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1 **Temperature and  $p\text{CO}_2$  effect on the bioaccumulation of radionuclides and**  
2 **trace elements in the eggs of the common cuttlefish, *Sepia officinalis***

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38 **Abstract.** The increasing release of CO<sub>2</sub> by human activities leads to ocean acidification and  
39 global warming. Both those consequences (*i.e.*, increase in seawater pCO<sub>2</sub> and temperature)  
40 may drastically affect physiology of marine organisms. The effects of low pH and elevated  
41 temperature on the bioaccumulation of radionuclides (<sup>241</sup>Am, <sup>134</sup>Cs) and trace elements (<sup>60</sup>Co,  
42 <sup>54</sup>Mn or <sup>75</sup>Se) were studied during the embryonic development of the common cuttlefish *Sepia*  
43 *officinalis*. The lowered accumulation of essential <sup>60</sup>Co and <sup>54</sup>Mn with decreasing pH was  
44 larger at 16°C than at 19°C. Se was not detected in the embryo whereas it penetrated through  
45 the eggshell, suggesting that an alternative pathway ensures the supply of this essential metal  
46 for the embryo. <sup>241</sup>Am was totally retained by the eggshell irrespective of pH and temperature.  
47 Finally, the amount of Cs found in the peri-vitelline fluid increased with decreasing pH likely  
48 because of an enhanced swelling of the cuttlefish egg under elevated CO<sub>2</sub>.

49

50 **Keywords:** cephalopod, ocean acidification, ocean warming, eggshell, embryonic  
51 development, metal.

52

## 53 INTRODUCTION

54

55 Climate change due to the increasing atmospheric carbon dioxide (CO<sub>2</sub>) concentrations in the  
56 atmosphere is now considered to be a major threat to biodiversity as well as to the structure  
57 and function of ecosystems (Mc Carthy, 2001). Although all the causes are still subjected to  
58 debate (Sharp, 2003), it is widely accepted that human activities such as fossil fuel  
59 combustion, cement production and change in land use (Hansen et al., 2007) drove the rising  
60 global atmospheric concentration of CO<sub>2</sub> from a pre-industrial value of approximately 280  
61 parts per million (ppm) to 384 ppm in 2007 (~387 ppm in 2009) (Anonymous, 2010).  
62 Moreover, models project that the CO<sub>2</sub> concentration will double by midcentury (> 500 ppm)  
63 and could reach between 730 and 1020 ppm in 2100 (IPCC, 2007). The accumulation of  
64 greenhouse gases will have profound consequences among which the global warming.  
65 Temperature is expected to increase on average by 3°C at the Earth's surface over the course  
66 of this century (IPCC, 2007). Furthermore, the precipitation and evaporation rates that drive  
67 the hydrologic cycle as well as the winds regimes will also be affected (Roessig et al., 2004).  
68 Regarding the Ocean, similar trends are expected for sea surface temperature due to the  
69 warming of the surface mixed layer (Levitus et al., 2005) and the sea level will rise mainly  
70 because of the melting ice. Moreover, as the oceans are major carbon sink absorbing about  
71 25% of anthropogenic CO<sub>2</sub>, the sea surface CO<sub>2</sub> partial pressure (*p*CO<sub>2</sub>) is also expected to  
72 increase (Sabine et al., 2004). This causes major shifts in seawater carbonate chemistry and is  
73 likely to reduce pH by 0.2-0.4 units, a phenomenon called “ocean acidification”, over the  
74 course of this century (Caldeira and Wickett, 2005).

75 In the northern Europe, the main scenario for climate change is milder, wetter and stormier  
76 winters (IPCC, 2007). Summers could be warmer and dryer, although the climatic effect is  
77 expected to be less pronounced than in winters (Jonsson and Jonsson, 2009). Simultaneously,

78 the increasing precipitations will directly and indirectly impact the marine coastal area,  
79 causing a decrease of the salinity and amplifying the anthropogenic factors, such as hypoxic  
80 events linked to increased agricultural runoff or contamination of the nearshore zones by  
81 organic and inorganic contaminants released in the environment (Harley et al., 2006). Thus,  
82 the marine biota will have to deal with interactions between increasing seawater temperature,  
83  $p\text{CO}_2$ , and well-known anthropogenic stressors such as the contamination by heavy metals.

