

1 Experimental shift of diet and DIC stable carbon isotopes: influence  
2 on shell  $\delta^{13}\text{C}$  values in the Manila clam *Ruditapes philippinarum*  
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## 26 Abstract

27 The influences of diet and seawater dissolved inorganic carbon (DIC) on the carbon isotope  
28 composition of shell aragonite ( $\delta^{13}\text{C}_{\text{shell}}$ ) in the Manila clam *Ruditapes philippinarum* reared  
29 under laboratory conditions were investigated. Clams were exposed to two successive  
30 negative carbon isotope shifts: a first shift in diet ( $\delta^{13}\text{C}_{\text{phytoplankton}}$ ) and a second shift, 35 days  
31 later, in DIC ( $\delta^{13}\text{C}_{\text{DIC}}$ ). Both successive shifts induced a decrease in  $\delta^{13}\text{C}_{\text{shell}}$ . These results are  
32 the first to experimentally confirm an incorporation of respired carbon derived from food and  
33 carbon from DIC into shell carbonate of adult bivalves. Skeletal  $\delta^{13}\text{C}$  responded to changes in  
34 the  $\delta^{13}\text{C}$  of both diet and DIC in less than 7 days. Consequently, proxies based on  $\delta^{13}\text{C}_{\text{shell}}$   
35 may be used with high temporal resolution. Using  $\delta^{13}\text{C}_{\text{phytoplankton}}$  as a proxy for the carbon  
36 isotope composition of respired carbon ( $\delta^{13}\text{C}_{\text{R}}$ ) resulted in a rather constant percentage of  
37 metabolic carbon ( $C_{\text{M}}$ ) into the shell carbonate over time (close to 12 %). However, an  
38 accurate estimation of  $\delta^{13}\text{C}_{\text{R}}$  is required in order to precisely estimate the percentage of  
39 metabolic carbon incorporated into the shell. Despite the significant incorporation of  
40 metabolic carbon into shell carbonate, our experimental results revealed that  $\delta^{13}\text{C}_{\text{shell}}$  was  
41 highly correlated with  $\delta^{13}\text{C}_{\text{DIC}}$  ( $r^2 = 0.77$ ,  $p < 0.0001$ ). Thus it seems that  $\delta^{13}\text{C}_{\text{shell}}$  is a  
42 promising proxy of large scale variations in  $\delta^{13}\text{C}_{\text{DIC}}$  and therefore of salinity in estuarine  
43 water.

44  
45

46 Keywords:  $\delta^{13}\text{C}_{\text{shell}}$ , DIC, salinity, metabolic carbon, *Ruditapes philippinarum*, estuarine  
47 water, proxy

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49

## 51 1 Introduction

52 Bivalves record environmental information in the form of chemical or structural messages  
53 archived in their calcareous exoskeleton during growth. For this reason, they are widely used  
54 for paleo-climatic and paleo-oceanographic reconstructions (e.g. Davenport, 1938, Weidman  
55 et al., 1994, Tripathi et al., 2001, Dutton et al., 2002, Schöne et al., 2003, Lazareth et al.,  
56 2006, Ivany et al., 2008, Goewert and Surge, 2008, Wanamaker et al., 2008). Epstein et al.  
57 (1953) showed that oxygen isotope composition of mollusk shells ( $\delta^{18}\text{O}_{\text{shell}}$ ) reflect the  
58 temperature and oxygen isotope composition of the water ( $\delta^{18}\text{O}_{\text{w}}$ ) in which they were formed.  
59 This allows the use of shell carbonate  $\delta^{18}\text{O}$  as a temperature proxy at least in cases where  
60  $\delta^{18}\text{O}_{\text{w}}$  remains constant and is known (e.g. Weidman et al., 1994, Chauvaud et al., 2005,  
61 Andrus and Rich, 2008). However,  $\delta^{18}\text{O}_{\text{w}}$  is often strongly dependant on salinity (Epstein and  
62 Mayeda, 1953, Craig and Gordon, 1965, Mook and Tan, 1991, Dettman et al., 2004).  
63 Therefore, the estimation of  $\delta^{18}\text{O}_{\text{w}}$  is difficult without an independent salinity proxy in  
64 environments displaying large salinity variations such as estuaries. In well flushed estuaries  
65 with short residence times, stable carbon isotope composition of dissolved inorganic carbon  
66 ( $\delta^{13}\text{C}_{\text{DIC}}$ ) is well correlated with salinity year round, especially when salinity is higher than 25  
67 (Mook, 1971, Surge et al., 2001, Fry, 2002, Gillikin et al., 2006). As carbon precipitating  
68 during shell mineralization is known to originate mainly from dissolved inorganic carbon  
69 (DIC) of seawater (Mook and Vogel, 1968, McConnaughey et al., 1997, Gillikin et al., 2006,  
70 McConnaughey and Gillikin, 2008, Owen et al., 2008), the carbon isotope composition of  
71 bivalve shells ( $\delta^{13}\text{C}_{\text{shell}}$ ) living in estuaries may possibly be used for reconstructing past  
72 variations of salinity. A paleo-salinity proxy would also be useful for correcting paleo-  
73 temperature based on  $\delta^{18}\text{O}$  of estuarine bivalve shells (see Gillikin et al., 2006).  
74 The carbon isotope composition of bivalve shell carbonates is, however, not only affected by  
75  $\delta^{13}\text{C}_{\text{DIC}}$ , but also by the physiology of the bivalve (Dillaman and Ford, 1982, Tanaka et al.,  
76 1986, McConnaughey et al., 1997, Lorrain et al., 2004, Gillikin et al., 2006, 2007, 2009,  
77 McConnaughey and Gillikin, 2008). Previous studies have shown that isotopically light  
78 metabolic carbon, derived from food, is incorporated into shell carbonate (e.g. Tanaka et al.,  
79 1986, McConnaughey et al., 1997, Wanamaker et al., 2007, Owen et al., 2008, Gillikin et al.,  
80 2006, 2007, 2009). However, the processes of metabolic carbon incorporation into the shell  
81 remain poorly known and the estimation of metabolic carbon contribution to the shell appears  
82 highly variable between studies. McConnaughey et al. (1997), Lorrain et al. (2004) and

83 Gillikin et al. (2006) estimated the percentage of metabolic carbon integrated into the shell to  
84 be lower than 10 % in deep-sea mollusks, *Pecten maximus* and *Mytilus edulis*, whereas  
85 Gillikin et al. (2007, 2009) found values between 25 and 40 % in marine *Mercenaria*  
86 *mercenaria* and freshwater Unionid shells, respectively. Consequently, development of  
87 environmental proxies based on  $\delta^{13}\text{C}_{\text{shell}}$  requires a better understanding of the processes  
88 involved in the incorporation of metabolic carbon into the bivalve shell.

