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**Ocean acidification modulates the incorporation of radio-labeled heavy metals in the larvae of the Mediterranean sea urchin *Paracentrotus lividus***

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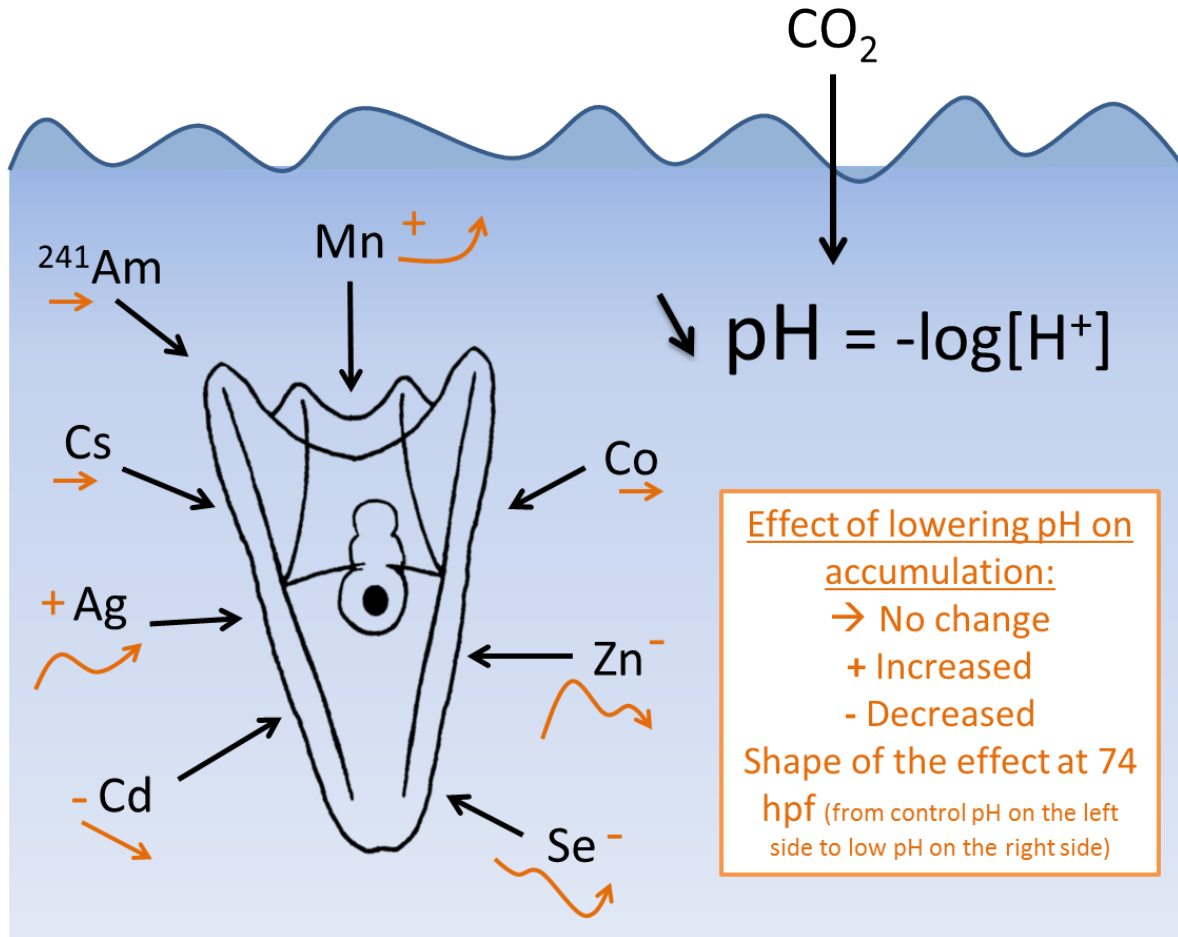
## ABSTRACT

The marine organisms which inhabit the coastline are exposed to a number of anthropogenic pressures that may interact. For instance, the accumulation of toxic metals present in coastal waters is expected to be modified by ocean acidification through e.g. changes in physiological performance and/or elements availability. Changes in bioaccumulation due to lowering pH are likely to be differently affected depending on the nature (essential vs. non-essential) and speciation of each element. The Mediterranean is of high concern for possible cumulative effects due to strong human influences on the coastline.

The aim of this study was to determine the effect of ocean acidification (from pH 8.1 down to -1.0 pH units) on the incorporation kinetics of six trace metals (Mn, Co, Zn, Se, Ag, Cd, Cs) and one radionuclide ( $^{241}\text{Am}$ ) in the larvae of an economically- and ecologically-relevant sea urchin of the Mediterranean coastline: *Paracentrotus lividus*. The radiolabelled metals and radionuclides added in trace concentrations allowed precise tracing of their incorporation in larvae during the first 74 hours of their development.

Independently of the expected indirect effect of pH on larval size/developmental rates, *Paracentrotus lividus* larvae exposed to decreasing pHs incorporated significantly more Mn and Ag and slightly less Cd. The incorporation of Co, Cs and  $^{241}\text{Am}$  was unchanged, and Zn and Se exhibited complex incorporation behaviors. Studies such as this are necessary prerequisites to the implementation of metal toxicity mitigation policies for the future ocean.

+ GRAPHICAL ABSTRACT



+ **HIGHLIGHTS**

- The radionuclide method allowed accurate tracing of the metal incorporation within this microscopic planktonic species.
- Metal incorporation in this sea urchin larvae is strongly correlated with the indirect delaying effect of seawater acidification on larval size.
- Independently of the size effect, acidification additionally directly affects the incorporation behavior of four metals (Mn, Ag, Se, Zn).
- The nature of the modulation is specific to each metallic element (see graphical abstract).
- The relationship between speciation, bioaccumulation and toxicity in the context of changing seawater pH requires more research.

+ **KEYWORDS:** Developmental biology, Bioaccumulation kinetics, Metals, Radionuclide, Pollution, CO<sub>2</sub>, Ocean acidification

## 1        1. INTRODUCTION

2        The marine organisms which inhabit the coastline are exposed to a number of anthropogenic  
3 pressures. On a local scale, metallic trace elements – originating from activity occurring along  
4 river basins (e.g. mine discharge, agriculture, wastewater treatment) – are harmful contaminants  
5 of the coastal marine environments and of concern to humans for ocean exploitation (Förstner  
6 and Wittmann, 1981). The French Mediterranean coastline, which is strongly influenced by the  
7 discharges of the Rhône river that drains industrialized and urban basins, is particularly exposed  
8 to metal pollution (UNEP/MAP, 2013). Metals can be highly toxic at low levels, especially to  
9 early development stages of marine invertebrates, and they have long been shown to alter (for  
10 example) sea urchin gametogenesis, larval growth, morphological development (e.g. Cu: Bougis,  
11 1965; Cd: Pagano et al., 1982 and Filosto et al., 2008; Mn: Pinsino et al., 2010) and  
12 biomineralization (reviewed by Matranga et al., 2011).

13        In parallel, the ocean is facing global changes, a consequence of the increase in  
14 anthropogenic carbon dioxide (CO<sub>2</sub>) in the atmosphere (280 to 400 ppm between 1850 and 2013,  
15 Etheridge et al., 1996 and Scripps CO<sub>2</sub> Program). On top of a warming (average + 0.1°C per  
16 decade between 1970 and 2010, IPCC, 2013), the surface ocean waters also experience an  
17 acidification (average -0.1 pH units - i.e. 30% increase in acidity - between pre-industrial levels  
18 and 2000: Caldeira and Wickett, 2008, -0.0044 units per year in the northwestern Mediterranean:  
19 Flecha et al., 2015). This ocean acidification is projected to decrease the current average pH of  
20 the global oceans (≈ 8.1) by a further 0.3 to 0.4 pH units by the end of the 21<sup>st</sup> century (IPCC,  
21 2013). Ocean acidification has been demonstrated to be a major threat for marine organisms,  
22 impacting their physiology (e.g. sea urchin larvae disrupted in their acid-base regulation: Stumpp  
23 et al., 2011 and feeding: Stumpp et al., 2013, increased metabolism: Dorey et al., 2013 and

24 modulated gene expression, in functions such as immunity: Runcie et al., 2017) as well as their  
25 fitness (e.g. negative effect on sea urchin larval growth: Dorey et al., 2013; Martin et al., 2011;  
26 Stumpp et al., 2011 and sea urchin gonadal growth: Siikavuopio et al., 2007; Stumpp et al.,  
27 2012b: exposure time  $\leq$  56 days, but see Dupont et al., 2013: no difference after 16 months).  
28 Ocean acidification has also been demonstrated to modify larval behavior (e.g. settlement  
29 behavior of *Bugula neritina*: Pecquet et al., 2017) and cognitive performances (e.g. sensory  
30 capacities and spatial navigation in larvae of fish: Munday et al., 2009) and more largely  
31 interactions within communities (e.g. Asnaghi et al., 2013). Early life stages, often considered as  
32 bottlenecks for population dynamics, have however been shown to be more resistant than  
33 previously thought (Kroeker et al., 2013). For instance, sea urchin larvae are resistant to a  
34 seawater acidification down to 7.1, albeit with larval growth being generally reduced when pH is  
35 lowered (*Paracentrotus lividus*: Martin et al., 2011, *Strongylocentrotus droebachiensis*: Dorey et  
36 al., 2013). Overall, studies show that responses are however species- and stage-specific (Kroeker  
37 et al., 2013), with for instance the larvae of another echinoderm, *Ophiotrix fragilis*, experiencing  
38 100% mortality at pH<sub>NBS</sub> 7.9 (vs. 70% in control pH: Dupont et al., 2008).