84 In ecotoxicology and risk assessment studies, it is widely accepted that the early life stages  
85 are the most sensitive to metallic contamination (*e.g.* Calabrese et al., 1973; Warnau et al.,  
86 1996; Oral et al., 2010). Recently, a growing body of works also showed that embryos and  
87 larvae were affected by hypercapnia (*e.g.* Kurihara et al., 2008; Dupont et al., 2008; Walther  
88 et al., 2010) as their developing systemic acid-base regulation is not yet fully efficient  
89 (Pörtner and Farrell, 2008). As the highly productive coastal zones serve as nursery grounds  
90 for many marine species of ecological and commercial importance (Dunn et al., 1999;  
91 Selleslagh and Amara, 2008), studying interaction between multiple stressors is critical to  
92 project the future of coastal ecosystems (Harley et al., 2006; Dupont et al., 2010).

93 In Europe, the common cuttlefish *Sepia officinalis* is seasonally exploited in the English  
94 Channel (Royer et al., 2006) when adults come in the shallow waters to mate and spawn their  
95 eggs before massive die-offs (Boucaud-Camou and Boismery, 1991). The eggs laid in  
96 shallow waters are subject to chronic exposure to various contaminants among which, heavy  
97 metals that bioaccumulate in the cuttlefish during its ontogenic development (Miramand et  
98 al., 2006; Villanueva and Bustamante, 2006). Experimental studies demonstrated that the  
99 cuttlefish embryo is partially protected because of the selective permeability of the eggshell to  
100 trace elements, depending on the developmental stage (Bustamante et al., 2002, 2004, 2006a;  
101 Lacoue-Labarthe et al., 2008a, 2009a, 2010). Moreover, these shielding properties of the egg  
102 capsule against Ag, Cd and Zn penetration and the metal accumulation efficiencies of the

103 embryo seemed to be affected when eggs develop under warmer and hypercapnic conditions  
104 (Lacoue-Labarthe et al., 2009b).

105 This study addresses the combined effects of elevated temperature and  $p\text{CO}_2$  on the  
106 bioaccumulation of selected radionuclides and trace elements in the cuttlefish embryo.  
107 Indeed, the development of nuclear facilities and fallout from nuclear weapon testing resulted  
108 in the release of several medium- and long-lived radionuclides into aquatic environments such  
109 as  $^{241}\text{Am}$ ,  $^{60}\text{Co}$ ,  $^{134}\text{Cs}$ ,  $^{54}\text{Mn}$  or  $^{75}\text{Se}$  (e.g. Warnau et al., 1999, Ke et al., 1999). Although these  
110 radionuclides are generally considered as micropollutants in the oceans, they are known to  
111 accumulate in marine organisms (e.g. Miramand and Guary, 1981; Bustamante et al. 2006b,  
112 Lacoue-Labarthe et al., 2010; Metian et al., 2009, 2011). In addition, radionuclides are of  
113 specific interest given the ecotoxicological concern regarding their stable isotopes. Indeed,  
114 Co, Mn and Se are trace elements that have essential functions in physiology but also toxic  
115 effect if they are excessively accumulated in organisms (e.g. Rainbow, 2002). In this study,  
116 we therefore use radiotracers to investigate the bioaccumulation behaviour of these  
117 corresponding stable elements that are present in marine waters (Warnau and Bustamante,  
118 2007).