89 In the present study we performed a laboratory experiment using the Manila clam, *Ruditapes*  
90 *philippinarum* (Adams and Reeve, 1850). This bivalve species, which has an aragonitic shell,  
91 was chosen for several reasons. First, it is an euryhaline bivalve living mainly at salinity  
92 levels ranging from 16 to 36 (Nie, 1991), buried a few centimeters in sandy and muddy  
93 sediments in intertidal to subtidal zones. Because of its importance for aquaculture and  
94 fisheries this species is well studied regarding its biology and physiology (e.g. Gouletquer et  
95 al., 1989, Kim et al., 2001, Richardson, 1987, Marin et al., 2003, Flye-Sainte-Marie et al.,  
96 2007). Moreover, clams, and particularly *Ruditapes* genus, are frequently found in shell-  
97 middens, and are therefore suitable candidates as archives of past coastal human settlements  
98 (Dupont and Marchand, 2008).

99 During this laboratory experiment, clams were reared under controlled values of  
100  $\delta^{13}\text{C}_{\text{phytoplankton}}$  which were more depleted than those encountered in the field. Moreover, after  
101 35 days of experiment, three salinity conditions and therefore three  $\delta^{13}\text{C}_{\text{DIC}}$  were tested. The  
102 aims of this study are (1) to demonstrate the incorporation of the two carbon sources (DIC  
103 from surrounding seawater and DIC originating from respiration) into adult bivalve shells,  
104 which, to our knowledge, has never been demonstrated experimentally, (2) to study the  
105 response time of these carbon incorporations into the shell, and (3) to investigate the potential  
106 use of  $\delta^{13}\text{C}_{\text{shell}}$  as a proxy of high  $\delta^{13}\text{C}_{\text{DIC}}$  variations and therefore of salinity in estuarine  
107 water. In parallel, this experiment was designed to assess the effect of salinity variations on  
108 clam physiology (i.e. condition index, shell growth rate and incorporation of carbon into soft  
109 tissues) which has potential impacts on shell mineralization processes.

110

## 111 **2 Material and methods**

### 112 **2.1 Biological material**

113 A total of 250 Manila clams (*R. philippinarum*; two to three-years old; average length 27 mm;  
114  $1\sigma = 2\text{mm}$ ) were collected by hand at low tide in an estuary located in the Gulf of Morbihan

115 (Bay of Kerdréan, 47°37'N, 2°56'W; Brittany; France; semidiurnal tidal regime) on the 18<sup>th</sup>  
116 of August 2008. Clams were transferred to French Research Institute for Exploitation of the  
117 Sea (IFREMER) Argenton Shellfish Laboratory (North Finistère, France).

118

## 119 **2.2 Experimental conditions**

120 Clams were randomly divided into three batches of 80 individuals and each batch was placed  
121 into a 25 L tank without sediment. Water within the tanks was homogenized with an aquarium  
122 pump. Each tank was supplied with UV sterilized water from a 300 L buffer tank allowing  
123 renewal rate of 25% h<sup>-1</sup> and complete water changes were made once a week. The 300 L  
124 buffer tanks were filled every 2 to 3 days with 1 µm filtered, UV sterilized water at room  
125 temperature. The experimental system is illustrated in Fig. 1.

126 Water flowing into the 25 L tanks was supplemented continuously during the entire  
127 experiment with cultured microalgae (50% *Isochrysis affinis galbana* (Tahitian strain *T.iso*)  
128 and 50% *Chaetoceros calcitrans*) depleted in <sup>13</sup>C ( $\delta^{13}\text{C} = -58\text{‰}$ ;  $1\sigma = 4\text{‰}$ ). <sup>13</sup>C depleted  
129 microalgae were obtained by bubbling <sup>13</sup>C-depleted industrial CO<sub>2</sub> into the culture medium  
130 (see Paulet et al., 2006 for elaboration). Algae were cultured at a salinity of 35. The  
131 experiment was performed over 64 days, during which temperature (20°C) and photoperiod  
132 (12/12) were kept constant.

133

134 During the first 35 days, salinity of the three tanks was maintained constant at 35. From days  
135 35 to 64, one tank was kept at 35, while salinity was modified to 28 and 20 in the other two  
136 tanks. Salinities of 20 and 28 were obtained by mixing seawater and tap water within the 300  
137 L buffer tanks, and salinity was checked and adjusted using a conductivity meter (WTW, LF  
138 197-S). Salinity was recorded every five minutes in the three tanks using an autonomous data  
139 logger (YSI-600 OMS) from day 7 (t<sub>7</sub>) to the end of the experiment (t<sub>64</sub>).

140

## 141 **2.3 Sampling**

### 142 **2.3.1 Biological sampling**

#### 143 **Soft tissue sampling**

144 During the first 35 days, while salinity was similar in all three tanks, two clams per tank were  
145 collected each week. After the salinity change, five clams were randomly collected from each  
146 tank weekly. Adductor and posterior muscle, digestive gland (without purging the digestive

147 tract) and mantle (without siphon) were dissected from each individual and were frozen at -  
148 20°C. In order to determine initial isotope composition of tissues at the beginning of the  
149 experiment ( $t_0$ ), five clams from the field were processed in the same way. In addition, five  
150 clams per tank were collected at  $t_{35}$  and  $t_{64}$ , and total soft tissues from each individual were  
151 frozen at -20°C. At the end of the experiment, tissues were freeze-dried (48h) and shells were  
152 air-dried. Tissues and shell were weighed in order to estimate the condition index (CI),  
153 following Lucas and Beninger, (1985) :

$$154 \text{ CI} = (\text{Soft Tissues Dry Weight} / \text{Shell Dry Weight}) \times 100.$$

155

### 156 **Calcein marking and shell sampling**

157 Two calcein markings were used to establish a temporal scale in the shells. Clams in each  
158 salinity treatment were exposed to a 150 mg L<sup>-1</sup> calcein solution during 4 hours (Rowley and  
159 Mackinnon, 1995, Thébault et al., 2005) at days 15 ( $t_{15}$ ) and 35 ( $t_{35}$ ). At the end of the  
160 experiment ( $t_{64}$ ), 30 clams from each salinity were sacrificed and their shells were used for  
161 growth rate determination and calcium carbonate sampling.

