

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

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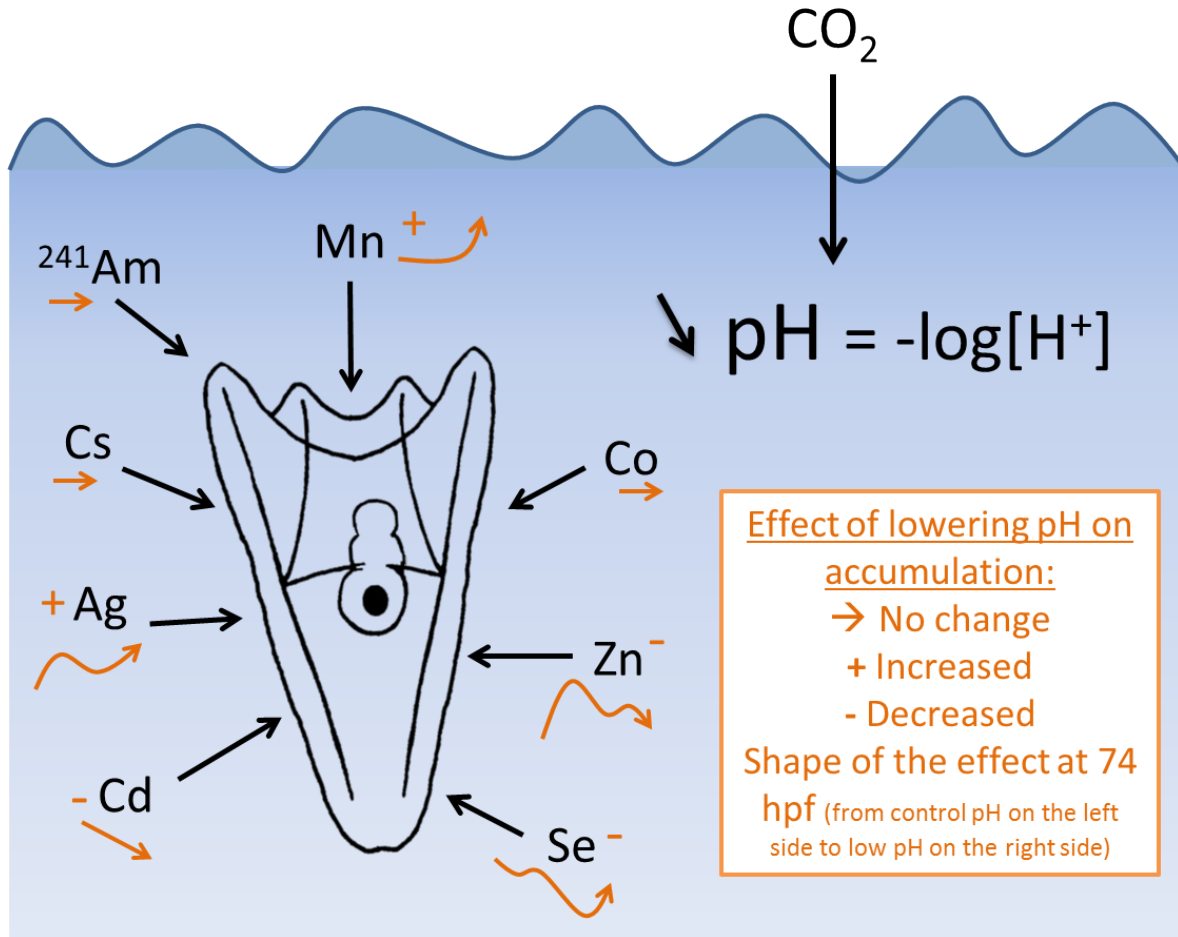
ABSTRACT