39 The resulting interactions of multiple stressors in the future ocean could lead to non-independent  
40 effects (e.g. Harley et al., 2006). The Mediterranean Sea is of high concern for possible  
41 cumulative effects (global change, over-fishing, pollution; UNEP/MAP, 2013). Although an  
42 important body of works demonstrated that increasing temperature arises metal toxicity (see  
43 review by Sokolova and Lannig, 2008) together with an elevation of the organism's metabolism  
44 and a modification of its physiology (e.g. feeding, osmoregulation), few studies focused on the  
45 effects of ocean acidification on the bioaccumulation and toxicity of metallic contaminants (see  
46 review **Table 1**). Yet, the changes of pH and seawater chemistry caused by increased CO<sub>2</sub> can

47 modify the speciation of metals, because element's speciation is largely dependent on  
48 physicochemical parameters (salinity, pH, redox potential: Millero et al., 2009; Stockdale et al.,  
49 2016), and therefore their bioavailability for organisms. CO<sub>2</sub>-driven acidification has been shown  
50 to modulate trace metal accumulation in both cephalopods early-life stages (Lacoue-Labarthe et  
51 al., 2011a, 2009) and the mantle cells of clams (Ivanina et al., 2013). A change in  
52 bioaccumulation efficiencies could lead to a change of metal toxicity.

53 Additionally, pH can impact the organism's abilities to deal with metal bioaccumulation and  
54 toxicity, as both are likely to interfere with metabolism and more specifically ionoregulation  
55 activities (Pörtner et al., 2004; Stumpp et al., 2012a). While sea urchin larvae have been shown to  
56 be relatively tolerant to lowered seawater pH - even for values well below the average projections  
57 for 2100 -, the effects are strong at the sub-lethal level. Decreasing pH resulted in larval growth  
58 delay (but conserved calcification in the form of calcium accumulation and relative arm length:  
59 Martin et al., 2011), increased metabolism (e.g. respiration rates increased by 9% per 0.1 pH unit  
60 decrease in Dorey et al., 2013) and changes in the expression of traits relating to cell division,  
61 metabolism and immune activity (pH 7.7: Runcie et al., 2017). These resulting sub-lethal changes  
62 could possibly be linked to the increase in energy demand (Stumpp et al., 2011) in order to  
63 maintain intracellular medium, via the use of energy-demanding membrane pumps (Stumpp et  
64 al., 2012a). Within these processes, ionic pumps such as -ATPase pumps play a key role by  
65 creating electrochemical gradients used by secondary active transporters (Stumpp and Hu, 2017).  
66 Yet, in marine organisms, these ion channels represent a major pathway for the entrance of  
67 metallic positively-charged ions (e.g. fish: Webb and Wood, 2000). Ocean acidification is  
68 therefore likely to increase metal pollutant bioaccumulation in organisms possessing acid-base  
69 regulation systems such as the sea urchin larvae. More information on the interactions between



70 pH and metal toxicity is thus required to improve predictive mathematical models and coastal  
71 management (Harley et al., 2006).

72 The aim of this study was to determine the effect of ocean acidification on the  
73 incorporation kinetics of six trace metals and one radionuclide (Mn, Co, Zn, Se, Ag, Cd, Cs,  
74 <sup>241</sup>Am) in an economically- and ecologically-relevant sea urchin of the Mediterranean coastline:  
75 *P. lividus*. These elements were chosen for their essential (e.g. Mn, Co, Se and Zn function as  
76 catalyst or activators of enzymatic reactions) and non-essential characters (e.g. Ag, Cd and  
77 <sup>241</sup>Am), their toxicity (“very toxic and relatively accessible” for Co, Se, Zn, Ag and Cd according  
78 to Förstner and Wittmann, 1981) and their occurrence in the coastal areas (e.g. <sup>241</sup>Am is a toxic  
79 and radioactive isotope associated with nuclear waste). The radiolabelled metals and radionuclide  
80 added in trace concentrations allowed precise tracing of their incorporation in larvae exposed to  
81 six different pH conditions (from pH<sub>T</sub> 8.1 down to -1.0 pH units) during the first 74 hours of  
82 development. We expected to observe decreased accumulation of radionuclides when pH was  
83 lowered due to the impaired larval developmental rates and we wished to detangle the more direct  
84 effects of pH on bioaccumulation due to e.g. changes in metal speciation, the larval  
85 physiology/metabolism and/or impairment of detoxification processes. We also expected  
86 differences in how metals would react to pH changes to be based on the nature of the metals  
87 (essential vs. non-essential) and their major form in the seawater (e.g. chloride-dominated vs. free  
88 form) and hence their expected speciation changes.

89

## 90 **2. MATERIAL AND METHODS**

### 91 *2.1 Biological material, spawning and experimental conditions*

92 In March and April 2009, specimens of adult sea urchin (*P. lividus*) were collected by  
93 divers from subtidal rocky shores ( $\approx 10\text{m}$  deep) in the Bay of Villefranche-sur-mer (NW  
94 Mediterranean coastline, France). This period corresponds to a few months prior to natural  
95 spawning in this area (mid-May to July, Boudouresque and Verlaque, 2001). The adults were  
96 maintained in a flow of unfiltered natural seawater (depth=10m, salinity=38) in the Laboratoire  
97 d'Océanographie de Villefranche and fed with *Posidonia oceanica* until fertilization (end of  
98 April). Three females and one male were opened around the peristome to retrieve gametes from  
99 the ripe gonads. Eggs were rinsed three times with filtered seawater (FSW;  $0.22\ \mu\text{m}$ , salinity=38)  
100 and pooled together. Sperm was collected dry using a Pasteur pipette and stored at  $4^\circ\text{C}$  in a small  
101 tube before use. Within an hour after collection the gametes were transferred to the Radioecology  
102 Laboratory (Environment Laboratories of the International Atomic Energy Agency, Monaco)  
103 premises for fertilization. A concentrated egg suspension ( $\approx 4\ 000\ 000$  eggs) was transferred to  
104 six 1-l beakers filled with FSW ( $20^\circ\text{C}$ ) and diluted sperm (1:50 ratio in FSW) was added to the  
105 egg suspension. Eggs were gently agitated for 15 min to optimize fertilization and then  
106 distributed to six replicates 4.5-l glass beakers per pH level (N=36 beakers) filled with  
107 radiolabeled FSW (seven radionuclides, detailed below). Embryos (start density of ca. 130 per  
108 ml) were grown in aerated FSW at their respective pH levels (pH<sub>T</sub>: 7.1, 7.3, 7.5, 7.7, 7.9, 8.1;  
109 **Table 2**).

110 Natural variability in pH has been described to be broad in certain coastal areas (e.g.  
111 Thomsen et al., 2010) and the shift of the pH range as a whole in the future (e.g. Hauri et al.,  
112 2012) is highlighted as an important reason to investigate of wide ranges of pH ( $\Delta\text{pH}$  down to -  
113 1.0 unit; e.g. Dorey et al., 2013). Yearly surface pH variations are much tamer near our site, with  
114 extreme pH values recorded ranging from 7.95 to 8.20 (DYFAMED station in the Ligurian Sea,

115 50 km from the coast, monthly data from 1995-2011: Geri et al., 2014, and SOMLIT  
116 Villefranche-sur-Mer Station, 500 m from the shore, April-December 2016, n=37: [http://somlit-](http://somlit-db.epoc.u-bordeaux1.fr/bdd.php)  
117 [db.epoc.u-bordeaux1.fr/bdd.php](http://somlit-db.epoc.u-bordeaux1.fr/bdd.php)) as well as in general in the northwestern Mediterranean Sea  
118 (e.g.  $\text{pH}_T = 7.893 \pm 0.007$  near the Strait of Gibraltar in Aug 2012-June 2015, n=15937, Flecha et  
119 al., 2015). This small pH seasonal variation window has been consistently shifting at a rate of -  
120 0.0044 units per year (Flecha et al., 2015). Nevertheless, a wide pH range approach also allows  
121 for a more precise understanding of the shape and intensity of the biological response to ocean  
122 acidification, with a focus in this study on the metal bioaccumulation efficiency in sea urchin  
123 early life stages.

## 124 *2.2 Experimental set-up and carbonate chemistry*

125 Temperature ( $\approx 20^\circ\text{C}$ ) was controlled to within  $\pm 0.5^\circ\text{C}$  in the 300-l bath surrounding the  
126 culture beakers (300W submersible heater coupled to a temperature controller: IKS, Karlsbad,  
127 Germany). Seawater pH was maintained to the desired levels within each culture beaker within  
128  $\pm 0.05$  pH units using a continuous pH-stat system (IKS) that controlled the addition of  $\text{CO}_2$  into  
129 the beakers (see Martin *et al.*, 2011 for detailed description). The pH values of the pH-stat system  
130 were adjusted from the measurements of pH on the total scale ( $\text{pH}_T$ ) in each beaker using a pH-  
131 meter (Metrohm 826 pH mobile, Metrohm, Herisau, Switzerland) calibrated using TRIS  
132 (Tris/HCl) and AMP (2-aminopyridine/HCl) buffer solutions with a salinity of 38. In order to  
133 avoid contamination of the lab equipment used for alkalinity determination with radioactive  
134 agents, total alkalinity (TA) was assessed potentiometrically in seawater from radiotracer-free  
135 culture beakers maintained in the same pH/temperature conditions and done in parallel in spring  
136 2009 (see below in 2.4; detailed description in Martin et al. 2011). The carbonate system  
137 parameters ( $p\text{CO}_2$ ,  $\Omega_a$  and  $\Omega_c$ ) were calculated from these measurements using the R package

138 *seacarb* (Lavigne and Gattuso, 2011) with the dissociation constants from Mehrbach et al. (1973)  
139 as refitted by Dickson and Millero (1987).