162

### 163 **2.3.2 Food and DIC sampling**

164 In order to determine the carbon isotope composition of the diet during the experiment, 15 mL  
165 of the algal mix was filtered on a precombusted Whatman GF/F filter every week. Following  
166 Lorrain et al. (2003), filters were immediately dried for 12h at 60°C and stored until analysis.  
167 When the clams were collected, water was sampled in the field and 200 mL was filtered on a  
168 precombusted Whatman GF/F filter to determine the carbon isotope composition of  
169 particulate organic matter ( $\delta^{13}\text{C}_{\text{POM}}$ ) using the method described above. The suspended POM  
170 pool is a mixture of different sources of carbon (e.g. phytoplankton, microphytobenthos,  
171 resuspended sediment, terrestrial carbon, marine micro-algae detritus). However,  $\delta^{13}\text{C}_{\text{POM}}$   
172 may be used as a proxy for  $\delta^{13}\text{C}_{\text{phytoplankton}}$  (see Gillikin et al., 2006). Following Gillikin and  
173 Bouillon (2007), water from the field and water within the 25 L clam tanks were sampled at  $t_0$   
174 and weekly respectively using 12 mL glass vials and poisoned with 20  $\mu\text{L}$  of saturated  
175 mercuric chloride ( $\text{HgCl}_2$ ) solution until analysis of the DIC isotope composition.

176

## 177 **2.4 Shell preparation and calcium carbonate sampling**

178 For the purposes of calcium carbonate sampling, 30 shells were collected from each tank at  
179 day 64 (end of experiment). A 8×10 mm piece of the shell, including the new shell material

180 formed during the experiment, was cut from the shell edge along the major growth axis and  
181 embedded in epoxy resin (Araldite 2020, DIL, France). A cross-section (800  $\mu\text{m}$  thick) was  
182 then cut using a low speed diamond saw and glued on a glass slide with epoxy resin. Thick  
183 sections were then ground with wet sandpaper (1200, 2400 and 4000  $\mu\text{m}$  grit size) and  
184 polished with a suspended diamond solution (3  $\mu\text{m}$ ). Shell sections were observed under a  
185 fluorescence microscope (OLYMPUS BX41) equipped with a 50 W high-pressure Hg lamp  
186 and a calcein filter. Photographs were acquired using a Hamamatsu C4742-95 digital camera  
187 fitted on the microscope. The *R. philippinarum* shell consists of two aragonitic layers, an  
188 inner homogeneous layer and an outer prismatic layer. Four evident marks in the outer layer  
189 allowed assigning calendar dates on the shell sections: a cleft presumably due to the shell  
190 growth stop induced by clam's collection in the field, the two calcein marks and the ventral  
191 margin which sets the date the experiment was ended. For the 30 clams per tank collected on  
192 the final day, growth between the four growth checks was measured on the shell section  
193 photograph using the software Visilog 6.6. Five of the 30 shells (per batch) were selected  
194 based on age (two-year old clams were selected to avoid any effect of ontogeny) and on high  
195 shell growth rate to obtain sufficient material for carbon isotopic analysis. Since the tidal  
196 periodicity of shell growth increment formation at intertidal flats is not lost in *R.*  
197 *philippinarum* even after several months in constant laboratory conditions without emersion  
198 (Richardson, 1987), it was possible to assign a calendar date to each growth increment (with  
199 a precision of 3 days due to the behavioral disturbance induced by handling). Calcium  
200 carbonates samples (average weight = 70  $\mu\text{g}$ ) were drilled to a depth of 350  $\mu\text{m}$  in the middle  
201 of the prismatic layer using a MicroMill (Merchantek) fitted with a 300  $\mu\text{m}$  diameter drill bit.  
202 One carbonate sample was drilled just before the cleft induced by clam collection in order to  
203 measure the field isotopic value ( $t_0$ ), one sample was drilled between the cleft and the first  
204 calcein mark, two samples were drilled between the first and the second calcein mark and  
205 three samples were drilled after the second calcein mark (see Fig. 2). Aragonite powder  
206 samples were stored in glass vials until analysis.

207

## 208 **2.5 Isotopic analyses**

209 Freeze-dried tissues were ground to a homogeneous fine powder. 500  $\mu\text{g}$  of the powder was  
210 packed into 5 $\times$ 9 mm precombusted silver cups. Carbon stable isotope analysis was performed  
211 at the Earth System Sciences laboratory (Vrije Universiteit Brussel) using an Elemental  
212 Analyzer (Flash 1112 EA Thermo Finnigan) coupled via a CONFLO III to a Thermo Delta V

213 Plus isotope ratio mass spectrometer (IRMS). A total of 42 samples of the international  
214 isotopic reference standard IAEA-CH-6 ( $-10.45 \pm 0.030$  ‰) were analyzed with the samples  
215 and yielded a reproducibility ( $1 \sigma$ ) of 0.36 ‰ for  $\delta^{13}\text{C}$ .

216 Filters were decarbonated via a 4-h exposure to HCl fumes in a dessicator (Lorrain et al.,  
217 2003) and were packed into 5×9 mm tin cups. Isotopic analysis was performed as described  
218 for the tissues.

219 The  $\delta^{13}\text{C}$  of the total DIC was analyzed according to the protocol of Gillikin and Bouillon,  
220 (2007). Briefly, 9.5 mL of water sample were acidified with 200  $\mu\text{L}$  of pure orthophosphoric  
221 acid in a 12 mL helium-flushed headspace vial, followed by overnight equilibration and  
222 injection of 500  $\mu\text{L}$  headspace into the carrier gas stream of the continuous flow EA-IRMS  
223 (same instrument used for tissues and diet). The Miyajima et al. (1995) algorithm was used to  
224 correct for the partitioning of  $\text{CO}_2$  between headspace and water phase and to calculate the  
225  $\delta^{13}\text{C}_{\text{DIC}}$ . Along with the headspace injections a  $\text{CO}_2$  house standard was injected ( $n = 28$ ,  $1 \sigma$   
226  $= 0.2$  ‰). Based on this and an average reproducibility of DIC sample measurements of 0.11  
227 ‰, a precision of the  $\delta^{13}\text{C}_{\text{DIC}}$  better than 0.2 ‰ ( $1 \sigma$ ) can be expected (also see Gillikin and  
228 Bouillon, 2007).

229 All carbonate isotopic analyses were performed on a Finningan MAT 252 IRMS equipped  
230 with a Kiel III automated sampling device at the University of Arizona, USA. Samples were  
231 reacted with  $> 100$  % orthophosphoric acid at  $70^\circ\text{C}$ . The standard deviation of replicate  
232 carbonate standards was 0.08‰ ( $1 \sigma$ ). All carbon isotopic results are reported relative to  
233 VPDB (Vienna Pee Dee Belemnite) by calibration to the NBS-19 reference standard ( $\delta^{13}\text{C} =$   
234  $+1.95$  ‰).

