). Adding a strong acid (HCl) to a  
300 system does not alter the concentration of dissolved inorganic carbon but does modify total  
301 alkalinity. This method of acidification is thus more artificial because alkalinity is not  
302 expected to change over this century. Consequently at similar pH values, water acidified with  
303 CO<sub>2</sub> will contain more dissolved CO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> than water acidified with a strong  
304 acid. CO<sub>2</sub> diffuses easily into intracellular compartments and rapidly reacts with water to form  
305 H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup>. This intracellular acidosis can affect many physiological processes and may  
306 explain why the strongest toxic effects were observed when CO<sub>2</sub> was used to manipulate  
307 acidity in the present study. Stronger toxicity of early developmental stages of sea bream was  
308 also observed when acidity was manipulated by CO<sub>2</sub> (Kikkawa et al., 2004). These results  
309 emphasize that the addition of strong acid does not fully mimic the changes in carbonate



310 chemistry expected with ocean acidification and would likely lead to an underestimation of  
311 the toxic effect.

#### 312 4.3 *S. knabeni* and *A. atopus* sensitivity

313 Even though ocean acidification is expected to reduce biodiversity (Widdicombe and  
314 Spicer, 2008), some species may benefit from these new environmental conditions (Dupont  
315 and Thorndyke, 2009a). In several experiments, increased  $p\text{CO}_2$  has modified the meiofaunal  
316 community composition of deep-sea (Bernhard et al., 2009; Thistle et al., 2006) and shallow  
317 (Widdicombe et al., 2009) sediments. Those experiments indicate that not all species within a  
318 community have the same sensitivity to  $\text{CO}_2$ . Differences were also observed between species  
319 of deep-sea copepod in their ability to escape  $\text{CO}_2$  (Thistle et al., 2007). Such natural  
320 differences could explain the observed lower sensitivity of *S. knabeni* relative to *A. atopus*.

321 Different physiological mechanisms allow organisms to counteract acidification via: (i)  
322 passive buffering of intra- and extracellular fluids, (ii) transport of  $\text{CO}_2$  in the blood of species  
323 that have respiratory pigments and (iii) metabolic suppression during periods of elevated  $\text{CO}_2$ .  
324 Species adapted to hypercapnic environments have evolved such mechanisms to counteract  
325  $\text{CO}_2$  more efficiently than other species (Seibel and Walsh, 2001, 2003).

326 The two copepod species studied are associated with different environments. The  
327 natural habitat and the distribution of *A. atopus* are unknown, but species in this genus are  
328 exclusively found on beaches comprised of large particles (large sand grains to small cobble)  
329 (Lotufo and Fleeger, 1995). The porosity of such sediment being high, the porewater pH is  
330 similar to the pH of overlying water with little change over space and time. Cultures of *S.*  
331 *knabeni* were initiated with specimens collected from estuarine sediments in Louisiana  
332 (Lotufo, 1997). The pH of sediment porewater in this environment is approximately 7.55 (Ho  
333 and Lane, 1973). This type of muddy sediment is poorly permeable and pH decreases strongly  
334 with depth at the millimetre scale (e.g. Fisher and Matisoff, 1981). Moreover, in muddy

335 sediments, the majority of meiofauna lives in the uppermost surficial layer. Bioturbation  
336 increases water penetration to depth (Aller and Aller, 1992), creating millimetre lateral  
337 heterogeneity in pH (Zhu et al., 2006). In addition to differences in spatial and temporal pH  
338 heterogeneity, muddy sediments have generally lower oxygen availability and higher CO<sub>2</sub>  
339 concentration than sandy sediment and cobble. Mud-dwelling meiofauna may consequently  
340 be better adapted to hypercapnic environments than the fauna of sand and cobble. Our  
341 observations are consistent with this hypothesis. Widdicombe et al. (2009) similarly observed  
342 that a nematode community from a mud environment was less sensitive to CO<sub>2</sub>-rich water  
343 than was a nematode community coming from sandy sediment.

#### 344 *4.4 Metal toxicity*

345 Anthropogenic activities have led to significant metal pollution in many parts of the  
346 world, especially in inshore sediments (Nixon, 1995). Physical adsorption and chemical  
347 binding contribute to heavy metal concentrations that are several orders of magnitude higher  
348 in sediment than in overlying water. As a result, sediments generally represent sinks for  
349 pollutants. Sediment dwellers like copepods can potentially be exposed by taking up  
350 dissolved pore-water metals across their thin exoskeletons and soft tissues and/or by ingesting  
351 sediment and associated metal.