### 140 *2.3 Radiolabelling and radioactivity analyses*

141 Radiotracers were purchased from Amersham, UK ( $^{109}\text{Cd}$ ), Polatom, Poland ( $^{110\text{m}}\text{Ag}$ ,  
142  $^{134}\text{Cs}$ ,  $^{75}\text{Se}$ ), Cyclotron, Russia ( $^{65}\text{Zn}$ ), and Isotope Product Laboratory, USA ( $^{241}\text{Am}$ ,  $^{60}\text{Co}$ ,  $^{54}\text{Mn}$ ).  
143 For determination of larval metal uptake, eggs were incubated in seawater labeled with dissolved  
144  $^{54}\text{Mn}$  (1 kBq l<sup>-1</sup> as  $^{54}\text{MnCl}_2$ ),  $^{60}\text{Co}$  (1 kBq l<sup>-1</sup> as  $^{60}\text{CoCl}_2$ ),  $^{65}\text{Zn}$  (1 kBq l<sup>-1</sup> as  $^{65}\text{ZnCl}_2$ ),  $^{75}\text{Se}$  (1 kBq  
145 l<sup>-1</sup> as  $\text{Na}_2^{75}\text{SeO}_3$ ),  $^{109}\text{Cd}$  (1 kBq l<sup>-1</sup> as  $^{109}\text{CdCl}_2$ ),  $^{110\text{m}}\text{Ag}$  (1 kBq l<sup>-1</sup> as  $^{110\text{m}}\text{AgNO}_3$ ),  $^{134}\text{Cs}$  (1 kBq l<sup>-1</sup>  
146 as  $^{134}\text{CsCl}$ ), and  $^{241}\text{Am}$  (0.35 kBq l<sup>-1</sup> as  $^{241}\text{AmNO}_3$ ) by spiking typically 5 to 10  $\mu\text{l}$  of stock  
147 solutions without affecting the tank seawater pH. These concentrations correspond to the addition  
148 of 21, 3, 133, 5, 6, and 0.7 ng l<sup>-1</sup> of Ag, Am, Cs, Co, Se and Zn stable element respectively.  
149 While the specific activities of  $^{109}\text{Cd}$  and  $^{54}\text{Mn}$  sources were not available from the providers, the  
150 use of these radionuclides usually results in the addition of ng l<sup>-1</sup> of metal or less (see Lacoue-  
151 Labarthe et al., 2011b), which correspond to environmentally-realistic trace metal concentrations  
152 (Bruland and Lohan, 2003; Yoon et al., 1999). This allows us to add simultaneously the eight  
153 radiotracers in each experimental microcosm, while being able to consider that the metals do not  
154 interact in this mixture at these concentrations.

155 At 28, 52 and 74 hpf (hours post-fertilization; i.e. until four-arm pluteus stage), culture  
156 samples of 300 to 1000 ml ( $\approx 10300$  to  $65500$  individuals depending on the culture density) were  
157 collected in triplicates in each pH treatment for radioactivity counting. Larvae were collected on  
158 20- $\mu\text{m}$  polycarbonate membranes, re-suspended and rinsed three times in radiotracer-free FSW.  
159 The filters with embryos or larvae were then radio-counted ( $\gamma$ -spectrometry system consisting of

160 Germanium detectors; see e.g. Lacoue-Labarthe et al., 2012). Sub-samples of 150 ml of the  
161 remaining filtered seawater were sampled and radio-counted in order to measure the radiotracer  
162 concentrations in the seawater at sampling time. In the culture beakers, the sampled seawater  
163 volume was replaced with pH-equilibrated FSW and spiked with adjusted concentrations of  
164 radiotracers to maintain activities as constant as possible. Measured concentrations of  
165 radiotracers in the seawater varied by  $5.9 \pm 11.5$  % of the desired value all along the experiment  
166 (N=30 for each metal).

167         During the experiment, larval density was followed every day for the first 74 hpf (count at  
168 the binocular microscope of six replicates of 1 or 2 ml - depending on larval density - of seawater  
169 for every pH treatment at 22, 47 and 74 hpf). Larval density decreased from 100-130 ind.ml<sup>-1</sup> at  
170 22 hpf to 10-25 ind.ml<sup>-1</sup> at 74 hpf. Total larval mortality (%) was calculated from the difference  
171 between initial (22 hpf) and final (74 hpf) larval density corrected for consecutive seawater  
172 dilutions (**Table 3**).

#### 173 *2.4 Data treatment and statistical analyses*

174         The accumulation of metals and radionuclide in sea urchin larvae was expressed as  
175 change of concentration factor (CF; Bq larvae<sup>-1</sup>), here defined as the radiotracer activities  
176 measured in the larval sample divided by the number of larvae per sample (estimated from larval  
177 density) and normalized by the time-integrated activity in the seawater over time. In order to limit  
178 total radiation exposure for experimental workers and waste management cost, only one 4.5-l  
179 tank was dedicated to each pH condition. Hence, triplicates of sampled larvae are considered as  
180 technical replicates according to Hurlbert (1984) and the bioaccumulation of radiotracers in

181 larvae has been tested considering the measured  $pH_T$  as a continuous covariate instead  
182 (Havenhand et al., 2010).

183 Larval body lengths ( $L$ ,  $\mu\text{m}$ ) at each sampling times (**Table 3**) were back-calculated from  
184 the growth rates measured in the same pH conditions during a parallel experiment (without  
185 radionuclides; spring 2009: Martin et al., 2011). This experiment was running at the same time as  
186 the present experiment and was identical (set-up:  $N=36$  4.5-l glass beakers, identical pool of  
187 embryos, pH, temperature and seawater origin) to the exception of the sampling times ( $\pm 1\text{h}$ ) and  
188 the initial density that was ca. 4 times lower at the start (32-35 embryos per ml vs. 130 per ml  
189 here). Although we are aware of the possible confounding effect this could have on growth rates,  
190 this difference was quickly reduced due to the large sampling required in the radio-labeled  
191 experiment (10 individuals per ml at 73h vs. 10-25 individuals per ml at 74h in the experiment  
192 presented here). To confirm the relationships between metal accumulation and larval size, we  
193 tested the fit of linear ( $CF = a \times L + b$ ) and logarithmic models ( $CF = a \times \log(L) + b$ ). The model  
194 comparison confirmed linear model to consistently have higher AIC (Akaike Information  
195 Criterion). Because of the well-documented developmental delay which sea urchin larvae  
196 encounter in acidified conditions (Dorey et al., 2013; Martin et al., 2011; Stumpp et al., 2011),  
197 larval body length was used instead of time during the analyses in order to avoid any growth-  
198 dependent effect. Since CF and larval size ( $L$ ) were significantly linearly correlated, we therefore  
199 performed statistical analyses on the CF normalized by larval size ( $CF/L$ :  $\text{Bq larvae}^{-1} \mu\text{m}^{-1}$ ).

200 In order to assess the effect of pH on the bioaccumulation of metals, we tested the effect  
201 of two continuous variables (mean  $pH_T$  and Time) on the CF normalized by larval size ( $CF/L$ ;  $\text{Bq}$   
202  $\text{larvae}^{-1} \mu\text{m}^{-1}$ ) for each metal using generalized linear model analyses. We then fitted generalized  
203 additive models (gam, R package *mgcv*) to the  $CF/L$  at the final time-point (74 hpf) to unravel the

204 different shapes of the relationships between each metal accumulation and pH. The basis  
205 dimensions for smooths were chosen visually and using the available function *gam.check* in the  
206 *mgcv* package. All the statistical analyses were performed using the *R* software (R Core Team,  
207 2009), with a significance level of  $\alpha=0.05$ . Results are expressed as mean  $\pm$  SD.

208

### 209 3. RESULTS

#### 210 3.1 Culture conditions

211 The mean  $\text{pH}_T$  values, as well as the calculated carbonate chemistry parameters, are  
212 shown in **Table 2**. Mean temperature was  $20.13 \pm 0.34$  °C (N=282). The  $\text{pH}_T$  values in every  
213 treatment were significantly different from each other (1-way ANOVA with Welch correction:  
214  $p < 0.0001$ ;  $F=30946$  and  $df=5$ , followed by pairwise t-test with Holm adjustment:  $p < 0.0001$ ).  
215 Because it was not possible to measure TA in radiolabelled samples, TA was measured in a  
216 similar experiment where the mean TA averaged between 2550 and 2565  $\mu\text{mol.kg}^{-1}$  depending  
217 on the culture conditions (changes of 5 to 35  $\mu\text{mol.kg}^{-1}$  from 0 to 73 hpf in each treatment, see  
218 Martin *et al.*, 2011). The mean TA ( $\approx 2558$   $\mu\text{mol.kg}^{-1}$ ) was therefore used for the carbonate  
219 chemistry calculations presented here.  $p\text{CO}_2$  values ranged from  $335 \pm 20$   $\mu\text{atm}$  in the 8.1 pH  
220 condition to  $4537 \pm 438$   $\mu\text{atm}$  in the lowest-pH condition. Seawater was under-saturated in the  
221 lowest-pH treatment ( $\text{pH}_T=7.12$ ) for both calcite and aragonite and only in the  $\text{pH}_T=7.32$   
222 treatment with respect to aragonite.

223 Larval densities were followed every day in each beaker (n=6 replicates per day) and  
224 decreased with time due to seawater dilution (**Table 3**: 130 to 10 larvae.ml<sup>-1</sup>). Larval mortality  
225 (%) was calculated from the difference between the expected larval density at 74 hpf taking into

226 account dilutions (i.e. 28.8 larvae.ml<sup>-1</sup>) and the measured density in each pH treatment at 74 hpf.  
227 Larval mortality was generally low (12-22%), except for pH<sub>T</sub>=7.12 where it reached 64%  
228 mortality in three days (**Table 3**).

### 229 3.2 Metal incorporation in larvae

230 Concentration factors (CF) increased with time for all the metals except Cs (**Fig. 1A**), as  
231 metals accumulated in the body of larvae in the first 74 hpf. Accordingly, the presence of Cs in  
232 the larval bodies was always relatively small (CF=  $6.8 \cdot 10^{-6} \pm 6.5 \cdot 10^{-6}$  Bq larvae<sup>-1</sup>). Between 28  
233 and 74 hpf, the CF increased on average by ~10-fold for Mn, Se and Am (respectively by 9.1,  
234 13.1 and 12.2 times) and by ~5-fold for the other four metals (4.8 times for Co, 5.3 times for Zn,  
235 6.4 times for Cd and 4.8 times for Ag).