119

## 120 MATERIALS AND METHODS

121

122 Adult cuttlefish were collected by net-fishing off Monaco in April 2008 and maintained in  
123 open-circuit tanks in the IAEA-MEL premises. After mating, the fertilized eggs that were laid  
124 by each female were immediately separated to optimise their oxygenation. The eggs ( $n = 300$ )  
125 were placed and kept in six 5-L plastic bottles (50 eggs per bottle; one bottle per treatment;  
126  $0.45\mu\text{m}$  and UV-sterilized seawater; constantly aerated closed-circuit; salinity 38 p.s.u.;  
127 light/dark cycle: 12h/12h) during the full development time in controlled conditions of  
128 temperature and pH in a crossed (2 temperatures x 3 pH levels) experimental design. The pH  
129 and temperature values were chosen consistent with those of realistic modelled scenarios of  
130 climate change that would occur at the end of the century (Orr et al., 2005; Solomon et al.,  
131 2007). Temperature was controlled in each bath to within  $\pm 0.5^\circ\text{C}$  using temperature  
132 controllers connected to 300 W submersible heaters. The pH was controlled in each bottle to  
133 within  $\pm 0.05$  pH unit with a continuous pH-stat system (IKS, Karlsbad) that bubbled pure  
134  $\text{CO}_2$  into the bottles that were continuously aerated with  $\text{CO}_2$ -free air. The pH was maintained  
135 at a mean ( $\pm$  SD) of  $7.61 \pm 0.11$ ,  $7.84 \pm 0.04$ , and  $8.09 \pm 0.04$ , at ambient temperature ( $16.0 \pm$   
136  $0.1^\circ\text{C}$ ), and of  $7.61 \pm 0.08$ ,  $7.84 \pm 0.04$ , and  $8.09 \pm 0.09$ , at elevated temperature ( $18.9 \pm$   
137  $0.3^\circ\text{C}$ ), corresponding to  $p\text{CO}_2$  of 1399, 781, and 404 ppm at ambient temperature and 1440,  
138 799, and 399 ppm at elevated temperature, respectively (Lacoue-Labarthe et al., 2009b). The  
139 eggs were exposed to dissolved radiotracer:  $0.27 \text{ kBq l}^{-1} {}^{241}\text{Am}$ ,  $0.95 \text{ kBq l}^{-1} {}^{60}\text{Co}$ ,  $1.18 \text{ kBq l}^{-1}$   
140  ${}^{134}\text{Cs}$  (Amersham, UK) and  $0.91 \text{ kBq l}^{-1} {}^{54}\text{Mn}$  and  $1.04 \text{ kBq l}^{-1} {}^{75}\text{Se}$  (Isotope Product  
141 Laboratory, USA). Briefly, seawater of each aquarium was spiked with typically  $5 \mu\text{l}$  of  
142 radioactive stock solution ( ${}^{241}\text{Am}$  dissolved in 1N HCl,  ${}^{60}\text{Co}$  in 0.1N HCl,  ${}^{134}\text{Cs}$  in  $\text{H}_2\text{O}$ ,  ${}^{54}\text{Mn}$   
143 in 0.1N HCl and  ${}^{75}\text{Se}$  in 0.1N HCl). Seawater and spikes were renewed daily during the first  
144 week and then every second day to maintain good water quality and radiotracer

145 concentrations as constant as possible. The detailed experimental procedure has been  
146 previously described in Lacoue-Labarthe et al. (2009b).

147 At hatching time (i.e., 62 d and 42 d at 16 and 19°C, respectively, when first hatching events  
148 occurred), in each treatment, 10 eggs were fresh weighed and dissected and the radiotracer  
149 activities were counted in the eggshell, the embryo and the perivitelline fluid (PVF). In  
150 parallel, 10 newly hatched cuttlefish in each bottle were weighted and counted.

151 Radioactivities were measured using a high-resolution  $\gamma$ -spectrometry system consisting of  
152 four coaxial Germanium (N- or P-type) detectors (EGNC 33-195-R, Canberra<sup>®</sup> and Eurysis<sup>®</sup>)  
153 connected to a multi-channel analyzer and a computer equipped with a spectra analysis  
154 software (Interwinner<sup>®</sup> 6). Radioactivities were determined by comparison with standards of  
155 known activity and of appropriate geometry. Measurements were corrected for counting  
156 efficiency and physical radioactive decay. Counting times were adapted to obtain relative  
157 propagated errors less than 5% (Metian et al., 2008).

158 Accumulation of radiotracers was expressed as concentration factors (CF), which is the ratio  
159 between radiotracer activity in the egg or egg compartment ( $\text{Bq g}^{-1}$ ) and the time-integrated  
160 activity in seawater ( $\text{Bq g}^{-1}$ ) (Metian et al., 2008). The distribution of metals was expressed a  
161 % of the total activity load. Results are expressed as mean  $\pm$  SD.