352 Compared to other heavy metals, Cd is relatively soluble, tends to bioaccumulate and is  
353 consequently considered to be a significant environmental threat (Goldberg, 1984). Cu can  
354 enter the aquatic environment primarily from mining activities (Lewis, 1995) but also from  
355 antifouling paints. Cu is an essential micronutrient for all organisms. However at high  
356 concentrations, it becomes toxic for plants and animals. In the present study, the Cu 96-h  
357 LC<sub>50</sub> of *A. atopus* was 650 µg.L<sup>-1</sup> at a pH of 8.2, which is in the range of LC<sub>50</sub>s found for the  
358 intertidal copepod genus *Tigriopus* (Barka et al., 2001; Kwok and leung, 2005; O'Brien et al.,  
359 1988).

360           4.5 Metal / CO<sub>2</sub> toxicity

361           In the future ocean, marine life will have to face acidification simultaneously with other  
362 anthropogenic stressors such as metal contamination. Prediction of the toxicology of multiple  
363 stressors is challenging when the combined effects of toxicants cannot be predicted from the  
364 individual effect of each. A review of studies of mixtures of heavy metals reveals that  
365 synergisms and antagonisms are more common than response-additive toxicity (Norwood et  
366 al., 2003). Such interactive toxicology was observed in copepods (Fleeger et al., 2007;  
367 Hagopian-Schlekat et al., 2001). However, the combined effect of CO<sub>2</sub> and metal toxicity for  
368 marine organisms have rarely been studied (Millero et al., 2009).

369           A consequence of ocean acidification is a decreased concentration of OH<sup>-</sup> and CO<sub>3</sub><sup>2-</sup>.  
370 These anions form strong complexes in ocean water with divalent and trivalent metals  
371 (Millero et al., 2009). This reduction is expected to change the speciation of numerous metal  
372 ions in seawater (Byrne, 2002). Metals such as Cd<sup>2+</sup> form strong complexes with chloride.  
373 However, because Cl<sup>-</sup> is pH insensitive, speciation of Cd will be minimally affected by ocean  
374 acidification. Indeed, modeling conducted in the present study revealed that the free-ion  
375 concentration of Cd was not affected by acidity. Other metals such as Cu<sup>2+</sup> form strong  
376 complexes with carbonate. Such metals will be more strongly affected by acidification and the  
377 concentration of their free ionic form will increase. In the present study, free-ion modeling  
378 with MINEQL+ revealed that in contrast to Cd, the free-ion form of Cu was increased with  
379 increasing acidity. Free-ion forms of metals are generally more toxic than complex forms  
380 (Allen et al., 1980). Comparison between LC<sub>50</sub> calculated using total Cu or free-ion Cu allows  
381 an evaluation of the influence of CO<sub>2</sub> on speciation. The approach using total Cu measured  
382 permits an evaluation of toxicity of combined (i) Cu, (ii) CO<sub>2</sub> and (iii) increased concentration  
383 of free-ion Cu according to CO<sub>2</sub> conditions. With the Cu approach, only Cu and CO<sub>2</sub> effects

384 are evaluated. The strongest antagonism observed with the free-ion approach confirmed that  
385 CO<sub>2</sub> increased the free-ion form of Cu, thus making it more toxic.