236 Seventy-four hours after fertilization, the larvae in the lowest pH treatment were ~40%  
237 smaller than the larvae grown at pH<sub>T</sub> 8.1 (**Table 2**, also see Martin et al. (2011) for complete  
238 description of the results). Because the CF was strongly correlated with larval size (L) for all  
239 metals but Cs (**Fig. 1B**: CF=a x L + b), we analyzed the effect of time and average pH<sub>T</sub> on the CF  
240 standardized by larval body length (CF/L, Bq larvae<sup>-1</sup> μm<sup>-1</sup>) using generalized linear models  
241 (**Table 4**). Taking into account the three time points, the pH<sub>T</sub> significantly affected the CF/L of  
242 the following three metals: Se, Cd and Ag. This analysis also confirmed that time had a  
243 significant effect on the standardized accumulation of all metals except for Cs (**Table 4**).

244 In order to understand how average pH<sub>T</sub> shaped the incorporation of metals in the larvae,  
245 we fitted generalized additive models to the CF/L data on 74 hpf (**Fig. 2**; the statistical results are  
246 detailed in **Table 5**). At this time-point, neither Co, Cs nor Am accumulations were significantly  
247 affected by pH<sub>T</sub>. However, pH<sub>T</sub> had a significant effect on the accumulation Se, Cd and Ag (the



248 same metals found significantly-affected in the generalized linear models analysis: **Table 4** and  
249 **5**), and two other additional metals: Mn and Zn (**Table 5**). The accumulation (CF/L) significantly  
250 decreased when pH<sub>T</sub> was lowered for three metals: Zn, Se and Cd, while it was significantly  
251 increased in Mn and Ag (**Table 5**). These trends were also observed at 24 and 58 hpf (data not  
252 shown).

253 The accumulation of Cd in larval tissues decreased linearly with lowering pH<sub>T</sub> (deviance  
254 explained by the model: D=25%; **Fig. 2** and **Table 5**): the larval content by size-unit was halved  
255 in the 7.1 pH treatment compared to pH 8.1 (CF/L=0.66.10<sup>-6</sup> ± 0.58.10<sup>-6</sup> vs. 1.2.10<sup>-6</sup> ± 0.06.10<sup>-6</sup>  
256 Bq larvae<sup>-1</sup> μm<sup>-1</sup> respectively). The larval content of Zn linearly decreased from pH 7.9 to 7.1  
257 (D=73%: CF/L= 5.0.10<sup>-6</sup> ± 0.2.10<sup>-6</sup> to 3.8.10<sup>-6</sup> ± 0.6.10<sup>-6</sup> Bq larvae<sup>-1</sup> μm<sup>-1</sup> respectively, i.e. -24%;  
258 **Fig. 2** and **Table 5**) but the larval content at the 7.1 pH treatment was comparable to that of the  
259 pH 8.1 (CF/L=3.7.10<sup>-6</sup> ± 0.2.10<sup>-6</sup> Bq larvae<sup>-1</sup> μm<sup>-1</sup>). Similarly, the larval content of Se at the 7.1  
260 pH treatment was comparable to that of pH 8.1 (CF/L=7.5.10<sup>-7</sup> ± 2.8.10<sup>-7</sup> and 8.8.10<sup>-7</sup> ± 1.6.10<sup>-7</sup>  
261 Bq larvae<sup>-1</sup> μm<sup>-1</sup> respectively) while the larval content linearly decreased by more than twice  
262 compared to pH 8.1 for the 7.3 pH treatment (CF/L= 2.6.10<sup>-7</sup> ± 0.8.10<sup>-7</sup> Bq larvae<sup>-1</sup> μm<sup>-1</sup>; D=66%;  
263 **Fig. 2** and **Table 5**). The differences observed could not be attributable to a change in larval size  
264 as the analyses were made on size-standardized CF.

265 The accumulation of Ag in larval tissues significantly (*p*=0.037; **Fig. 2** and **Table 5**) and  
266 gradually increased with lowering pH<sub>T</sub> (D=67%): the larval content (CF/L) was more than 3.5  
267 times higher in the 7.1 pH treatment than at pH 8.1 (CF/L=6.5.10<sup>-6</sup> ± 1.9.10<sup>-6</sup> vs. 1.8.10<sup>-6</sup> ±  
268 0.3.10<sup>-6</sup> Bq larvae<sup>-1</sup> μm<sup>-1</sup> respectively). The accumulation of Mn in larval tissues significantly  
269 (*p*=0.033; **Fig. 2** and **Table 5**) increased with lowering pH<sub>T</sub> (D=67%) although the main  
270 difference was visible for the 7.1 pH treatment where the larval content was ~2 times higher than

271 in the rest of the treatments ( $CF/L=4.3 \cdot 10^{-6} \pm 1.7 \cdot 10^{-6}$  for 7.1 while other ranged from  $1.5 \cdot 10^{-6} \pm$   
272  $0.4 \cdot 10^{-6}$  to  $1.9 \cdot 10^{-6} \pm 0.3 \cdot 10^{-6}$  Bq larvae<sup>-1</sup> μm<sup>-1</sup>).

273

#### 274 **4. Discussion**

##### 275 *4.1 Ocean acidification modulates the incorporation of radio-labeled heavy metals in sea urchin* 276 *larvae*

277 In this study, we observed that a decrease in seawater pH can alter the incorporation of  
278 essential and non-essential elements. A known effect of pH is the slowing down of  
279 developmental rates in sea urchin larvae, and here we standardized incorporation by size in order  
280 to better focus on the direct effects of pH on metal incorporation. *Paracentrotus lividus* larvae  
281 exposed to decreasing pHs from 8.1 to 7.1 for 74 hpf incorporated significantly more Mn (2.5  
282 fold increase in concentration at 7.1 compared to the other pHs), significantly more Ag (3.7 fold  
283 increase in concentration for a pH unit decrease) and slightly less Cd (1.9 fold decrease for a pH  
284 unit decrease to non-significant if one zero data point is taken out). Zn and Se exhibited complex  
285 incorporation behavior with pH: an initial increase for Zn (1.35 fold from pH 8.1 to 7.9) followed  
286 by a gradual decrease back to the initial (pH 8.1) values at pH 7.1, while Se concentration  
287 gradually decreased by 3.4 fold until pH 7.3 to sharply rise back nearly to the initial (pH 8.1)  
288 concentrations at pH 7.1. The incorporation of Co, Cs and <sup>241</sup>Am was unchanged, partly owing to  
289 low concentration detected in the larvae for e.g. Cs.

290 Remarkably, there was no obvious trend linked to the nature of the elements (essential vs.  
291 non-essential): lowering pH significantly increased the concentration of Ag and Mn in sea urchin  
292 larvae but slightly decreased Cd when Zn and Se concentration had opposite behaviors. There

293 was no evident impact on the other elements tested (Co, <sup>241</sup>Am, Cs). The bioaccumulation  
294 behaviors observed did also not appear obviously linked to the type of the element form (whether  
295 chloride-dominated for Cd and Ag, oxidized state for Se or mostly found as free form for Cs, Co,  
296 Zn, Mn) and the consequent speciation changes. For instance, Ag and Cd have similar speciation  
297 changes with lowering pH, resulting in a very little increase in the toxic form due to pH (Cd<sup>2+</sup>  
298 represents 20.1% of the Cd forms at pH 8.1 and 20.2% at pH 7.4 in the model by Millero et al.,  
299 2009), but end up having different bioaccumulation reactions to pH. It is thus unlikely that the  
300 observed disturbances in elemental bioaccumulation with pH are only due to speciation changes  
301 or to increased metabolism (increased incorporation) and these disturbances must be specific to  
302 each metal and their entrance pathways and mechanisms.

#### 303 *4.2 The nature of the modulation is specific to each metallic element*

304 Silver is toxic at low levels for sea urchin larvae (EC<sub>50</sub> of 40-85 µg l<sup>-1</sup> for *P. lividus*,  
305 Warnau et al., 1996). Here, <sup>110m</sup>Ag was taken up at higher concentrations when pH was lowered  
306 in the tissues of the sea urchin larvae. This observation is consistent with previous observations  
307 of increased Ag accumulation with lowering pH in whole eggs and newly hatched juveniles of  
308 cephalopods (Lacoue-Labarthe et al., 2011a, 2009). Although reasons specific to the embryonic  
309 development of the studied cephalopods had been discussed (peri-vitellin fluid properties  
310 affecting the specificity of Ag speciation and availability and higher metal translocation from the  
311 eggshell to the embryo), this study shows that increased Ag incorporation with lower pH was also  
312 visible in animals with a different life history. Silver is thought to enter marine invertebrates via  
313 Na<sup>+</sup>/K<sup>+</sup>-ATPase pumps (e.g. shrimp, sea hare, urchin: Bianchini et al., 2005). These pumps are  
314 likely to be more active in the sea urchin larvae exposed to acidified seawater as a way to  
315 maintain internal stasis (Stumpp and Hu, 2017). Therefore, this strengthens the hypothesis that the

316 increase in Ag incorporation at low pH is driven by increased activities in ionic regulatory  
317 mechanisms (Lacoue-Labarthe et al., 2011a): an increased metabolism could consequently  
318 increase the transport of Ag into the cells.