235

## 236 **2.6 Data analysis**

237 All statistical analyses were performed using the software Statgraphics. Homoscedasticity was  
238 tested using Bartlett's test ( $\alpha=0.05$ ). ANOVAs were performed to check: (1) the differences in  
239 mean shell growth of clams for the three salinity conditions between  $t_{35}$  and  $t_{64}$ , and (2) the  
240 differences in carbon isotope composition between the different salinity conditions for each  
241 organ at each sampling date. The differences in condition index between the three salinity  
242 conditions were tested by performing non-parametric Kruskal-Wallis test.

243

## 244 **3 Results**

### 245 **3.1 Animal growth and condition during the experiment**

246 All clams exhibited significant growth during the experiment with shell growth between the  
 247 1<sup>st</sup> calcein marking ( $t_{15}$ ) and the shell edge ( $t_{64}$ ) of 1986  $\mu\text{m}$  ( $1\sigma = 1037 \mu\text{m}$ ;  $n = 90$ ; see Fig.  
 248 2). The two bright calcein marks and the cleft induced by clam collection from the field were  
 249 readily identifiable in all shells. The three salinity treatments tested between day 35 (2<sup>nd</sup>  
 250 calcein marking) and the end of the experiment (shell edge) induced no significant differences  
 251 on shell growth (ANOVA,  $p = 0.32$ ) with a mean shell growth of 1402  $\mu\text{m}$  ( $1\sigma = 752 \mu\text{m}$ ;  $n =$   
 252 30), 1368  $\mu\text{m}$  ( $1\sigma = 598 \mu\text{m}$ ;  $n = 30$ ) and 1135  $\mu\text{m}$  ( $1\sigma = 663 \mu\text{m}$ ;  $n = 30$ ) at salinities 35, 28  
 253 and 20, respectively.

254 The condition index was almost constant during the entire experiment (average value = 8.80;  
 255  $1\sigma = 1.85$ ). Between days 35 and 64, at each sampling date, there were no significant  
 256 differences in average values of condition index between the three salinities conditions  
 257 (Kruskal-Wallis,  $p > 0.05$ ).

### 259 **3.2 Salinity and $\delta^{13}\text{C}_{\text{DIC}}$**

260 During the first 35 days, salinity of the three tanks was 35.42 ( $1\sigma = 0.21$ ), 35.77 ( $1\sigma = 0.11$ )  
 261 and 35.36 ( $1\sigma = 0.10$ ) respectively. From days 35 to 64, the first tank was maintained at 35.28  
 262 ( $1\sigma = 0.16$ ), while salinity was changed to 28.02 ( $1\sigma = 0.14$ ) and 20.76 ( $1\sigma = 0.13$ ) in the two  
 263 other tanks.

264 The carbon isotopic DIC values in the experimental tanks ranged between -1.28 ‰ and -3.34  
 265 ‰ at salinity 35, between -4.97 ‰ and -5.90 ‰ at salinity 28 and between -8.33 ‰ and -9.52  
 266 ‰ at salinity 20 (Fig. 3A). There was a high positive correlation between  $\delta^{13}\text{C}_{\text{DIC}}$  and salinity  
 267 ( $\delta^{13}\text{C}_{\text{DIC}} = 0.424 \times \text{salinity} - 17.04$ ,  $r^2 = 0.95$ ,  $p < 0.0001$ ,  $n = 16$ ) as shown in Fig. 3B.

### 269 **3.3 $\delta^{13}\text{C}_{\text{tissues}}$**

270 From days 35 to 64, there was no significant effect of salinity on  $\delta^{13}\text{C}$  values for digestive  
 271 gland and muscle at each sample date (ANOVA,  $p > 0.05$ ). Therefore, values were pooled per  
 272 date as shown in Fig. 4. The  $\delta^{13}\text{C}$  trend was similar for the different tissues (muscle, digestive  
 273 gland, mantle and total soft tissue) with a decrease over time. However,  $\delta^{13}\text{C}_{\text{muscle}}$  decreased  
 274 from -14.7 to -32.6 ‰ in 64 days whereas  $\delta^{13}\text{C}_{\text{digestive gland}}$  decreased from -17.5 to -38.5 ‰ in

275 only 7 days. Mantle  $\delta^{13}\text{C}$  values were not significantly different than the muscle  $\delta^{13}\text{C}$  value  
276 (ANOVA,  $p > 0.05$ ) at  $t_{35}$ . Total soft tissues  $\delta^{13}\text{C}$  values were between muscle and digestive  
277 gland  $\delta^{13}\text{C}$  values.  
278

### 279 **3.4 $\delta^{13}\text{C}_{\text{shell}}$**

280 Shell carbonate sampled before the cleft in the shell, which represents three to four days of  
281 growth in the field just before collection, had mean  $\delta^{13}\text{C}_{\text{shell}}$  values equal to  $-0.83\text{‰}$  ( $1\sigma =$   
282  $0.26\text{‰}$ ) as shown in Fig. 5. At day 7, shell material formed in experimental tanks showed a  
283 decreased in  $\delta^{13}\text{C}_{\text{shell}}$  values in comparison to the field, with a mean value of  $-6.48\text{‰}$  ( $1\sigma =$   
284  $0.80\text{‰}$ ; i.e., a  $5.65\text{‰}$  decrease on average). After the salinity changes, mean  $\delta^{13}\text{C}_{\text{shell}}$  values  
285 ranged from  $-5.03$  to  $-6.64\text{‰}$  for clams at salinity 35, from  $-8.35$  to  $-9.82\text{‰}$  for clams at  
286 salinity 28 and from  $-10.39$  to  $-12.11\text{‰}$  for clams at salinity 20 (Fig. 5). These shell  $\delta^{13}\text{C}$   
287 values were significantly different between the three salinities at  $t_{40}$ ,  $t_{52}$  and  $t_{62}$  (ANOVA,  $p <$   
288  $0.0001$ ). There was a significant positive linear relationship between  $\delta^{13}\text{C}_{\text{shell}}$  and  $\delta^{13}\text{C}_{\text{DIC}}$  ( $r^2 =$   
289  $0.77$ ,  $p < 0.0001$ ,  $n = 83$ ) as shown in Fig. 6.  
290

## 291 **4 Discussion**

292 At the beginning of the experiment, clams were transferred from the field where the  $\delta^{13}\text{C}_{\text{POM}}$   
293 value was  $-18\text{‰}$  to laboratory conditions where  $\delta^{13}\text{C}_{\text{phytoplankton}}$  was  $-58\text{‰}$ . This shift in  $\delta^{13}\text{C}$   
294 of the clams diet led to a decrease in  $\delta^{13}\text{C}$  in all tissues analyzed (muscle, digestive gland,  
295 mantle and total soft tissues). This indicates a successful and quick incorporation of the  
296 isotopically light dietary carbon from phytoplankton into clam tissues. In agreement with the  
297 observation of Paulet et al. (2006) on *Pecten maximus* and *Crassostrea gigas* and Malet et al.  
298 (2007) on *Crassostrea gigas*, our results indicate that the incorporation of dietary carbon is  
299 faster in the digestive gland than in the muscle. This illustrates that the tissue turnover rate is  
300 higher in the digestive gland than in the muscle.