386       Acidification of the ocean also leads to an increased concentration of H<sup>+</sup>. As a result,  
387 competition for binding sites increases between H<sup>+</sup> and metals. Surface sites become less  
388 available to adsorb metals in the presence of increasing H<sup>+</sup>, potentially making acidification  
389 and metal toxicity antagonistic. In the present study, an antagonism was observed between  
390 CO<sub>2</sub> and Cd and of CO<sub>2</sub> and the free-ion of Cu, suggesting a competition for binding sites  
391 with H<sup>+</sup> for both metals. CO<sub>2</sub> concentration decreased toxicity of both metals by the same  
392 order of magnitude, suggesting that the binding sites of both metals may be similar. However,  
393 other processes could lead to the observed antagonist effect. Indeed, one mechanism to  
394 counteract acidosis is to depress metabolism (Guppy and Withers, 1999), thus reducing the  
395 rate of metal transport in the animals. Cd mimics Ca, and a reduced calcification rate can, for  
396 instance, reduce the rate of Cd uptake. The extent to which such processes operate in  
397 harpacticoid copepods is not well understood. To our knowledge, only one other study  
398 dealing with CO<sub>2</sub> and metal toxicity has been performed in the marine environment. In this  
399 case, antagonism was also observed between CO<sub>2</sub> and Cd: when CO<sub>2</sub> concentration was  
400 increased, the uptake of Cd decreased in the hatchling tissue of cuttlefish *Sepia officinalis*  
401 (Lacoue-Labarthe et al., 2009). Studies of freshwater organisms are more common and reveal  
402 competition for binding sites between H<sup>+</sup> and metals in aquatic biota (e.g. Hare and Tessier,  
403 1996; Yu and Wang, 2002). Similarly, uptake of metal by soil-dwelling animals was reduced  
404 when pH was changed by adding lime (e.g. Oste et al., 2001).

#### 405       4.6 Extrapolation to natural environment

406       The present study was conducted in water and allows evaluation of pharmacological  
407 interactions between metals and CO<sub>2</sub>. However, exposure in sediments can produce other  
408 kinds of interactions that are difficult to predict. The effects of mixtures in sediments are

409 especially complex for deposit-feeding animals that are exposed to both dissolved  
410 contaminants and contaminated food/sediment. For example, the narcotic effect of PAH has  
411 been shown to slow the uptake of Cd-contaminated sediment in a bulk deposit-feeding  
412 oligochaete by reducing his feeding rate (Millward et al., 2001). CO<sub>2</sub> also has a narcotic effect  
413 but its toxic interactions with metals in sediment remains uncertain, as copepods are not  
414 considered as bulk deposit feeders (Green et al., 1993). Other interactions may differ between  
415 aquatic and sediment environments. High amounts of metal can potentially be bound to  
416 sediment and those complex forms of metals are not necessarily bioavailable and toxic for  
417 sediment dwelling animals such as harpacticoid copepods. Acidification releases bound  
418 metals from sediments (Ardelan et al., 2009) rendering them more toxic in a free form. The  
419 amount of free metal released from sediment may be more important than amount available  
420 from pelagic environments. Such mechanisms are largely unknown and therefore studies are  
421 needed to better evaluate such potential indirect effects of ocean acidification.

422

423

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426

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## 7. Figure captions

643 Figure 1. Mortality (%) of *A. atopus* ( $\pm$  s.d.,  $N = 3 \times 15$  specimens) versus  $p\text{CO}_2$  (matm) and  
644 versus pH (using either  $\text{CO}_2$  or HCl). Dose response and  $\pm 95\%$  confidence intervals (shaded  
645 areas) using log-probit analysis.

646 Figure 2. Mortality (%) of *S. knabeni* ( $\pm$  s.d.,  $N = 3 \times 15$  specimens) versus  $p\text{CO}_2$  (matm) and  
647 versus pH (using either  $\text{CO}_2$  or HCl). Dose response and  $\pm 95\%$  confidence intervals (shaded  
648 areas) using log-probit analysis.

649 Figure 3. Mortality (%) of *A. atopus* ( $\pm$  s.d.,  $N = 3 \times 15$  specimens) versus total Cd  
650 concentrations at 4 different pH values induced by  $\text{CO}_2$ .

651 Figure 4. Effect of Cd- $\text{CO}_2$  mixture on *A. atopus* mortality ( $\pm$  s.d.,  $N = 3 \times 15$  specimens).  
652 Dose-response curve represents the best fit to toxic-unit data (shaded area;  $\pm 95\%$  confidence  
653 intervals). Crosshair represents the theoretical point at which the center of the dose-response  
654 curve should pass if both components were to act in a dose-additive manner.

655 Figure 5. Mortality (%) of *A. atopus* ( $\pm$  s.d.,  $N = 3 \times 15$  specimens) versus Cu concentrations  
656 at 5 different pH levels induced by  $\text{CO}_2$ .