319 Cadmium has no known biological role, it is highly toxic to sea urchin larvae and has  
320 been effectively used as an inducer of skeleton malformation (Matranga et al., 2011). It also  
321 induces the expression of stress proteins (Roccheri et al., 2004), methallothionein genes (Russo et  
322 al., 2003) and even apoptosis (Filosto et al., 2008). Even 15 hours after Cd removal from the  
323 culture, negative effects can persist (Roccheri et al., 2004). In our study,  $^{109}\text{Cd}$  uptake did not  
324 seem to be strongly affected by pH (except from one point at 7.1), which is in accordance with  
325 the absence of strong speciation changes for this element. Cadmium is thought to enter the cell  
326 membrane in the form of  $\text{Cd}^{2+}$  passively via calcium channels (Rainbow and Black, 2005), and to  
327 mimic  $\text{Ca}^{2+}$  behavior. Accordingly to the behavior of Cd observed in our study (no effect of pH),  
328 Martin et al. (2011) found no effect of pH on the accumulation of  $^{45}\text{Ca}$  in the larvae. However,  
329 Cd uptake has been shown to be either decreased with lowering pH (isolated clam cells: Ivanina  
330 et al., 2013; cephalopods: Lacoue-Labarthe et al., 2011, 2009; phytoplankton: Xu et al., 2012),  
331 increased (bivalves: Shi et al., 2016) or to remain unaffected by pH (bivalves: Götze et al., 2014).  
332 The hypothesis that the lowered Cd accumulation in acidified water is only due to reduced  
333 metabolism in both clam cells and squid embryos does not corroborate our observations.  
334 However, one plausible mechanism for limited Cd incorporation at lower pH, even with the  
335 increased metabolic rates for sea urchin larvae, could be that there is a strong competition for  
336 binding sites with increased  $\text{H}^+$  ion concentration in low pH as suggested by Pascal et al. (2010).  
337 Ivanina et al. (2013) also discuss possibly strong interactions between Cd accumulation in the

338 cells and other essential metals (Zn), which may increase toxicity by replacement on the binding  
339 sites in case Cd pollution is present in the extra-cellular medium.

340 Zinc is an essential element to normal cellular function, as it acts as an enzyme co-factor  
341 for e.g. DNA and RNA-polymerase or carbonic anhydrase. However, zinc contamination of the  
342 environment from anthropogenic sources (e.g. discharges of mining products and wastes, use of  
343 fertilizers and wood preservatives: ATSDR, 2013), has been associated with several toxic effects.  
344 In sea urchin larvae, this metal has been shown to induce strong morphological abnormalities  
345 with delayed/blocked development at the blastula stage, exogastrula, spaceship Apollo-like  
346 gastrula, radicalized pluteus (Kobayashi and Okamura, 2004; Radenac et al., 2001). Ocean  
347 acidification is expected to increase the relative concentration of dissolved  $Zn^{2+}$  from 80% at  $pH_T$   
348 of 8.1 to 95% at  $pH_T$  7.4. Zn bioaccumulation was lowered by ~1.5 fold in coral tissues when pH  
349 decreased ( $pH_T$  7.5 and 7.8 vs. 8.1) and in coral skeleton ( $pH_T$  7.5 vs. 7.8 and 8.1; Houlbrèque et  
350 al., 2011). On the contrary, here we observed an initial increase (x1.3) for Zn in the first 0.2 pH  
351 unit decrease followed by a gradual decrease back to the initial (pH 8.1) values at pH 7.1. A  
352 similar bioaccumulation behavior (maximum CF found at  $pH_T$  7.85 vs.  $pH_T$  8.1 and 7.60) was  
353 found for cuttlefish newly hatched juveniles (Lacoue-Labarthe et al., 2009) and squid paralarvae  
354 (Lacoue-Labarthe et al., 2011a). As previously proposed, this bell-like behavior may be linked to  
355 the complex interactions between the requirement to maintain acid-base balance and the function  
356 of Zn as a co-factor of carbonic anhydrase, an enzyme involved in the skeletogenesis but also in  
357 the regulation of acid-base balance (see further details in Karakostis et al., 2016) and, in  
358 particular, for its putative role in proton excretion in the stomach of sea urchin larvae (Stumpp  
359 and Hu, 2017). Seawater acidification might therefore enhance carbonic anhydrase synthesis and  
360 consequently, Zn requirement in the tissues in the first acidification step. The decrease in Zn

361 accumulation at even lower pHs ( $\leq 7.7$ ) might be due to an increased competition with protons for  
362 the cells binding sites, which might limit Zn cation incorporation.

363 Selenium displayed the same kind of trends with a decreased accumulation with lowered  
364 pH, except at the extreme value of pH 7.1, suggesting again a competition between protons and  
365 Se free form ion. To date, little information on the mechanisms involved in dissolved Se uptake  
366 in marine animals is available to link physiological responses of sea urchin larvae to Se  
367 bioaccumulation processes. It is noteworthy that the accumulation values observed for Se  
368 compared to the other trace elements are relatively low which is congruent with the fact that Se is  
369 known to be mainly accumulated from the trophic route (Sandholm et al., 1973). Selenium plays  
370 an essential role as a co-factor for the reduction of anti-oxidant enzymes, especially in marine  
371 organisms. It is mostly found in the water under its oxidized state ( $\text{SeO}_4^{2-}$  or  $\text{SeO}_3^-$ ), which  
372 presumes that it is little affected by  $\text{CO}_2$ -driven seawater acidification (Millero et al., 2009). It  
373 enters the water from surface runoff, drainage, coal mining, treatment plants and historically oil  
374 refineries with concentrations in polluted effluents that can reach  $0.25 \text{ mg l}^{-1}$  (average seawater  
375 concentration:  $0.09 \text{ } \mu\text{g l}^{-1}$ ; ATSDR, 2003). Selenium toxicity to sea urchin has been seldom  
376 studied, to our knowledge; only one study found a significant effect of Se on larval abnormality  
377 ( $\text{EC}_{50}$  of  $26 \text{ } \mu\text{g l}^{-1}$  in plutei of *Diadema antillarum*; Bielmyer et al., 2005) and only a single study  
378 investigated the effect of pH on its bioaccumulation (undetected in cuttlefish newly hatched  
379 juveniles; Lacoue-Labarthe et al., 2012).

380 Finally, manganese is an essential element which is involved in enzyme activation and  
381 several regulatory functions such as e.g. bone mineralization, energetic metabolism or  
382 immunological and nervous system activities (see review by Pinsino et al., 2012). It is however  
383 an emerging pollutant in the marine environment which is present in several human-made waste

384 (e.g. metallic and chemical products, sewage) and it is a known neurotoxin in humans. In water, it  
385 is principally found as  $Mn^{2+}$  with a slight increase in the toxic form presence with decreasing pH  
386 (97.3% at pH 8.1 to 99.05% at pH 7.4: Millero et al., 2009). Extensive work on *P. lividus* larvae  
387 (Pinsino et al., 2014, 2011, 2010) demonstrated that, although high levels of Mn did not create  
388 lethal effects, Mn accumulated in the cells with time and led to strong sub-lethal effects. For  
389 instance, the authors observed that Mn exposure disturbed calcium uptake and increased  
390 morphological abnormalities (at a concentration of  $61.6 \text{ mg l}^{-1}$ , Mn prevented skeletal growth and  
391 modified gene expression linked to skeletogenesis), and also increased heat-shock protein  
392 expression. In this study, we observed that lowering pH increased Mn bioaccumulation in tissues,  
393 but this was mostly due to an increase at  $pH_T$  7.1. Until this extreme pH value, the absence of any  
394 change in Mn bioaccumulation remain consistent with the quasi-stable concentration of the free  
395 form ion, suggesting that responses of sea urchin larvae to decreasing pH do not affect its Mn  
396 metabolism. At  $pH_T$  7.1, it is difficult to interpret the sudden increase since larvae are usually  
397 highly abnormal in their morphology (pers. data:  $46 \pm 40\%$  abnormality vs.  $1.5 \pm 3.2\%$  for *S.*  
398 *droebachiensis* at  $pH_T$  7.1: Dorey et al., 2013).

## 399 **5. Conclusions**

400 In conclusion, we have shown that  $CO_2$ -induced pH changes can modify the  
401 bioaccumulation rates of metals in sea urchin larvae. The variance in accumulation was mostly  
402 explained by the indirect effect of pH on larval growth but effects independent of the size were  
403 also significant for five metals. The relationship between the changes in speciation,  
404 bioaccumulation and toxicity are not straightforward and urgently requires more research. In  
405 particular, studies combining toxicity with environmentally-relevant levels of metals and  
406 bioaccumulation data are vital to implement the necessary policies for water and fisheries

407 management. Combined pH/metal toxicity studies have been carried out mainly on Cd and Cu  
408 (Campbell et al., 2014; Ivanina et al., 2015, 2014, Ivanina and Sokolova, 2015, 2013; Lewis et  
409 al., 2013) and tend to show an increase of metal toxicity with lowering pH. In our study, we show  
410 that the responses in terms of bioaccumulation are specific to each metal and this highlights the  
411 current need to understand the mechanisms which operate in seawater with lowered pH (e.g.  
412 passive or active regulation of metal uptake, metal uptake as by-products of metabolic rates or  
413 acid-base balance regulation), which will in turn be essential to be able to implement more  
414 accurate water management policies.

415 Finally, this study did not consider the processes of detoxification (e.g. Pinsino et al.,  
416 2011) and the reversibility of trace metal effects (e.g. Morroni et al., 2018), which could play an  
417 important role in determining the sensitivity of sea urchin larvae to a future ocean where seawater  
418 acidification and metal pollution co-exist, and represents a challenging gap of knowledge.

419  
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686 **TABLE 1. Literature review** of the effects of CO<sub>2</sub>-induced acidification on metal bioaccumulation and toxicity in marine invertebrates  
687 using lab-based experiments. Article search was performed in Web of Science using the keywords 'ocean acidification' and 'metal' in  
688 July 2017. This review does not include publications on CSS-leakage experiments, field or sediment toxicity studies (but see e.g.  
689 Basallote et al., 2014) nor nano-metals (e.g. Hu *et al.*, 2017). For a review by metals, see also Ivanina & Sokolova (2015). Stage: S=  
690 sperm, F=fertilization, E=embryos, L=larvae, J=juveniles, A=adults, gen=generations. pCO<sub>2</sub> is indicated when it was measured, or an additional  
691 carbonate chemistry parameter was measured (e.g. TA, DIC).