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}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}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, CO₂, 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 (CO₂) in the atmosphere (280 to 400 ppm between 1850 and 2013,
15 Etheridge et al., 1996 and Scripps CO₂ 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 21st 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_{NBS} 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 CO₂ 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. CO₂-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 ²⁴¹Am) 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 ²⁴¹Am), 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. ²⁴¹Am 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_T 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°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°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_T: 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 (ΔpH 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 (≈ 20 °C) was controlled to within ± 0.5 °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 ± 0.05 pH units using a continuous pH-stat system (IKS) that controlled the addition of CO₂ 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 (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 ($p\text{CO}_2$, Ω_a and Ω_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}Cd), Polatom, Poland ($^{110\text{m}}\text{Ag}$,
142 ^{134}Cs , ^{75}Se), Cyclotron, Russia (^{65}Zn), and Isotope Product Laboratory, USA (^{241}Am , ^{60}Co , ^{54}Mn).
143 For determination of larval metal uptake, eggs were incubated in seawater labeled with dissolved
144 ^{54}Mn (1 kBq l⁻¹ as $^{54}\text{MnCl}_2$), ^{60}Co (1 kBq l⁻¹ as $^{60}\text{CoCl}_2$), ^{65}Zn (1 kBq l⁻¹ as $^{65}\text{ZnCl}_2$), ^{75}Se (1 kBq
145 l⁻¹ as $\text{Na}_2^{75}\text{SeO}_3$), ^{109}Cd (1 kBq l⁻¹ as $^{109}\text{CdCl}_2$), $^{110\text{m}}\text{Ag}$ (1 kBq l⁻¹ as $^{110\text{m}}\text{AgNO}_3$), ^{134}Cs (1 kBq l⁻¹
146 as $^{134}\text{CsCl}$), and ^{241}Am (0.35 kBq l⁻¹ as $^{241}\text{AmNO}_3$) by spiking typically 5 to 10 μl 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⁻¹ of Ag, Am, Cs, Co, Se and Zn stable element respectively.
149 While the specific activities of ^{109}Cd and ^{54}Mn sources were not available from the providers, the
150 use of these radionuclides usually results in the addition of ng l⁻¹ 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 (≈ 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- μm polycarbonate membranes, re-suspended and rinsed three times in radiotracer-free FSW.
159 The filters with embryos or larvae were then radio-counted (γ -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 ± 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⁻¹ at
170 22 hpf to 10-25 ind.ml⁻¹ 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⁻¹), 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 , μm) 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 ; 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 pH_T values, as well as the calculated carbonate chemistry parameters, are
212 shown in **Table 2**. Mean temperature was 20.13 ± 0.34 °C (N=282). The 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 (≈ 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 ± 20 μatm in the 8.1 pH
220 condition to 4537 ± 438 μatm 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⁻¹). 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⁻¹) and the measured density in each pH treatment at 74 hpf.
227 Larval mortality was generally low (12-22%), except for pH_T=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⁻¹). 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_T 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_T on the CF
240 standardized by larval body length (CF/L, Bq larvae⁻¹ μm⁻¹) using generalized linear models
241 (**Table 4**). Taking into account the three time points, the pH_T 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_T 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_T. However, pH_T 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_T 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_T (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=0.66.10⁻⁶ ± 0.58.10⁻⁶ vs. 1.2.10⁻⁶ ± 0.06.10⁻⁶
256 Bq larvae⁻¹ μm⁻¹ respectively). The larval content of Zn linearly decreased from pH 7.9 to 7.1
257 (D=73%: CF/L= 5.0.10⁻⁶ ± 0.2.10⁻⁶ to 3.8.10⁻⁶ ± 0.6.10⁻⁶ Bq larvae⁻¹ μm⁻¹ 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=3.7.10⁻⁶ ± 0.2.10⁻⁶ Bq larvae⁻¹ μm⁻¹). Similarly, the larval content of Se at the 7.1
260 pH treatment was comparable to that of pH 8.1 (CF/L=7.5.10⁻⁷ ± 2.8.10⁻⁷ and 8.8.10⁻⁷ ± 1.6.10⁻⁷
261 Bq larvae⁻¹ μm⁻¹ 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= 2.6.10⁻⁷ ± 0.8.10⁻⁷ Bq larvae⁻¹ μm⁻¹; 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_T (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=6.5.10⁻⁶ ± 1.9.10⁻⁶ vs. 1.8.10⁻⁶ ±
268 0.3.10⁻⁶ Bq larvae⁻¹ μm⁻¹ respectively). The accumulation of Mn in larval tissues significantly
269 (*p*=0.033; **Fig. 2** and **Table 5**) increased with lowering pH_T (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⁻¹ μm⁻¹).