162



## 163 RESULTS

164

165 At hatching time, only  $^{60}\text{Co}$ ,  $^{134}\text{Cs}$  and  $^{54}\text{Mn}$  were accumulated with sufficient activities to be  
166 detected in the newly hatched juveniles, showing that  $^{241}\text{Am}$  and  $^{75}\text{Se}$  were not efficiently  
167 accumulated during the embryonic development. The lower the pH of seawater where eggs  
168 were incubating, the less  $^{60}\text{Co}$  and  $^{54}\text{Mn}$  were accumulated in the tissues of hatchlings (Fig. 1;  
169 Table 1). Moreover, this effect tended to be reduced when the temperature increases, i.e.  $^{60}\text{Co}$   
170 and  $^{54}\text{Mn}$  CF values were 1.5 and 1.1 fold lower at pH 7.60 than at normal pH, when eggs  
171 developed at 16 and 19°C, respectively. Finally, neither the temperature nor the pH has an  
172 effect on the  $^{134}\text{Cs}$  CF values in the hatchlings.

173

174 At the end of the embryonic development, radiotracer distribution was determined among the  
175 different eggs compartments (Table 2). Regardless the temperature and  $p\text{CO}_2$ , over 80% of  
176 both radionuclides and trace elements were found associated with the eggshell, with the  
177 exception of  $^{134}\text{Cs}$  that was found in variable proportions in the eggshell, the embryo and the  
178 PVF. More precisely, the fraction of Cs in PVF was higher at lower pH levels than at normal  
179 pH (KW test;  $p < 0.05$ ), whereas the one in the eggshell was lower at  $\text{pH}_T$  7.60 than at  $\text{pH}_T$   
180 8.10 (KW test;  $p < 0.05$ ). No effect of the temperature was noted on the Cs fraction in PVF  
181 (KW test;  $p > 0.05$ ). Concerning Se, this element was slightly detected in the PVF but not in  
182 the embryo.

183

## 184 DISCUSSION

185

186 Studying the impact of the ocean acidification on the metal accumulation on water-breathing  
187 animals must raise attention on two aspects. On the one hand, the speciation of some metals

188 and their bioavailability could be affected as  $^{241}\text{Am}$  that form strong complexes with  
189 hydroxide and carbonate ions (Choppin, 2006), both of which being expected to decrease in  
190 seawater (Feely, 2004). Other metals mainly found as free forms ( $\text{Co}^{2+}$ ,  $\text{Cs}^+$ ,  $\text{Mn}^{2+}$ ), or  
191 oxydized state ( $\text{SeO}_4^{2-}$ ) will be more available by only few percent with decreasing pH  
192 (Byrne, 2002). Moreover, the increase in the concentration of  $\text{H}^+$  could reduce by competition  
193 for the binding site the metal adsorption on the eggshell surface or epithelia (Millero et al.,  
194 2009). On the other hand, the acid-base balance and ionoregulation impairments (Pörtner,  
195 2008) caused by hypercapnia could modify the active transport or passive diffusion of metals  
196 through the epithelial membrane (Rainbow, 1997) and therefore the accumulation efficiencies  
197 in the animal tissues.

198 As noticed in previous studies for different elements (Bustamante et al., 2002, 2004, 2006a;  
199 Lacoue-Labarthe et al., 2008a, 2009a, 2010) the behaviour of trace elements towards  
200 cuttlefish egg strongly varies according to the considered elements. Among the three element  
201 found in the embryo, Co and Mn accumulation decreased with pH as previously observed  
202 with Cd (Lacoue-Labarthe et al., 2009b). It is likely that both these “Class Borderline”  
203 elements (Nieboer and Richardson, 1980) might be subjected to increased competition with  
204 protons for the adsorption on binding sites on embryonic tissues. Nevertheless, regarding the  
205 direct effect of temperature with similar CF at 19°C than at 16°C despite of a shorter exposure  
206 duration, we assume that the presumably increased metabolic level of the embryo could  
207 contribute to these essential element uptake efficiencies (Rainbow and White, 1990;  
208 Barceloux, 1999). The seawater hypercapnia would in this case enhance the metabolic  
209 depression of the late-stage embryo (Rosa and Seibel, 2008; Melzner et al., sbm) and limit  
210 active metal accumulation. Contrasting to Co and Mn, Se passed slightly through the eggshell  
211 but was not significantly accumulated by the embryo, suggesting that the need of this  
212 essential metal (Bell et al., 1986) was entirely supplied by the maternal transfer through Se

213 incorporation in the yolk reserve (Lacoue-Labarthe et al., 2008b). More globally, these results  
214 raise the question of the impact of a potential depletion in essential elements during the  
215 sensitive early-life stages of this marine invertebrate.