Type of study	Group	Species	Stage	Chemical elements (nominal concentration)	Measured pH (pCO <sub>2</sub> ) treatments	Conclusions	Reference
Bioaccumulation (radiotracers)	Cephalopods	Cuttlefish	Embryos	Ag, Cd, Zn (trace)	pH <sub>i</sub> =8.09 (400), 7.84 (800), 7.61 (1400)	↓ pH ↑ Ag and ↓ Cd accumulation, modulates Zn accumulation	Lacoue-Labarthe et al., 2009
			Embryos	Am, Cs, Co, Mn, Se (trace)	pH <sub>i</sub> =8.04 (400), 7.84 (800), 7.61 (1400)	↓ pH ↓ Co and Mn accumulation	Lacoue-Labarthe et al., 2012
	Squid		Embryos, Juveniles	Ag, Cd, Co, Hg, Mn, Zn (trace)	pH <sub>i</sub> =8.1 (380), 7.85 (850), 7.60 (1500)	↓ pH ↑ Ag and Zn, ↓ Cd and Mn accumulation, modulates Hg accumulation in Embryos	Lacoue-Labarthe et al., 2011
			Adults	Zn (trace)	pH <sub>i</sub> =8.09 (ambient), 7.78 (900), 7.46 (2040)	↓ Zn accumulation in tissue (7.8) and skeleton (7.5)	Houlbréque et al., 2011
	Cnidarians	Coral	Adults	Mn, Co, Zn, Ag, Cd, <sup>241</sup> Am (trace)	pH <sub>i</sub> =8.1 (380), 7.8 (900), 7.5 (2040)	No effect of pH on metals' bioaccumulation	Belivermis et al., 2016
			Embryos, Larvae	Mn, Co, Zn, Se, Ag, Cd, Cs, <sup>241</sup> Am (trace)	pH <sub>i</sub> =8.1 (335), 7.9 (625), 7.7 (1020), 7.5 (1700), 7.3 (2840), 7.1 (4540)	↓ pH ↑ Ag accumulation, non-linear modulations of Mn, Zn and Se	Present study
	Echinoderms	Urchin	Adults		pH <sub>i</sub> NBS=8.17 (430), 7.42 (2980)	No effect of pH on Cd accumulation, ↓ pH ↑ Cd genotoxicity	Nardi et al., 2017
			Adults		pH <sub>i</sub> NBS=8.11 (485), 7.68 (1420)	↓ pH enhanced extracellular acidosis and ↑ Cu toxicity	Lewis et al., 2016
			Adults		8.05, 7.60	No/little effect of pH on Cd uptake or Cd toxicity	Benedetti et al., 2016
	Toxicity experiment and uptake	Bivalves	Mussel	Adults	Cd (20 µg l <sup>-1</sup> )		Intermediate pH ↑ Cu accumulation, interactive effects on gene expression
Adults				Cu (0.1 µM)			
		Scallop	Adults	Cd (40 µg l <sup>-1</sup> )			
		Oyster, Clam	Adults	Cu or Cd (50 µg l <sup>-1</sup> )	pH <sub>i</sub> NBS=8.11 (531), 7.93 (860), 7.76 (1330)		



	Mussel, Clams	Adults	Cd (50 µg l <sup>-1</sup> )	pH <sub>NBS</sub> =8.18 (580), 7.93 (1100), 7.60 (2590)	No effect of pH on Cd accumulation, ↓ pH ↑ Cd effects on immunology	Ivanina et al., 2014
		Adults	Cd or Cu (50 µg l <sup>-1</sup> )	pH <sub>NBS</sub> =8.14 (415), 7.83 (970), 7.52 (2090)	↓ pH ↑ Cu and Cd accumulation	Ivanina et al., 2015
		Adults	Cd (50 µg l <sup>-1</sup> )	8.07, 7.79, 7.42	↓ pH ↑ Cd accumulation	Shi et al., 2016
Crustaceans	Copepod	A, E, L, J, 2 gen. A, E, L, J, 3 gen.	Cu (20 µg l <sup>-1</sup> ) Hg (1 µg l <sup>-1</sup> )	8.06 (210), 7.95 (285), 7.82 (400), 7.67 (590) 8.02, 7.67	↓ pH ↑ Cu accumulation and toxicity ↓ pH ↓ Hg uptake, ↓ pH decreased Hg inhibitory effects	Fitzer et al., 2013 Li et al., 2017
Polychaetes	Arenicola	S, F, E, L	Cu (0.2 µM, 2 µM or 20 µM)	pH <sub>NBS</sub> =8.28 (390), 7.77 (1450), 7.47 (3090) pH <sub>NBS</sub> =8.17 (320), 7.60 (1490) and 7.41 (2410)	↓ pH ↑ Cu toxicity ↓ pH ↑ Cu lethal toxicity	Campbell et al., 2014 Lewis et al., 2013
Cnidarians	Anemone	Adults	Zn, Ni (10, 50, 100 µg l <sup>-1</sup> )	7.91 (n.a. ambient), Ni: 7.78 (1080) -Zn: 7.75 (1135)	↓ pH ↑ Zn and Ni accumulation No effect of pH on As uptake, pH affects ability to detoxify	Duckworth et al., 2017 Moreira et al., 2016
		Adult	As (4 mg l <sup>-1</sup> )	7.80 (760), 7.27 (3170)		
Echinoderms	Urchin	Adults	Cu (0.1 µM)	pH <sub>NBS</sub> =8.13 (500), 7.71 (1390)	↓ pH ↑ Cu toxicity	Lewis et al., 2016
<i>In vitro</i> study		Adult tissues: mantle cells	Cd (25 or 100 µM) or Cu (1 or 5 µM)	pH <sub>NBS</sub> =8.3 (0.05 kPa, ≈490), 7.4 (1.52 kPa, ≈15 000), 7.2 (3.01 kPa, ≈29 700)	↓ pH ↓ Cd and ↑ Cu accumulation	Ivanina et al., 2013
Bivalves	Clam					

693 **TABLE 2. Experimental conditions:** Measured  $\text{pH}_T$  and calculated carbonate chemistry in each  
 694 of the pH treatments. Measurements in italic ( $p\text{CO}_2$  and saturation states: Omega calcite and  
 695 aragonite) are calculated from  $\text{pH}_T$  (measurements taken every 15 min from 0 to 74 hpf, N=282  
 696 for each pH treatment), temperature ( $20.13 \pm 0.34$  °C, n=282, measured every 15 min in one  
 697 tank), salinity (38) and a mean total alkalinity (TA) of  $2558 \mu\text{mol.kg}^{-1}$  using the R package  
 698 *seacarb*. Data are expressed as mean  $\pm$  sd. Larval body length (L;  $\mu\text{m}$ ) was calculated for each  
 699 sampling time (hpf) and pH treatment used in the experiment (lengths calculated from Martin et  
 700 al., 2011).

pH treatment	$\text{pH}_T$	$p\text{CO}_2$ ( $\mu\text{atm}$ )	<i>Omega calcite</i>	<i>Omega aragonite</i>	Body length (L)		
					28 hpf	52 hpf	74 hpf
7.1	$7.12 \pm 0.06$	<i><math>4537 \pm 438</math></i>	<i><math>0.75 \pm 0.20</math></i>	<i><math>0.49 \pm 0.13</math></i>	128.13	255.67	328.37
7.3	$7.32 \pm 0.04$	<i><math>2840 \pm 261</math></i>	<i><math>1.14 \pm 0.09</math></i>	<i><math>0.74 \pm 0.06</math></i>	186.39	346.26	437.37
7.5	$7.53 \pm 0.03$	<i><math>1697 \pm 109</math></i>	<i><math>1.79 \pm 0.10</math></i>	<i><math>1.17 \pm 0.07</math></i>	208.46	382.72	482.05
7.7	$7.73 \pm 0.03$	<i><math>1017 \pm 75</math></i>	<i><math>2.75 \pm 0.15</math></i>	<i><math>1.79 \pm 0.10</math></i>	234.76	407.19	505.46
7.9	$7.92 \pm 0.04$	<i><math>625 \pm 60</math></i>	<i><math>3.98 \pm 0.24</math></i>	<i><math>2.60 \pm 0.16</math></i>	240.61	429.83	537.67
8.1	$8.14 \pm 0.02$	<i><math>335 \pm 20</math></i>	<i><math>5.99 \pm 0.19</math></i>	<i><math>3.91 \pm 0.12</math></i>	249.27	435.25	541.25

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702

703 **TABLE 3. Larval densities and mortality:**

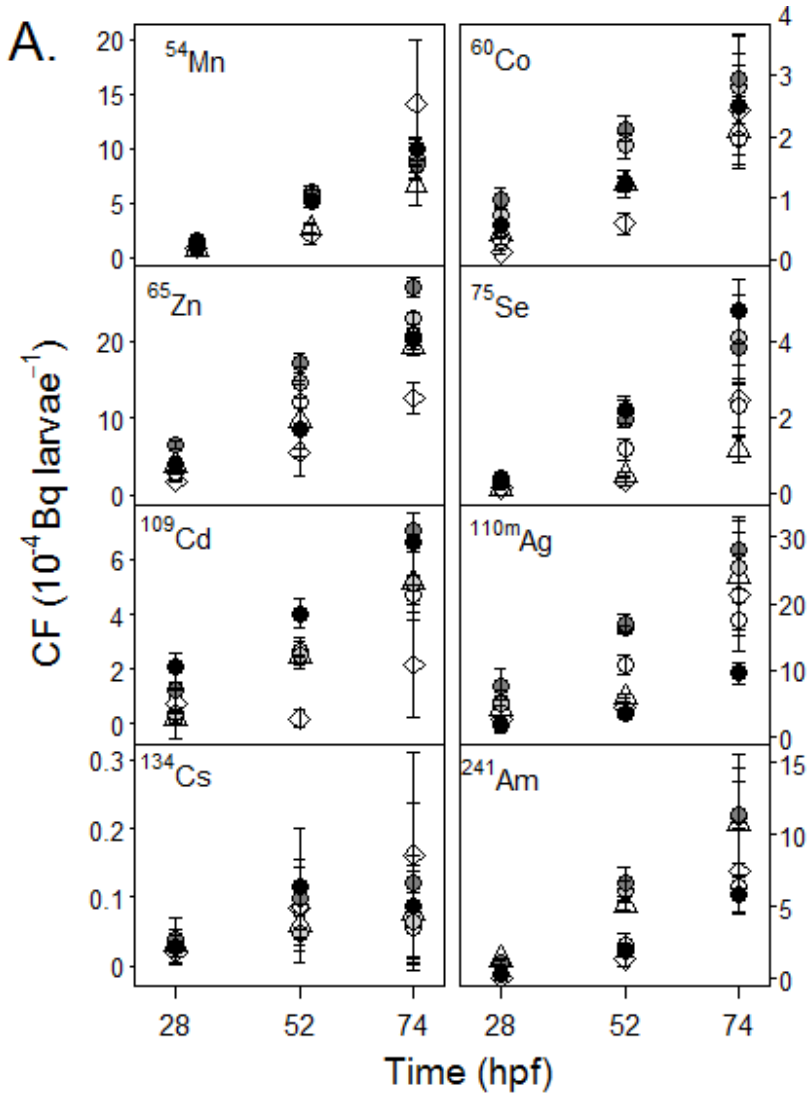
704 Larval density (larvae.ml<sup>-1</sup>; mean ± SD) in each of the six pH treatments and three time-points  
 705 (N=6 replicates of 1 to 2 ml of seawater per pH treatment and time point) and total mortality (%  
 706 larvae) after 74 hpf.