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 ²⁴¹Am 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, ²⁴¹Am, 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 (Cd²⁺
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 (EC₅₀ of 40-85 µg l⁻¹ for *P. lividus*,
305 Warnau et al., 1996). Here, ^{110m}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⁺/K⁺-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}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 Cd^{2+} passively via calcium channels (Rainbow and Black, 2005), and to
327 mimic 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}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 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 (≤ 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 (SeO_4^{2-} or SeO_3^-), which
372 presumes that it is little affected by 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 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 (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 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 CO₂-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. pCO₂ 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 ₂) treatments	Conclusions	Reference
Bioaccumulation (radiotracers)	Cephalopods	Cuttlefish	Embryos	Ag, Cd, Zn (trace)	pH _i =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 _i =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 _i =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 _i =8.09 (ambient), 7.78 (900), 7.46 (2040)	↓ Zn accumulation in tissue (7.8) and skeleton (7.5)	Houlbrèque et al., 2011
	Cnidarians	Coral	Adults	Mn, Co, Zn, Ag, Cd, ²⁴¹ Am (trace)	pH _i =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, ²⁴¹ Am (trace)	pH _i =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 _i NBS=8.17 (430), 7.42 (2980)	No effect of pH on Cd accumulation, ↓ pH ↑ Cd genotoxicity	Nardi et al., 2017
			Adults		pH _i 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 ⁻¹)		Intermediate pH ↑ Cu accumulation, interactive effects on gene expression
Adults				Cu (0.1 µM)			
		Scallop	Adults	Cd (40 µg l ⁻¹)			
		Oyster, Clam	Adults	Cu or Cd (50 µg l ⁻¹)	pH _i NBS=8.11 (531), 7.93 (860), 7.76 (1330)		

	Mussel, Clams	Adults	Cd (50 µg l ⁻¹)	pH _{NBS} =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 ⁻¹)	pH _{NBS} =8.14 (415), 7.83 (970), 7.52 (2090)	↓ pH ↑ Cu and Cd accumulation	Ivanina et al., 2015
		Adults	Cd (50 µg l ⁻¹)	8.07, 7.79, 7.42	↓ pH ↑ Cd accumulation	Shi et al., 2016
	Crustaceans	Copepod	A, E, L, J, 2 gen. Cu (20 µg l ⁻¹)	8.06 (210), 7.95 (285), 7.82 (400), 7.67 (590)	↓ pH ↑ Cu accumulation and toxicity	Fitzer et al., 2013
			A, E, L, J, 3 gen. Hg (1 µg l ⁻¹)	8.02, 7.67	↓ pH ↓ Hg uptake, ↓ pH decreased Hg inhibitory effects	Li et al., 2017
	Polychaetes	Arenicola	S, F, E, L Cu (0.2 µM, 2 µM or 20 µM)	pH _{NBS} =8.28 (390), 7.77 (1450), 7.47 (3090)	↓ pH ↑ Cu toxicity	Campbell et al., 2014
		Arenicola	F, E, L Cu (0.002 µM)	pH _{NBS} =8.17 (320), 7.60 (1490) and 7.41 (2410)	↓ pH ↑ Cu lethal toxicity	Lewis et al., 2013
				7.91 (n.a. ambient), Ni: 7.78 (1080) -Zn:		
	Cnidarians	Anemone	Zn, Ni (10, 50, 100 µg l ⁻¹)	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 ⁻¹)	7.80 (760), 7.27 (3170)		
		Adults	Cu (0.1 µM)	pH _{NBS} =8.13 (500), 7.71 (1390)	↓ pH ↑ Cu toxicity	Lewis et al., 2016
<i>In vitro</i> study	Echinoderms	Urchin	Adult tissues: mantle cells Cd (25 or 100 µM) or Cu (1 or 5 µM)	pH _{NBS} =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 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 pH_T (measurements taken every 15 min from 0 to 74 hpf, N=282
696 for each pH treatment), temperature (20.13 ± 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; μm) 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	pH_T	$p\text{CO}_2$ (μatm)	<i>Omega calcite</i>	<i>Omega aragonite</i>	Body length (L)		
					28 hpf	52 hpf	74 hpf
7.1	7.12 ± 0.06	<i>4537 ± 438</i>	<i>0.75 ± 0.20</i>	<i>0.49 ± 0.13</i>	128.13	255.67	328.37
7.3	7.32 ± 0.04	<i>2840 ± 261</i>	<i>1.14 ± 0.09</i>	<i>0.74 ± 0.06</i>	186.39	346.26	437.37
7.5	7.53 ± 0.03	<i>1697 ± 109</i>	<i>1.79 ± 0.10</i>	<i>1.17 ± 0.07</i>	208.46	382.72	482.05
7.7	7.73 ± 0.03	<i>1017 ± 75</i>	<i>2.75 ± 0.15</i>	<i>1.79 ± 0.10</i>	234.76	407.19	505.46
7.9	7.92 ± 0.04	<i>625 ± 60</i>	<i>3.98 ± 0.24</i>	<i>2.60 ± 0.16</i>	240.61	429.83	537.67
8.1	8.14 ± 0.02	<i>335 ± 20</i>	<i>5.99 ± 0.19</i>	<i>3.91 ± 0.12</i>	249.27	435.25	541.25