216 Regarding the radionuclide behaviours, the absence of  $^{241}\text{Am}$  neither in the embryo nor in the  
217 PVF confirms that the eggshell, retaining more than 98% of the Am amount, totally prevents  
218 against  $^{241}\text{Am}$  penetration (Bustamante et al., 2006a; Lacoue-Labarthe et al., 2010) and that  
219 pH and temperature variations did not affect these shielding properties although the  
220 proportion of bioavailable Am increase with decreasing pH (Choppin, 2006). Oppositely, the  
221 eggshell displayed an evident permeability toward  $^{134}\text{Cs}$  (Bustamante et al., 2006a) as CF  
222 values were  $> 10$  in the embryo. Interestingly, decreasing pH and increasing temperature did  
223 not affect Cs uptake in the embryo but enhanced the proportion associated with the PVF  
224 compartment (Table 2). This probably results from the enhanced egg swelling illustrated by  
225 higher egg weight under hypercapnic conditions and warm water (Lacoue-Labarthe et al.,  
226 2009b). Therefore the fraction of  $^{134}\text{Cs}$ , already described as a tracer of water movement  
227 during the cuttlefish egg development (Lacoue-Labarthe et al., 2010), increased with the peri-  
228 vitelline volume.

229 In conclusion, this study demonstrates that both temperature and pH have distinct effects on  
230 the bioaccumulation in cuttlefish embryos. Both the chemical properties of the elements and  
231 the physiological responses of the organism to ocean warming and acidification could account  
232 for the observed effects. The impacts of pollutants in the context of the global change, such as  
233 the potential depletion of essential elements in embryo, should to be further assessed in  
234 marine organisms that use coastal areas to complete their life cycle.

235

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243

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401 Table 1. *Sepia officinalis*. Two-way ANOVA parameters testing the effects of three pH (7.60, 7.85  
 402 and 8.10) and two temperatures (16 and 19°C) on the weight of the eggs (from Lacoue-Labarthe et al  
 403 [33]), and on the concentration factor (CF) of <sup>60</sup>Co, <sup>134</sup>Cs and <sup>54</sup>Mn in the hatchlings at the end of the  
 404 embryonic development (see Figures 1).

405

Parameter	pH			Temperature			pH X Temperature		
	<i>df</i>	MS	F	<i>df</i>	MS	F	<i>df</i>	MS	F
Egg weight	2	0.587	8.8 ***	1	1.486	22.2 ***	2	0.018	0.3 ns
<sup>60</sup> Co CF	2	20424	17.3 ***	1	454	0.7 ns	2	7465	6.3 **
<sup>134</sup> Cs CF	2	168	0.9 ns	1	345	1.8 ns	2	138	0.7 ns
<sup>54</sup> Mn CF	2	2152	17.3 ***	1	1316	10.6 **	2	2230	18.0 ***

406 *df* = degree of freedom; MS = mean squares. Probability levels for significant effects: p < 0.001 (\*\*\*), p < 0.01 (\*\*), p <  
 407 0.05 (\*), p < 0.1 (°); ns = non significant.

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409 Table 2. *Sepia officinalis*. Distribution  $^{241}\text{Am}$ ,  $^{60}\text{Co}$ ,  $^{134}\text{Cs}$ ,  $^{54}\text{Mn}$  and  $^{75}\text{Se}$  expressed as % among the  
 410 different egg compartments, at the end of development (after 62 d and 42 d of incubation at 16°C and  
 411 19°C, respectively) following three different pH levels at two different temperatures.  
 412