301 In our study, there was no significant effect of salinity on the clams physiology as indicated  
302 by the incorporation of carbon into the tissues, the condition index and shell growth rates.  
303 These observations are in agreement with the results of Kim et al. (2001), who showed that  
304 reasonable changes in salinity (35 to 20) have a small effect on activity rhythm and oxygen  
305 consumption. Kim et al. (2001) report that Manila clams cannot maintain a normal metabolic

306 activity at salinities lower than 15 and all clams died after an exposure at a salinity of 5 for 7  
307 days.

308  
309 Our results showed that the transfer from field to experimental conditions was followed by a  
310 decrease from -0.83 to -6.48 ‰ in  $\delta^{13}\text{C}_{\text{shell}}$  in just 7 days (Fig. 5). Since the difference  
311 between field and laboratory  $\delta^{13}\text{C}_{\text{DIC}}$  was less than 1 ‰, the change in  $\delta^{13}\text{C}_{\text{shell}}$  was most likely  
312 due to the diet  $\delta^{13}\text{C}$  shift. This strongly suggests an incorporation of carbon derived from food  
313 into the shell. After 35 days, three salinity conditions were tested (20, 28 and 35). As  
314 expected, shifts in salinity resulted in shifts in  $\delta^{13}\text{C}_{\text{DIC}}$  (as we mixed waters with different  
315  $\delta^{13}\text{C}_{\text{DIC}}$  values), which was followed by significant changes of  $\delta^{13}\text{C}_{\text{shell}}$  values. These results  
316 experimentally confirmed an integration into the shell of both carbon from seawater DIC and  
317 metabolic DIC derived from food as suggested by number of authors (e.g. Tanaka et al., 1986,  
318 McConnaughey et al., 1997, Lorrain et al., 2004, Wanamaker et al., 2007, McConnaughey  
319 and Gillikin, 2008, Owen et al., 2008, Gillikin et al., 2006, 2007, 2009).

320  
321 For both successive shifts ( $\delta^{13}\text{C}_{\text{phytoplankton}}$  shift at  $t_0$  and  $\delta^{13}\text{C}_{\text{DIC}}$  shift at  $t_{35}$ ), the incorporation  
322 of carbon into the shell was very rapid: the first carbonate samples following both switches,  
323 which correspond to an average of nearly 7 days, had significantly different  $\delta^{13}\text{C}_{\text{shell}}$  than  
324 carbonate samples before the shifts. Because seawater DIC is presumed to be supplied in  
325 extrapallial fluids directly from the surrounding seawater through mantle epithelium and  
326 periostracum (see McConnaughey and Gillikin, 2008), a short incorporation time is expected.  
327 Interestingly the incorporation of carbon from food was also very rapid, which is not  
328 necessarily expected because assimilation and metabolic processes may occur at a longer time  
329 scale. This result suggests that during this experiment most respired C used in shell  
330 construction is derived from the direct oxidation of food and is not from stored C sources.

331  
332 During this experiment,  $\delta^{13}\text{C}_{\text{DIC}}$  was not as constant as expected over time for the three  
333 salinities (Fig. 3A). As salinity was constant during this experiment (except the voluntary shift  
334 at  $t_{35}$ ),  $\delta^{13}\text{C}_{\text{DIC}}$  fluctuations are not explained by salinity variations. Two other hypotheses may  
335 be considered. (1) Clam respiration and calcification will modify the  $\text{CO}_2$  fluxes and then  
336  $\delta^{13}\text{C}_{\text{DIC}}$ , however, aquaria water was continuously renewed at a rate of  $25\% \text{ h}^{-1}$  (see Materials  
337 and methods), which would greatly reduce or even eliminate this effect on  $\delta^{13}\text{C}_{\text{DIC}}$ .  
338 Nevertheless, future studies should consider this and analyze pH, total DIC,  $\text{pCO}_2$  and even

339 O<sub>2</sub> levels as all these parameters have been proposed to influence shell  $\delta^{13}\text{C}$  (see  
 340 McConnaughey and Gillikin, 2008 for a review); (2) Variable quantities of food supplied to  
 341 the clams is a more probable cause of these  $\delta^{13}\text{C}_{\text{DIC}}$  fluctuations. In order to obtain the  
 342 appropriate phytoplankton concentration within the clam tanks, the flow of algae culture  
 343 supplied was adjusted daily as a function of algae culture concentration and the number of  
 344 clams in the tank. The  $\delta^{13}\text{C}_{\text{DIC}}$  value in this algae culture was -22 ‰ during this experiment.  
 345 Therefore, when variable quantities of algae solutions are supplied to the clams,  
 346 corresponding variable  $^{13}\text{C}$ -depleted DIC in the culture medium might induce differential  
 347  $\delta^{13}\text{C}_{\text{DIC}}$  values within the clam tanks. Despite the relative instability of  $\delta^{13}\text{C}_{\text{DIC}}$ ,  $\delta^{13}\text{C}_{\text{shell}}$   
 348 followed the same variations as  $\delta^{13}\text{C}_{\text{DIC}}$ . There was a positive linear relationship between  
 349  $\delta^{13}\text{C}_{\text{DIC}}$  and  $\delta^{13}\text{C}_{\text{shell}}$  during the experiment with a high correlation coefficient value ( $r^2 = 0.77$ ,  
 350  $p < 0.0001$ ,  $n = 83$ , Fig. 6). It seems that, in this species,  $\delta^{13}\text{C}_{\text{shell}}$  may be a promising proxy of  
 351 large scale variations in  $\delta^{13}\text{C}_{\text{DIC}}$  and therefore of salinity in estuarine water.