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702

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

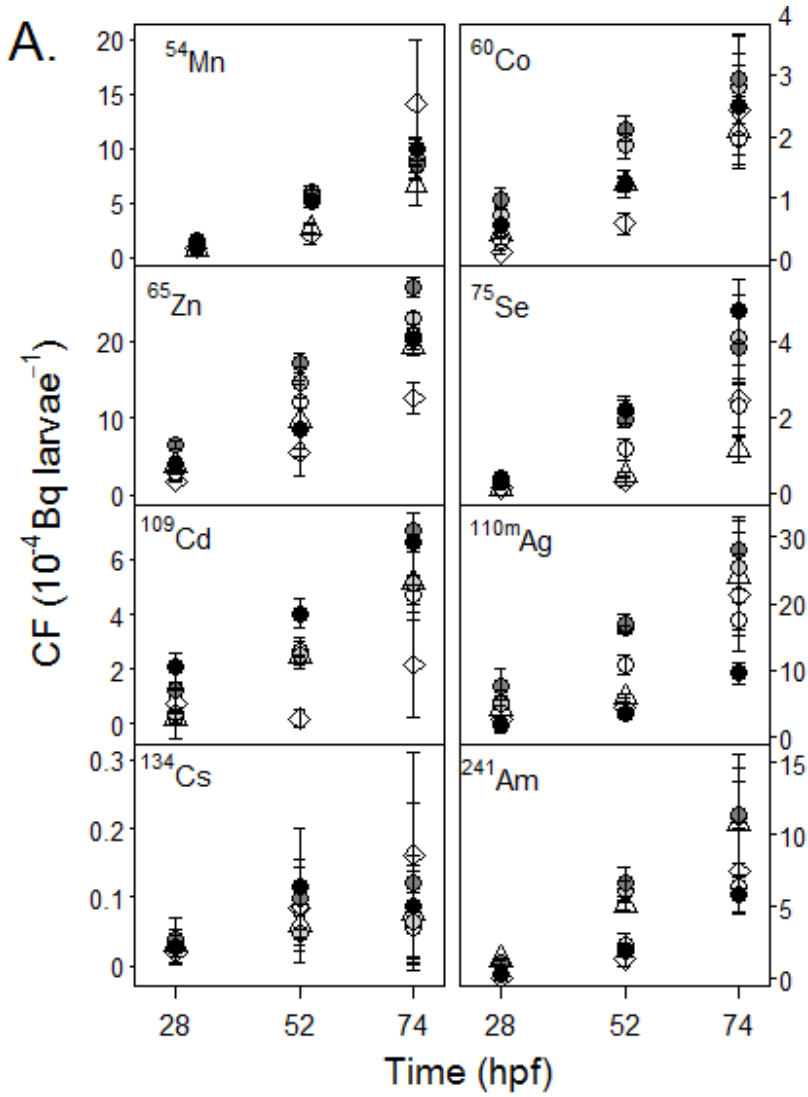
704 Larval density (larvae.ml⁻¹; mean ± SD) in each of the six pH treatments and three time-points
 705 (N=6 replicates of 1 to 2 ml of seawater per pH treatment and time point) and total mortality (%
 706 larvae) after 74 hpf.