Experiment	16°C			19°C		
	7.6	7.85	8.1	7.6	7.85	8.1
(a) $^{241}\text{Am}$						
Eggshell	98.8 ± 0.2	98.8 ± 0.7	98.2 ± 1.2	96.9 ± 2.2	98.8 ± 0.8	98.3 ± 1.0
Embryo	< 1	< 1	< 1	< 1	< 1	< 1
PVF	< 1	< 1	< 1	< 1	< 1	< 1
(b) $^{60}\text{Co}$						
Eggshell	88.4 ± 0.9	87.2 ± 2.1	90.5 ± 2.6	83.1 ± 1.8	89.7 ± 1.0	91.4 ± 0.4
Embryo	10.2 ± 0.8	11.5 ± 2.1	8.5 ± 2.6	15.0 ± 1.3	9.0 ± 0.9	8.4 ± 0.5
PVF	1.4 ± 0.1	1.3 ± 0.2	0.9 ± 0.2	1.9 ± 0.5	1.3 ± 0.4	0.2 ± 0.1
(c) $^{134}\text{Cs}$						
Eggshell	27.8 ± 11.7	33.0 ± 8.0	52.7 ± 17.6	19.2 ± 3.1	24.9 ± 14.1	48.6 ± 19.5
Embryo	30.6 ± 14.9	34.0 ± 7.1	30.3 ± 14.6	39.4 ± 5.0	36.0 ± 14.3	42.5 ± 18.1
PVF	41.6 ± 8.9	33.0 ± 1.7	17.0 ± 4.9	41.3 ± 5.7	39.1 ± 6.1	8.9 ± 2.1
(d) $^{54}\text{Mn}$						
Eggshell	88.9 ± 1.3	88.8 ± 0.3	88.0 ± 2.6	84.8 ± 2.1	90.2 ± 1.7	83.4 ± 1.9
Embryo	8.7 ± 0.8	8.9 ± 0.5	10.0 ± 3.0	12.8 ± 2.0	8.1 ± 1.3	15.5 ± 1.7
PVF	2.4 ± 1.0	2.3 ± 0.4	2.0 ± 0.7	2.4 ± 0.5	1.7 ± 0.4	1.1 ± 0.4
(e) $^{75}\text{Se}$						
Eggshell	97.2 ± 0.4	97.5 ± 0.4	98.3 ± 0.3	95.0 ± 1.3	96.1 ± 0.4	98.0 ± 0.6
Embryo	< 1	< 1	< 1	< 1	< 1	< 1
PVF	2.0 ± 0.2	2.0 ± 0.5	1.1 ± 0.3	3.6 ± 0.8	2.7 ± 0.3	1.0 ± 0.4

413 PVF: perivitelline fluid. Letters denote statistically significant differences (Kruskall-Wallis test;  $p <$   
 414 0.05) between pHs for each temperature.  
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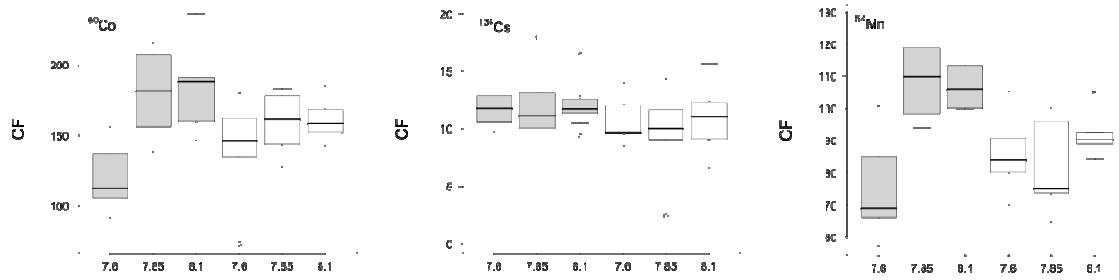


Figure 1. *Sepia officinalis*. Concentration factors of  $^{60}\text{Co}$ ,  $^{134}\text{Cs}$  and  $^{54}\text{Mn}$  (CF; n = 10), in the newly hatched juvenile exposed at three different pH at 16°C (grey) and 19°C (white) during the whole development time, *i.e.* 62 d and 42 d, respectively.  $^{241}\text{Am}$  and  $^{75}\text{Se}$  activities levels were under detection limits. Results of the statistical analysis were reported on the Table 1.