352  
 353 In order to estimate the percentage of metabolic carbon in the shell carbonate ( $C_M$ ), previous  
 354 studies used the classical two end-member mixing equation proposed by McConnaughey et al.  
 355 (1997):  $C_M = 100 \times (\delta^{13}\text{C}_{\text{shell}} - \epsilon_{\text{ar-b}} - \delta^{13}\text{C}_{\text{DIC}}) / (\delta^{13}\text{C}_R - \delta^{13}\text{C}_{\text{DIC}})$  where  $\epsilon_{\text{ar-b}}$  is the enrichment  
 356 factor between bicarbonate and aragonite (+ 2.7 ‰; Romanek et al., 1992) and  $\delta^{13}\text{C}_R$  is the  
 357 carbon isotope composition of respired carbon which has never been measured in bivalves.  
 358 According to Tanaka et al. (1986) and McConnaughey et al. (1997),  $\delta^{13}\text{C}_{\text{tissues}}$  values provide  
 359 a good approximation of  $\delta^{13}\text{C}_R$ . Nevertheless, there is no consensus on the tissue used to  
 360 estimate  $\delta^{13}\text{C}_R$ ; consequently we performed this estimation with all potential  $\delta^{13}\text{C}_R$  values  
 361 ( $\delta^{13}\text{C}$  of muscle, digestive gland, mantle, total soft tissues and cultured phytoplankton). There  
 362 was no significant difference between  $C_M$  values calculated for the three salinity conditions  
 363 with all potential  $\delta^{13}\text{C}_R$  values and at any sampling date (Kruskal-Wallis,  $p > 0.05$ ). The  
 364 similarity between  $C_M$  values at the three salinities seems to reject a strong impact of carbonic  
 365 anhydrase (CA) activity on shell  $\delta^{13}\text{C}$ . Low salinity could result in decreased carbonic  
 366 anhydrase (CA) activity in the animal, as has been described for some bivalves (Henry and  
 367 Saintsing, 1983). In addition, CA activity has also been shown to be inhibited by  $\text{Cl}^-$  ions  
 368 (Pocker and Tanaka, 1978) and therefore by salinity levels. Gillikin et al. (2006) hypothesized  
 369 that CA, which catalyses the reaction of bicarbonate to  $\text{CO}_2$ , thereby facilitating diffusion of  
 370 DIC through membranes (Paneth and O'Leary, 1985), may add or remove carbon species  
 371 from the internal calcifying fluids. A reduction in CA activity due to low salinity could cause  
 372 a reduction in environmental DIC entering the animal, resulting in a larger relative

373 contribution of metabolic DIC and more negative  $\delta^{13}\text{C}$  in the calcifying fluid and shell. Since  
 374 there was no significant difference between  $C_M$  values calculated for the three salinity  
 375 conditions, it appears that the salinity effect on CA activity and the ability of the animal to  
 376 move environmental DIC into their calcification space is small or non-existent. The similarity  
 377 between  $C_M$  values at the three salinities also seems to reject a strong impact of possible  
 378 varying  $\text{O}_2$  and  $\text{CO}_2$  levels on shell  $\delta^{13}\text{C}$  (see McConnaughey and Gillikin, 2008) that could  
 379 arise with changing salinities. However, future studies should measure these parameters to  
 380 estimate their role.

381 The estimated values of  $C_M$  shown in Fig. 7 were highly sensitive to the type of tissue used  
 382 for calculation and, at  $t_7$ ,  $C_M$  values ranged from 21 % using digestive gland to 50 % using  
 383 muscle (note that we calculated  $C_M$  for each date based on  $\delta^{13}\text{C}_{\text{tissues}}$  values pooled from all  
 384 salinities as there was no salinity effect on  $C_M$ ). The estimations using  $\delta^{13}\text{C}_{\text{mantle}}$  and  $\delta^{13}\text{C}_{\text{total}}$   
 385 *soft tissues* resulted in intermediate values that ranged between the estimations made using  
 386 muscle and digestive gland  $\delta^{13}\text{C}$  values. At the end of this experiment, these differences were  
 387 reduced (12 % using digestive gland to 20 % using muscle). Interestingly  $C_M$  values  
 388 calculated with digestive gland or muscle, as a proxy for respired carbon  $\delta^{13}\text{C}$ , between the  
 389 clams collected at the end of the experiment and clams collected in the field, did not greatly  
 390 differ (see Fig. 7, 17 vs 12 % or 21 vs 20 % using the digestive gland and muscle  
 391 respectively). As clams in the laboratory were growing without sediments, this would suggest  
 392 that  $^{13}\text{C}$  depleted porewater plays little to no role in biomineralization (see McConnaughey  
 393 and Gillikin, 2008). Indeed, incorporation of  $^{13}\text{C}$  depleted porewater in the field would lead to  
 394 more negative shell  $\delta^{13}\text{C}$  and then an overestimation of  $C_M$  in the field. In our lab experiment,  
 395 without such depleted porewaters,  $C_M$  values should have been much smaller than the field if  
 396 carbon from these waters was strongly incorporated into the shells.

397 The  $C_M$  fluctuations through time were higher using  $\delta^{13}\text{C}_{\text{muscle}}$  as  $\delta^{13}\text{C}_R$  than using  $\delta^{13}\text{C}_{\text{digestive}}$   
 398 *gland* (50 to 20 % using muscle; 21 to 12 % using digestive gland). Interestingly, the estimation  
 399 using  $\delta^{13}\text{C}_{\text{phytoplankton}}$  (which is lower than  $\delta^{13}\text{C}_{\text{tissues}}$ , especially at the beginning of the  
 400 experiment) results in an even more constant estimation of  $C_M$  over time than using  $\delta^{13}\text{C}_{\text{digestive}}$   
 401 *gland*, with a value close to 12 %. Since salinity variations didn't affect the clams physiology  
 402 and other experimental conditions such as temperature and food quantity were constant during  
 403 this experiment, such a constant value can be expected. Moreover, a  $C_M$  value of 12 % is near  
 404 the typical value of 10 % recorded for most aquatic mollusks (see McConnaughey and  
 405 Gillikin, 2008). Assuming that  $\delta^{13}\text{C}_R$  is close to  $\delta^{13}\text{C}_{\text{phytoplankton}}$  during this experiment  
 406 illustrates that metabolic  $\text{CO}_2$  originates from very reactive tissues or directly from food

407 oxidation when animals are fed continuously, and that muscle tissues were not appropriate to  
408 estimate  $C_M$ .