pH treatment	Density (larvae.ml ⁻¹)			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

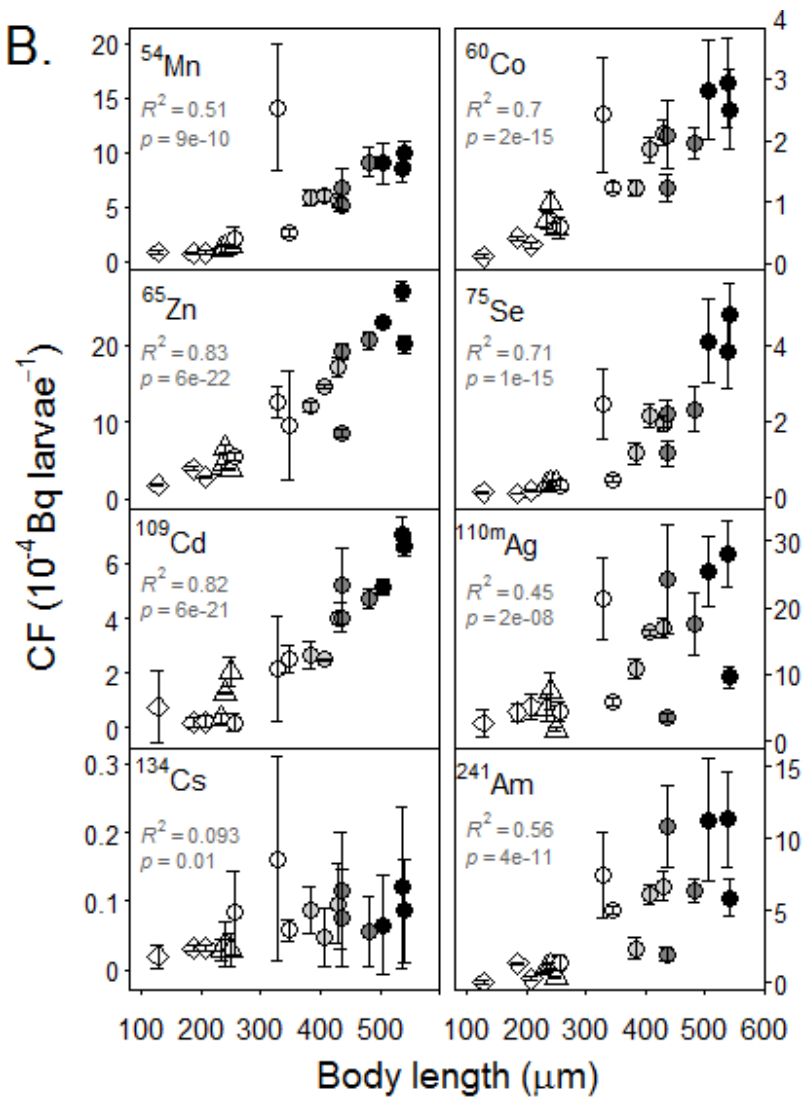
708

709 **FIGURE 1:** Concentration factor (CF±SD; 10^{-4} Bq larvae⁻¹) 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 (μ m). 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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714