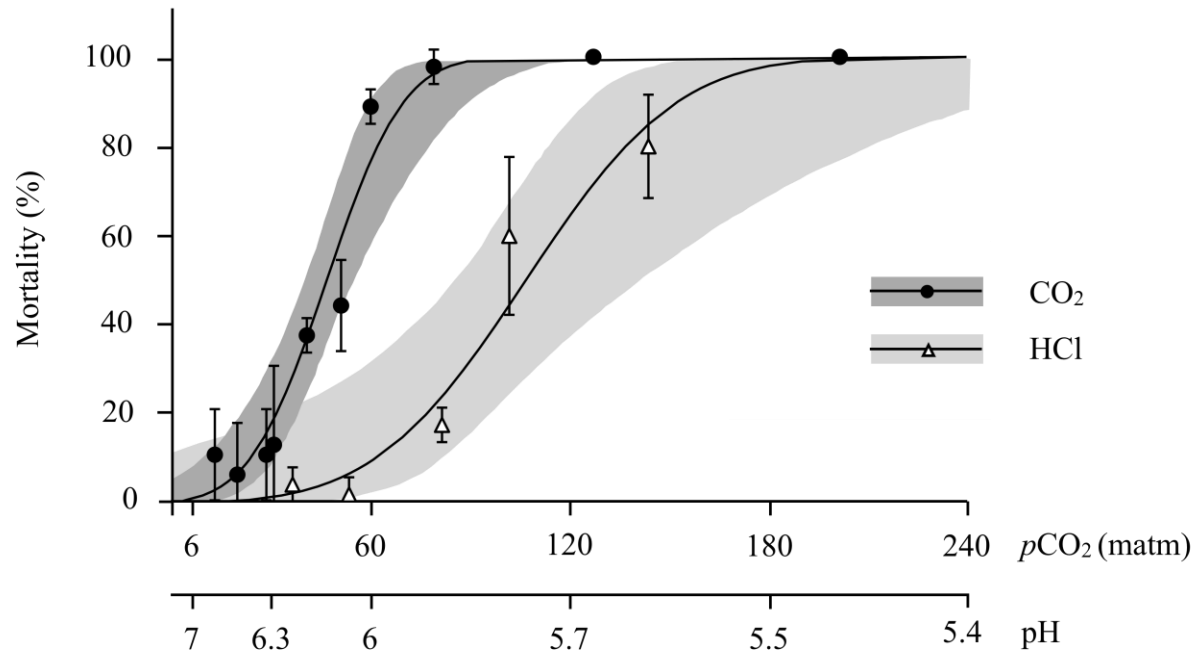
657 Figure 6. Effect of Cu- $\text{CO}_2$  mixture on *A. atopus* mortality ( $\pm$  s.d.,  $N = 3 \times 15$  specimens).  
658 Dose-response curve represents fit to toxic-unit data (shaded area;  $\pm 95\%$  confidence  
659 intervals). Crosshair represents the theoretical point at which the center of the dose-response  
660 curve should pass if both components act in dose-additive manner.

661 Figure 7. Effect of free  $\text{Cu}^{2+}$  ion- $\text{CO}_2$  mixture on *A. atopus* mortality ( $\pm$  s.d.,  $N = 3 \times 15$   
662 specimens). Dose-response curve represents fit to toxic-unit data (shaded area;  $\pm 95\%$   
663 confidence intervals). Crosshair represents the theoretical point at which the center of the  
664 dose-response curve should pass if both components act in dose-additive manner.