409 In the field, availability of energy (food) and energy allocation within bivalves is highly  
410 variable throughout the year (Paulet et al., 2006). During phytoplankton blooms, when  
411 metabolism is generally high, more of the carbon used for shell mineralization may be derived  
412 from metabolic  $CO_2$  which mainly results from oxidation of food. In such a case,  $\delta^{13}C_R$  may  
413 be closer to  $\delta^{13}C$  values of phytoplankton. During less productive periods, carbon contribution  
414 may mainly come from DIC and because metabolism is mainly fuelled from reserves,  $\delta^{13}C_R$   
415 should be closer to  $\delta^{13}C$  values of storage organs (e.g. muscle). Thus, in bivalves, both  
416 proportion and carbon isotope value of metabolic carbon may be variable over the year. If this  
417 hypothesis is true, then studies investigating high frequency variations in  $\delta^{13}C_{shell}$  should use  
418 different tissues to estimate  $\delta^{13}C_R$  according to the time period studied (digestive gland or  
419 phytoplankton during productive periods and muscle during less productive period). Studies  
420 that average more time (e.g. annual) can use tissues with a slower turnover rate, such as the  
421 muscle. However, under natural conditions the effect would be much smaller than in this  
422 study as in the field food items are not likely to vary as much as the 40 ‰ shift imposed in  
423 this study, and muscle and digestive gland  $\delta^{13}C$  values would not be so isotopically different  
424 (e.g. Lorrain et al., 2002).

425

426 Our study highlights that  $\delta^{13}C$  values of respired carbon are necessary to accurately determine  
427 the percentage of metabolic carbon integrated into bivalve shells. This respired  $CO_2$  is  
428 however very difficult to sample and has never been measured in bivalves.

429

430

## 431 **Conclusion**

432 This study experimentally highlights the incorporation of two carbon sources into shell  
433 carbonate: dissolved inorganic carbon from seawater and respired carbon derived from food.

434 The incorporation time of both carbon sources into the shell is very short (less than 7 days).

435 These short incorporation times indicate that proxies based on  $\delta^{13}C_{shell}$  may be used with high  
436 temporal resolution. Our experimental results also demonstrate that (1) the physiology of this

437 euryhaline clam was not affected by the salinity treatments ranging between 20 and 35 during  
438 this experiment; (2) salinity does not seem to affect carbon cycling within the clam; and (3)

439  $\delta^{13}C_{shell}$  is highly correlated to  $\delta^{13}C_{DIC}$ . Consequently,  $\delta^{13}C_{shell}$  of *R. philippinarum* is a

440 promising proxy of large scale variations in  $\delta^{13}\text{C}_{\text{DIC}}$  and therefore of salinity in estuarine  
441 water.

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#### 445 **Acknowledgements**

446 This work is a contribution to the PaleoSalt project funded by the European Science  
447 Fondation (ESF) under the EUROCORES Program EUROCLIMATE. The authors thank all  
448 the staff of the IFREMER shellfish laboratory at Argenton, particularly Pierrick Le Souchu,  
449 Christian Mingant, Luc Lebrun and Isabelle Queau; Jérémy Querné, Antoine Carlier and  
450 Jean-Marie Munaron for clam collection, technical assistance in clam dissection and water  
451 filtration; Mickael Korntheuer for technical support with the EA-IRMS and David Dettman  
452 for the carbonate isotopic analysis. This manuscript was greatly improved by comments from  
453 Jonathan Flye-Sainte-Marie and Julien Thébault and by reviews from Ted McConnaughey  
454 and Alan Wanamaker. We also thank the GDR IRD and IFREMER ACOMAR “Analyse,  
455 Compréhension et Modélisation des Archives biologiques marines” for funding. Support for  
456 this work was obtained also from the Science for Sustainable Development Program, Brussels  
457 via contract SD/CS/02A.

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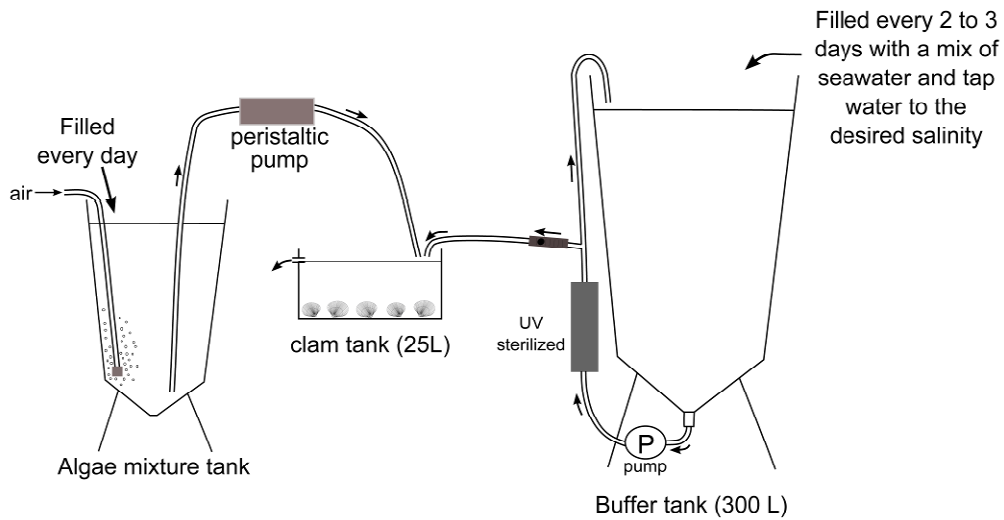
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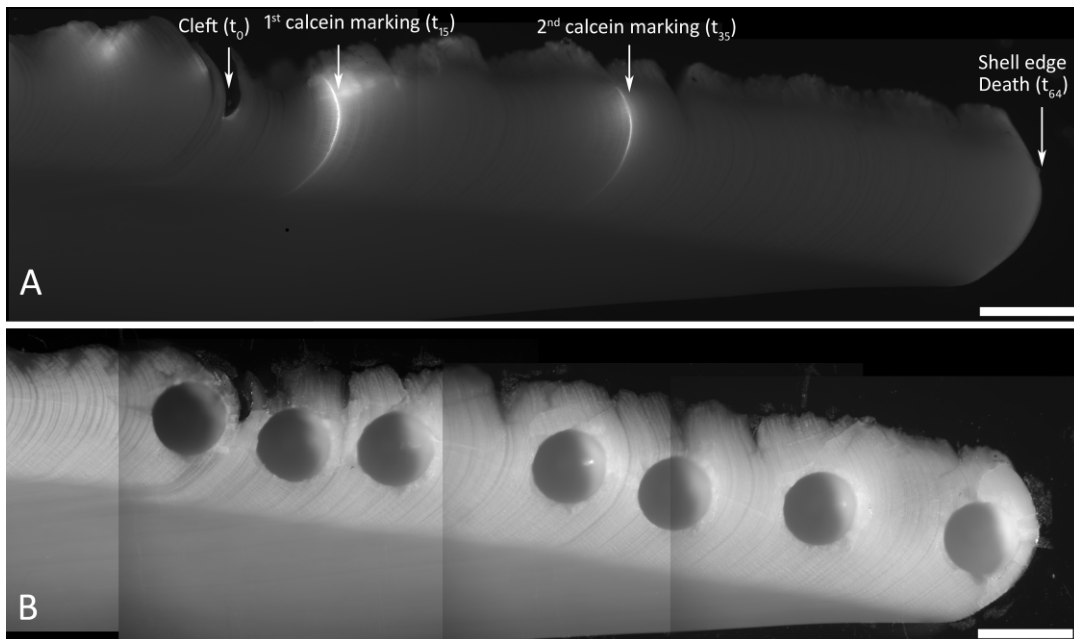
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609 **Figures**  
610