pH treatment	Density (larvae.ml <sup>-1</sup> )			Mortality at 74 hpf (% larvae)
	22 hpf	47 hpf	74 hpf	
7.1	119.7 ± 18.0	42.8 ± 5.5	10.3 ± 1.3	<i>64.1</i>
7.3	122.0 ± 5.9	49.8 ± 2.9	23.8 ± 4.3	<i>17.4</i>
7.5	131.0 ± 5.0	50.3 ± 6.8	23.6 ± 1.7	<i>21.5</i>
7.7	119.8 ± 10.0	54.5 ± 6.5	25.2 ± 5.4	<i>12.5</i>
7.9	103.8 ± 6.9	53.5 ± 4.9	23.9 ± 4.0	<i>16.8</i>
8.1	126.0 ± 13.7	75.7 ± 7.9	24.7 ± 4.1	<i>14.2</i>

707

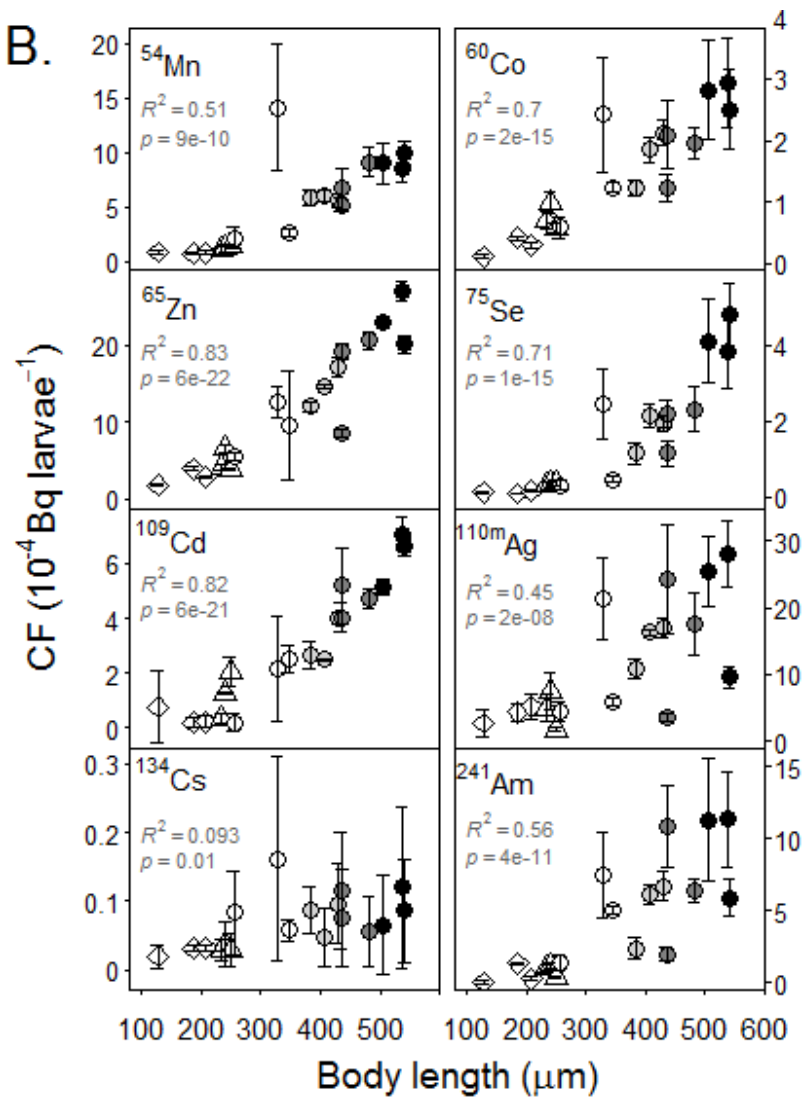
708

709 **FIGURE 1:** Concentration factor (CF±SD;  $10^{-4}$  Bq larvae<sup>-1</sup>) of metals in the different pH treatments:  
 710 diamond (7.1), triangle (7.3), circles: hollow (7.5), light-grey (7.7), grey (7.9), black (8.1); as a function of  
 711 **A.** time (three time points: 28, 52 and 74 hpf) and **B.** body length (μm). The results of the linear regression  
 712 between CF and body length for each metal are presented in grey ( $R^2$  and  $p$  for the  $p$ -value).



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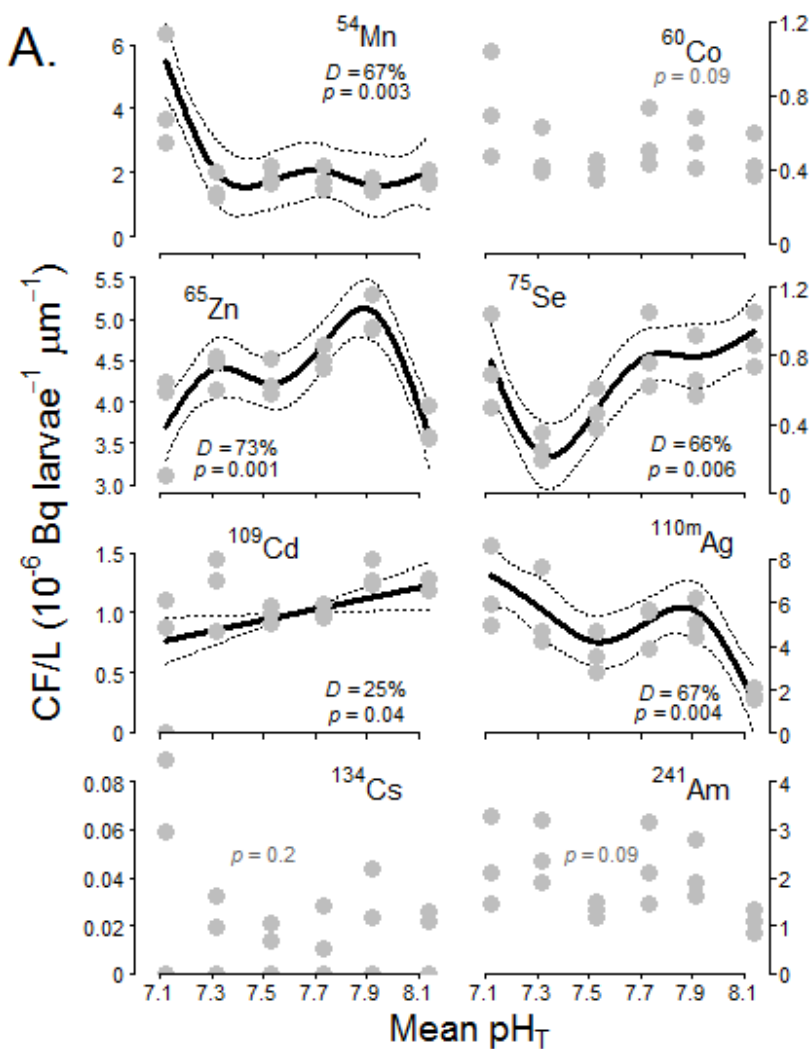


717 **TABLE 4. Generalized linear models results** testing the effect of two continuous variables  
718 (mean pH<sub>T</sub> and Time) on the CF/L (Bq larvae<sup>-1</sup> μm<sup>-1</sup>), for each metal. The best model was always  
719 without interaction term (pHT: Time) and so it is the one presented here. The variables that had a  
720 significant effect in CF/L are shown in bold for each metal.