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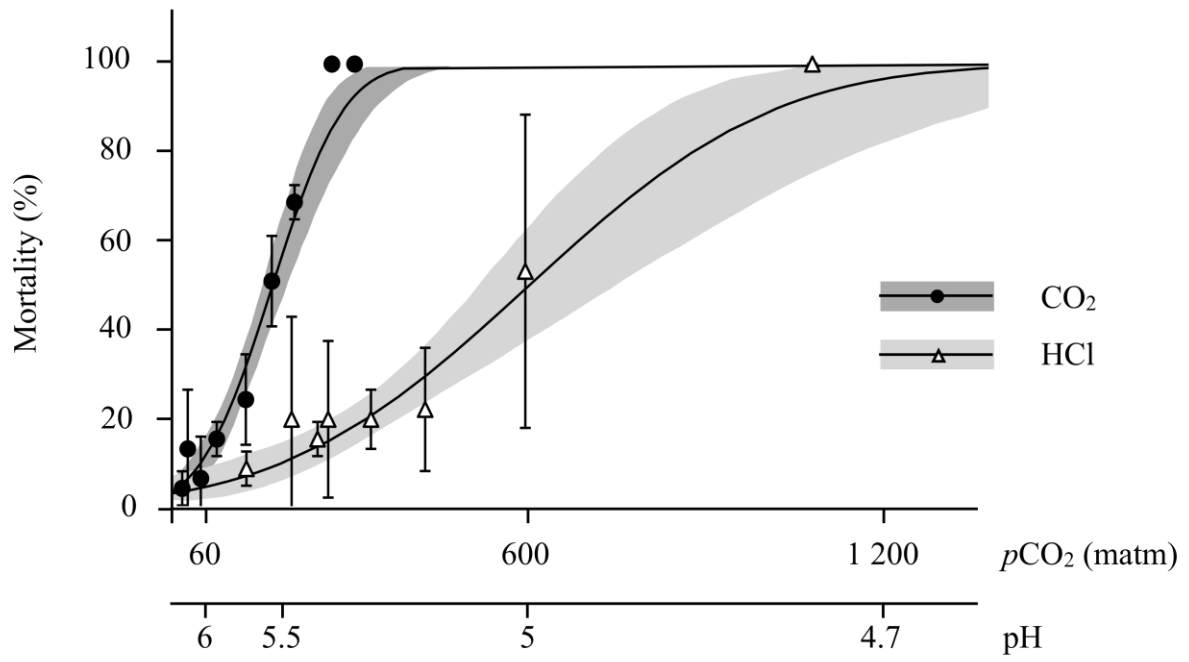
### 8. Figure



667

668 Figure 1.

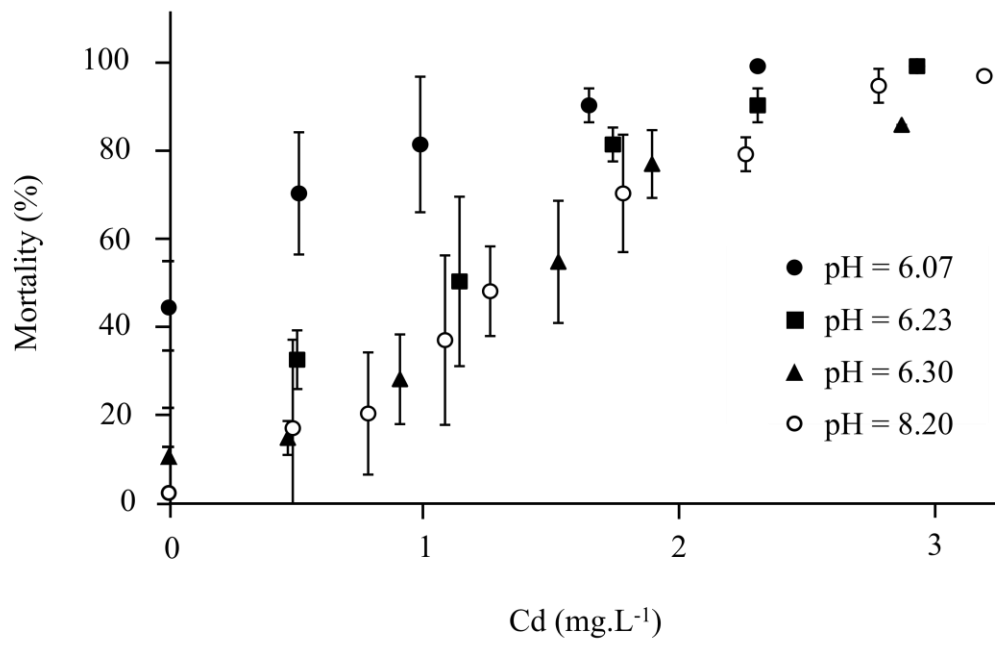
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671 Figure 2.