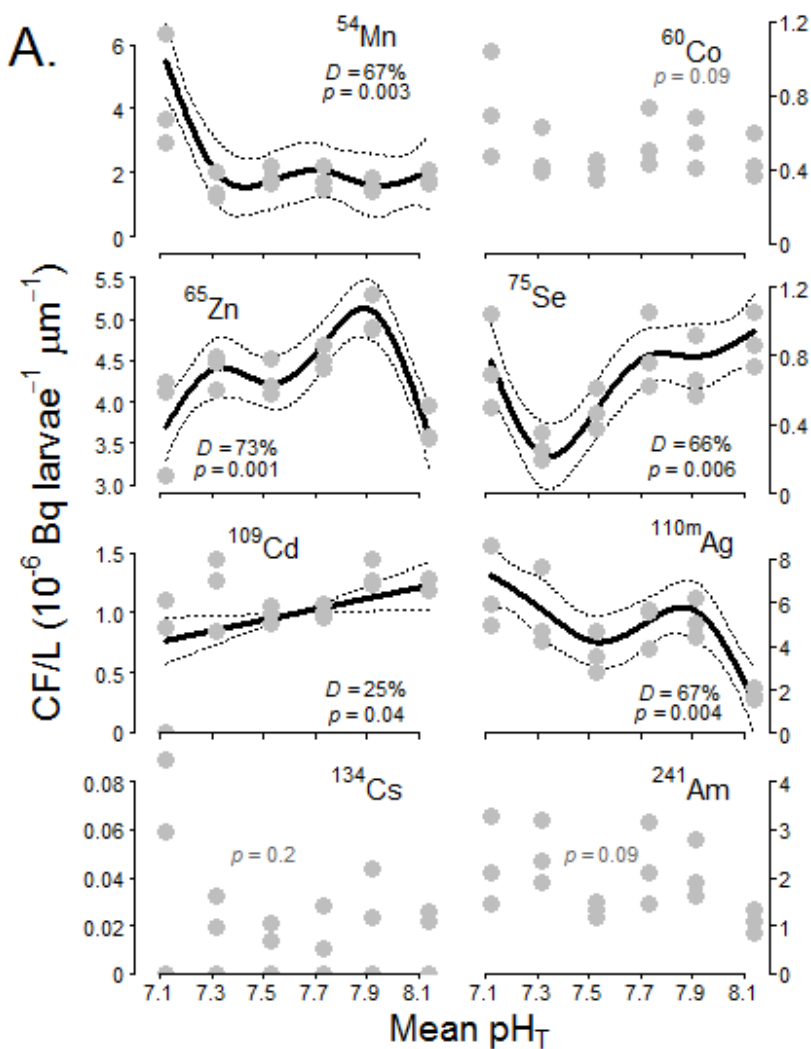


717 **TABLE 4. Generalized linear models results** testing the effect of two continuous variables
718 (mean pH_T and Time) on the CF/L (Bq larvae⁻¹ μm⁻¹), for each metal. The best model was always
719 without interaction term (pHT: Time) and so it is the one presented here. The variables that had a
720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

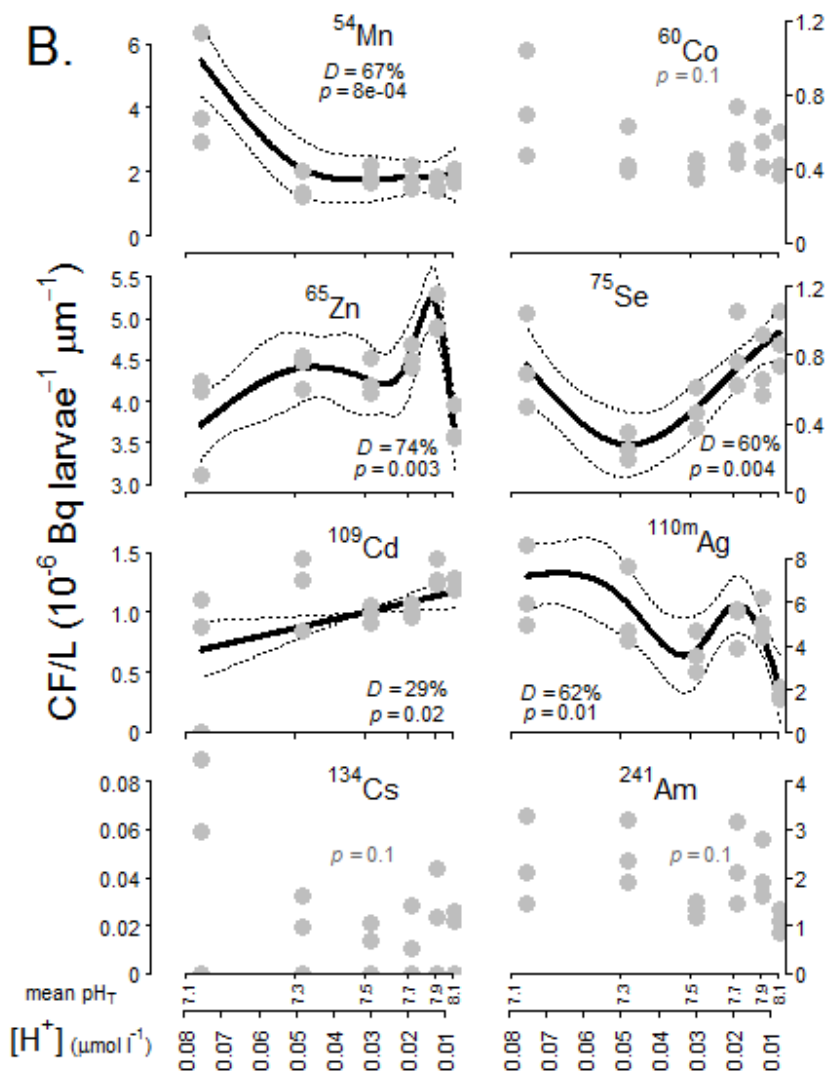
Metal	Variable	Estimate	Std Error	t-value	p-value	AIC	Df (null, residual)
⁵⁴ Mn	(Intercept)	2.65.10 ⁻⁶	2.15.10 ⁻⁶	1.23	0.224	-1370.6	3,51
	pH _T	-4.18.10 ⁻⁷	2.80.10 ⁻⁷	-1.50	0.141		
	Time	3.57.10 ⁻⁸	5.16.10 ⁻⁹	6.91	<0.0001		
⁶⁰ Co	(Intercept)	-3.81.10 ⁻⁷	4.04.10 ⁻⁷	-0.94	0.350	-1551.3	3,51
	pH _T	5.51.10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