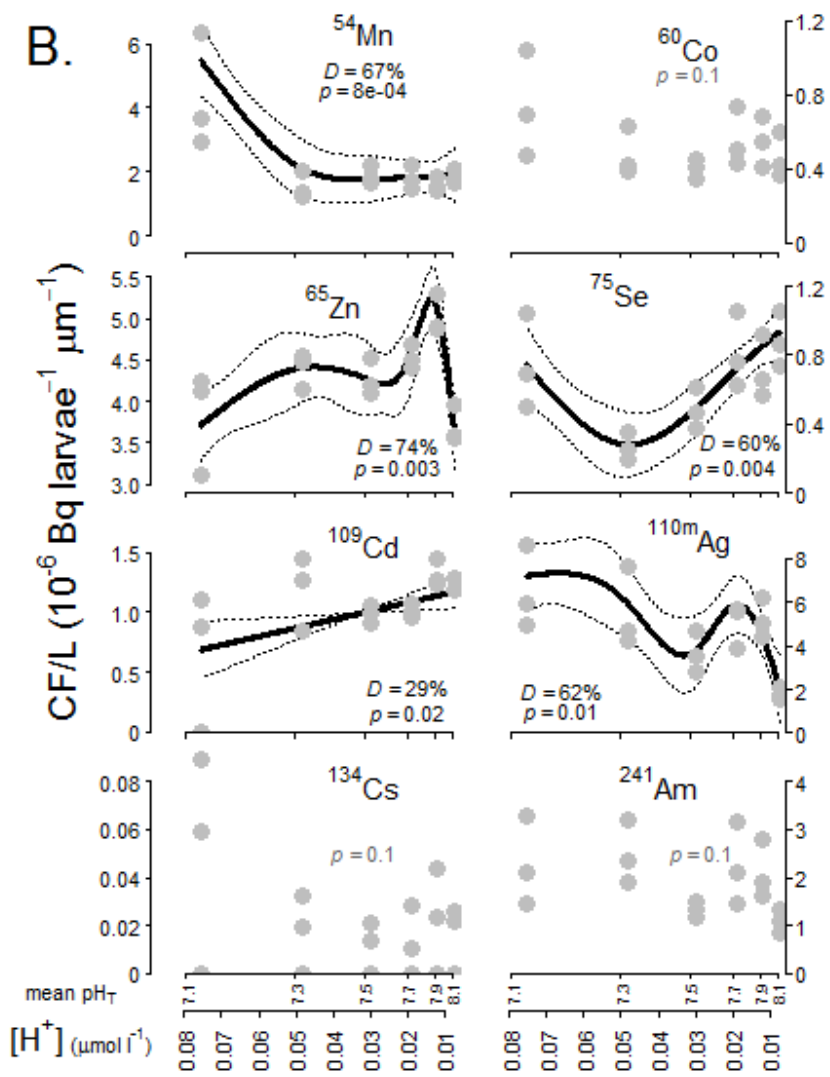
Metal	Variable	Estimate	Std Error	t-value	p-value	AIC	Df (null, residual)
<sup>54</sup> Mn	(Intercept)	2.65.10 <sup>-6</sup>	2.15.10 <sup>-6</sup>	1.23	0.224	-1370.6	3,51
	pH <sub>T</sub>	-4.18.10 <sup>-7</sup>	2.80.10 <sup>-7</sup>	-1.50	0.141		
	<b>Time</b>	3.57.10 <sup>-8</sup>	5.16.10 <sup>-9</sup>	6.91	<b>&lt;0.0001</b>		
<sup>60</sup> Co	(Intercept)	-3.81.10 <sup>-7</sup>	4.04.10 <sup>-7</sup>	-0.94	0.350	-1551.3	3,51
	pH <sub>T</sub>	5.51.10 <sup>-8</sup>	5.25.10 <sup>-8</sup>	1.05	0.298		
	<b>Time</b>	6.48.10 <sup>-9</sup>	9.68.10 <sup>-10</sup>	6.69	<b>&lt;0.0001</b>		
<sup>65</sup> Zn	(Intercept)	-2.3.10 <sup>-6</sup>	2.2.10 <sup>-6</sup>	-1.04	0.303	-1368.9	3,51
	pH <sub>T</sub>	3.3.10 <sup>-7</sup>	2.8.10 <sup>-7</sup>	1.17	0.249		
	<b>Time</b>	5.4.10 <sup>-8</sup>	5.2.10 <sup>-9</sup>	10.23	<b>&lt;0.0001</b>		
<sup>75</sup> Se	<b>(Intercept)</b>	-2.4.10 <sup>-6</sup>	4.8.10 <sup>-7</sup>	-4.97	<b>&lt;0.0001</b>	-1531.9	3,51
	<b>pH<sub>T</sub></b>	2.8.10 <sup>-7</sup>	6.3.10 <sup>-8</sup>	4.52	<b>&lt;0.0001</b>		
	<b>Time</b>	1.2.10 <sup>-8</sup>	1.2.10 <sup>-9</sup>	10.12	<b>&lt;0.0001</b>		
<sup>109</sup> Cd	<b>(Intercept)</b>	-3.8.10 <sup>-6</sup>	1.0.10 <sup>-6</sup>	-3.77	<b>&lt;0.0005</b>	-1452.8	3,51
	<b>pH<sub>T</sub></b>	4.9.10 <sup>-7</sup>	1.3.10 <sup>-7</sup>	3.75	<b>&lt;0.0005</b>		
	<b>Time</b>	1.5.10 <sup>-8</sup>	2.4.10 <sup>-9</sup>	6.10	<b>&lt;0.0001</b>		
<sup>110m</sup> Ag	(Intercept)	9.5.10 <sup>-6</sup>	4.4.10 <sup>-6</sup>	2.13	0.038	-1292.3	3,51
	<b>pH<sub>T</sub></b>	-1.2.10 <sup>-6</sup>	5.8.10 <sup>-7</sup>	-2.07	<b>0.043</b>		
	<b>Time</b>	5.4.10 <sup>-8</sup>	1.1.10 <sup>-8</sup>	5.04	<b>&lt;0.0001</b>		
<sup>134</sup> Cs	(Intercept)	8.2.10 <sup>-8</sup>	5.0.10 <sup>-8</sup>	1.65	0.105	-1777.5	3,51
	pH <sub>T</sub>	-9.3.10 <sup>-9</sup>	6.5.10 <sup>-9</sup>	-1.44	0.157		
	Time	1.6.10 <sup>-10</sup>	1.2.10 <sup>-10</sup>	1.32	0.194		
<sup>241</sup> Am	(Intercept)	1.30.10 <sup>-6</sup>	1.,65.10 <sup>-6</sup>	0.79	0.434	-1399.1	3,51
	pH <sub>T</sub>	-2.58.10 <sup>-7</sup>	2.15.10 <sup>-7</sup>	-1.20	0.235		
	<b>Time</b>	3.43.10 <sup>-8</sup>	3.96.10 <sup>-9</sup>	8.66	<b>&lt;0.0001</b>		

721 *Std Error = standard error; AIC = Akaike information criterion; Df = degree of freedom.*

722 **FIGURE 2: Larvae metal content (CF/L) at 74 hpf changes with A. pH and B. calculated H<sup>+</sup>**  
 723 **concentration** - Generalized additive models (lines, gam, R package *mgcv*) fitted to the  
 724 concentration factor divided by larval size (CF/L:10<sup>-6</sup> Bq larvae mm<sup>-1</sup> μm<sup>-1</sup>) as a function of the  
 725 mean pH<sub>T</sub> for each metal investigated, at the final time-point (74 hpf). When the lines of the  
 726 generalized additive model are not present, the models were not significant. The proportion of  
 727 deviance explained by the model is given as *D* (%) and the significance for the smooth by *p*. The  
 728 detailed results for pH are presented in **Table 5**.



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733 **TABLE 5. General additive models results** testing for the effect of a continuous variable (mean pH<sub>T</sub>) on the CF/L (Bq larvae<sup>-1</sup> μm<sup>-1</sup>) at

734 74 hpf, for each metal. Only the fittest model for K is presented. The significant models are shown in bold for each metal.

Metal	K	Intercept		Mean pH <sub>T</sub> (approximate significance of the smooth term)										
		Estimate	SE	tvalue	p-value	edf	Rdf	F	p-value	R <sup>2</sup>	D	GCV	Sc Est	N
<sup>54</sup> Mn	5	2.16.10 <sup>-6</sup>	1.81.10 <sup>-7</sup>	11.91	<0.0001	3.59	3.90	7.018	<b>0.0033</b>	0.586	67%	7.9.10 <sup>-13</sup>	5.9.10 <sup>-13</sup>	18
<sup>60</sup> Co	4	5.30.10 <sup>-7</sup>	3.58.10 <sup>-8</sup>	14.82	<0.0001	2.88	2.99	2.767	0.091	0.245	37%	2.9.10 <sup>-14</sup>	2.3.10 <sup>-14</sup>	18
<sup>65</sup> Zn	5	4.29.10 <sup>-6</sup>	7.35.10 <sup>-8</sup>	58.33	<0.0001	3.84	3.99	8.167	<b>&lt;0.0001</b>	0.651	73%	1.3.10 <sup>-13</sup>	9.7.10 <sup>-14</sup>	18
<sup>75</sup> Se	5	6.51.10 <sup>-7</sup>	4.20.10 <sup>-8</sup>	15.50	<0.0001	3.72	3.95	6.438	<b>0.0055</b>	0.560	66%	4.3.10 <sup>-14</sup>	3.2.10 <sup>-14</sup>	18
<sup>109</sup> Cd	3	1.06.10 <sup>-6</sup>	6.77.10 <sup>-8</sup>	15.69	<0.0001	1.00	1.00	5.278	<b>0.035</b>	0.201	25%	9.3.10 <sup>-14</sup>	8.3.10 <sup>-14</sup>	18
<sup>110m</sup> Ag	5	4.61.10 <sup>-6</sup>	2.93.10 <sup>-7</sup>	15.75	<0.0001	3.72	3.95	6.164	<b>0.0037</b>	0.574	67%	2.1.10 <sup>-12</sup>	1.5.10 <sup>-12</sup>	18
<sup>134</sup> Cs	4	2.15.10 <sup>-8</sup>	5.17.10 <sup>-9</sup>	4.164	0.0008	1.88	2.26	1.868	0.22	0.147	24%	5.7.10 <sup>-16</sup>	4.8.10 <sup>-16</sup>	18
<sup>241</sup> Am	4	1.92.10 <sup>-6</sup>	1.67.10 <sup>-7</sup>	11.50	<0.0001	1.00	1.00	3.284	0.089	0.118	17%	5.6.10 <sup>-13</sup>	5.0.10 <sup>-13</sup>	18

735 *K* = specified knot values to be used for basis construction; *SE* = standard error; *edf* = degree of freedom; *Rdf* = reference df; *R<sup>2</sup>* =

736 adjusted *r*-squared; *D* = % deviance explained; *GCV* = method of generalized cross validation for smoothing parameter estimation; *Sc*

737 *Est* = scale estimate

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