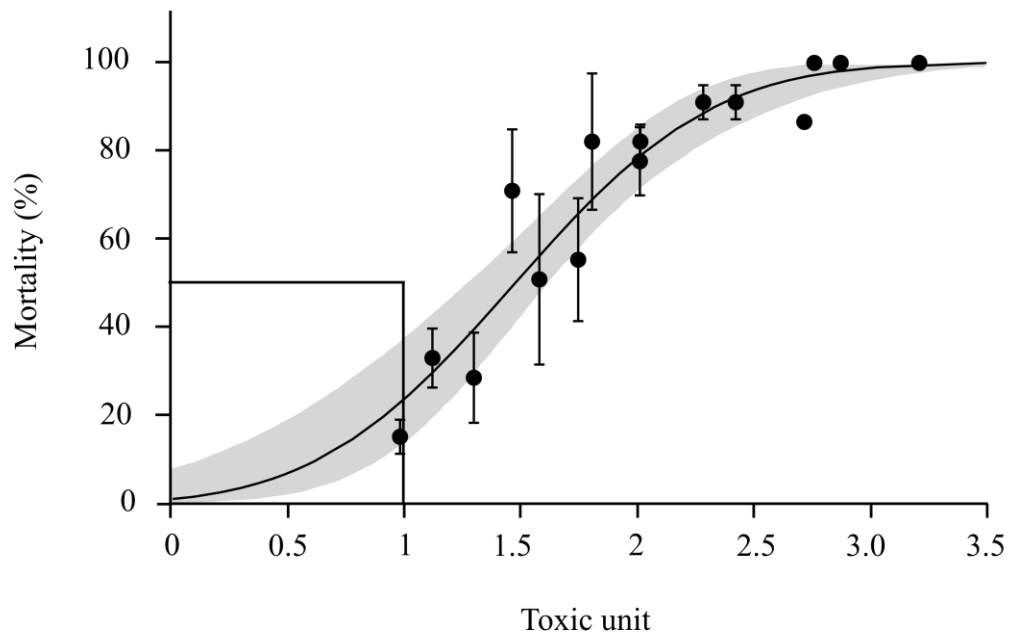
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674 Figure 3.

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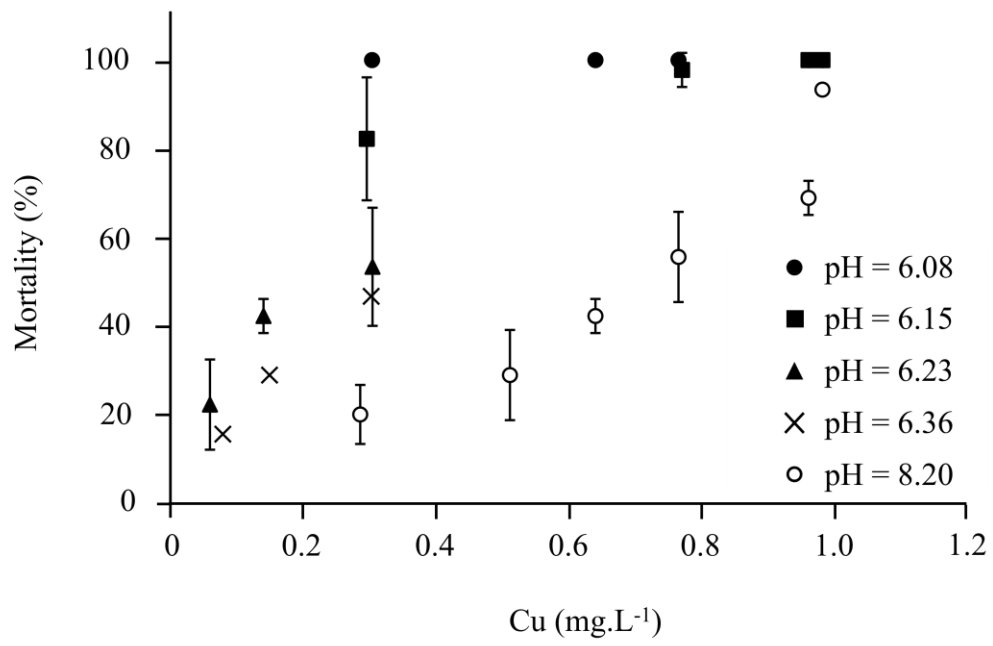


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677 Figure 4.