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⁺**
 723 **concentration** - Generalized additive models (lines, gam, R package *mgcv*) fitted to the
 724 concentration factor divided by larval size (CF/L:10⁻⁶ Bq larvae mm⁻¹ μm⁻¹) as a function of the
 725 mean pH_T 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_T) on the CF/L (Bq larvae⁻¹ μm⁻¹) 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 _T (approximate significance of the smooth term)										
		Estimate	SE	tvalue	p-value	edf	Rdf	F	p-value	R ²	D	GCV	Sc Est	N
⁵⁴ Mn	5	2.16.10 ⁻⁶	1.81.10 ⁻⁷	11.91	<0.0001	3.59	3.90	7.018	0.0033	0.586	67%	7.9.10 ⁻¹³	5.9.10 ⁻¹³	18
⁶⁰ Co	4	5.30.10 ⁻⁷	3.58.10 ⁻⁸	14.82	<0.0001	2.88	2.99	2.767	0.091	0.245	37%	2.9.10 ⁻¹⁴	2.3.10 ⁻¹⁴	18
⁶⁵ Zn	5	4.29.10 ⁻⁶	7.35.10 ⁻⁸	58.33	<0.0001	3.84	3.99	8.167	<0.0001	0.651	73%	1.3.10 ⁻¹³	9.7.10 ⁻¹⁴	18
⁷⁵ Se	5	6.51.10 ⁻⁷	4.20.10 ⁻⁸	15.50	<0.0001	3.72	3.95	6.438	0.0055	0.560	66%	4.3.10 ⁻¹⁴	3.2.10 ⁻¹⁴	18
¹⁰⁹ Cd	3	1.06.10 ⁻⁶	6.77.10 ⁻⁸	15.69	<0.0001	1.00	1.00	5.278	0.035	0.201	25%	9.3.10 ⁻¹⁴	8.3.10 ⁻¹⁴	18
^{110m} Ag	5	4.61.10 ⁻⁶	2.93.10 ⁻⁷	15.75	<0.0001	3.72	3.95	6.164	0.0037	0.574	67%	2.1.10 ⁻¹²	1.5.10 ⁻¹²	18
¹³⁴ Cs	4	2.15.10 ⁻⁸	5.17.10 ⁻⁹	4.164	0.0008	1.88	2.26	1.868	0.22	0.147	24%	5.7.10 ⁻¹⁶	4.8.10 ⁻¹⁶	18
²⁴¹ Am	4	1.92.10 ⁻⁶	1.67.10 ⁻⁷	11.50	<0.0001	1.00	1.00	3.284	0.089	0.118	17%	5.6.10 ⁻¹³	5.0.10 ⁻¹³	18

735 *K* = specified knot values to be used for basis construction; *SE* = standard error; *edf* = degree of freedom; *Rdf* = reference df; *R²* =

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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