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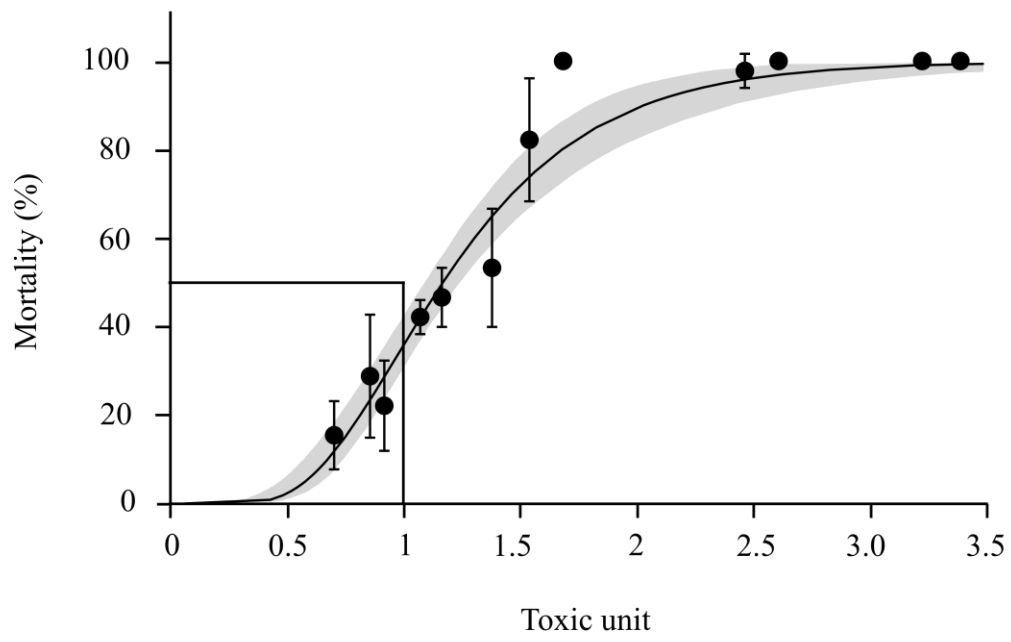




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680 Figure 5.

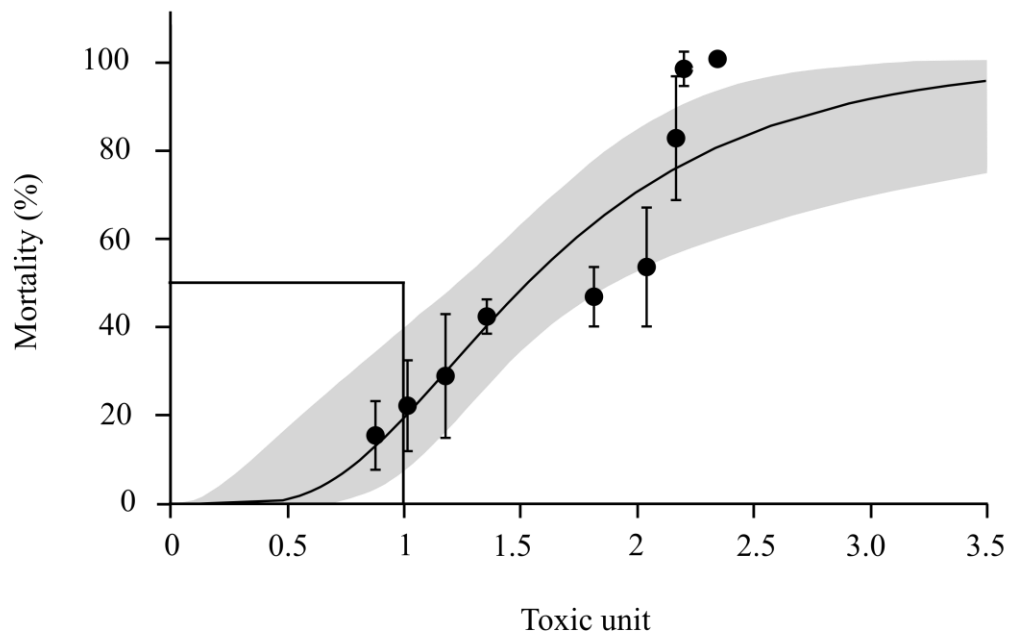
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683 Figure 6.

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686 Figure 7.