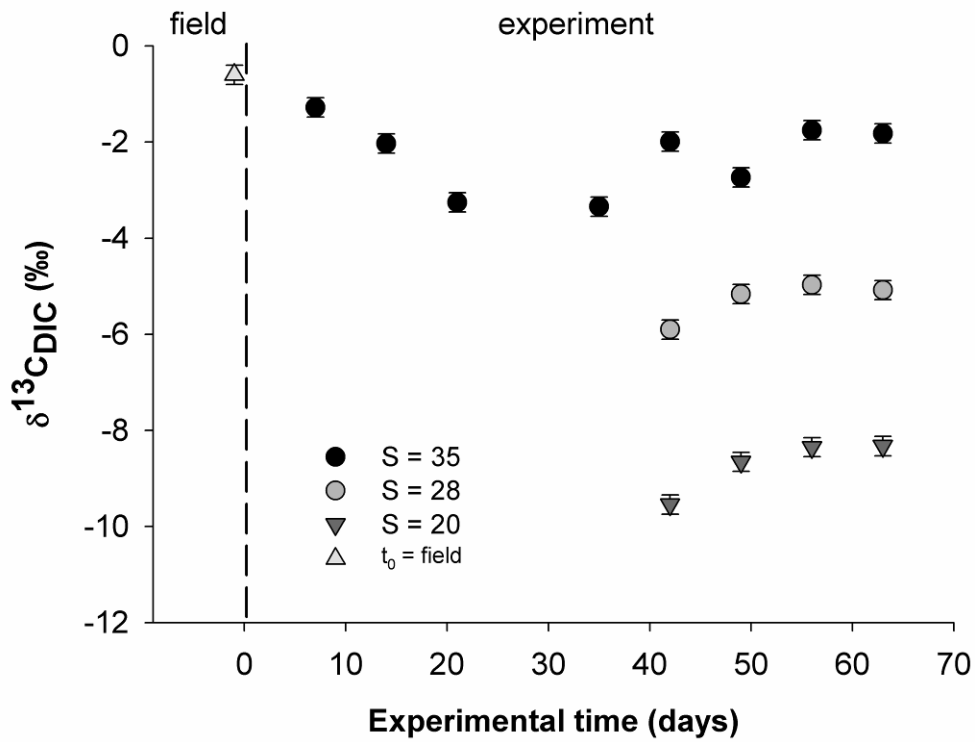


611  
612 Fig. 1. Schematic diagram of the experimental system. The complete experimental system  
613 consisted of three clam and buffer tanks, one for each clam batch. The same algae culture was  
614 used for the three clam batches.  
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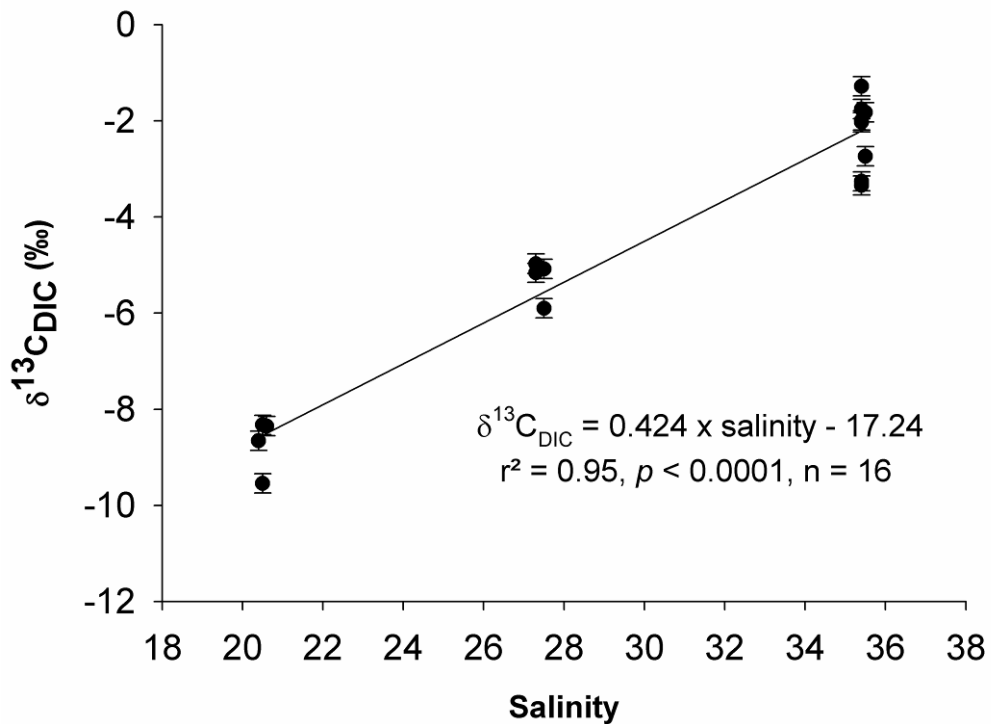


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617 Fig. 2. Photographs of shell transversal section. Growth direction is left to right, the outside of  
618 the shell is toward the top of picture. A: The cleft corresponds to a growth stop due to clam  
619 harvesting in the field on the 18<sup>th</sup> of August 2008. Clams were kept in laboratory for 64 days.  
620 The two calcein stains at  $t_{15}$  and  $t_{35}$  are visible. Shell edge corresponds to the end of the  
621 experiment. B: Drill holes (300  $\mu\text{m}$  diameter; 350  $\mu\text{m}$  depth) made in the middle of the outer  
622 layer. Scale bar = 500  $\mu\text{m}$ .

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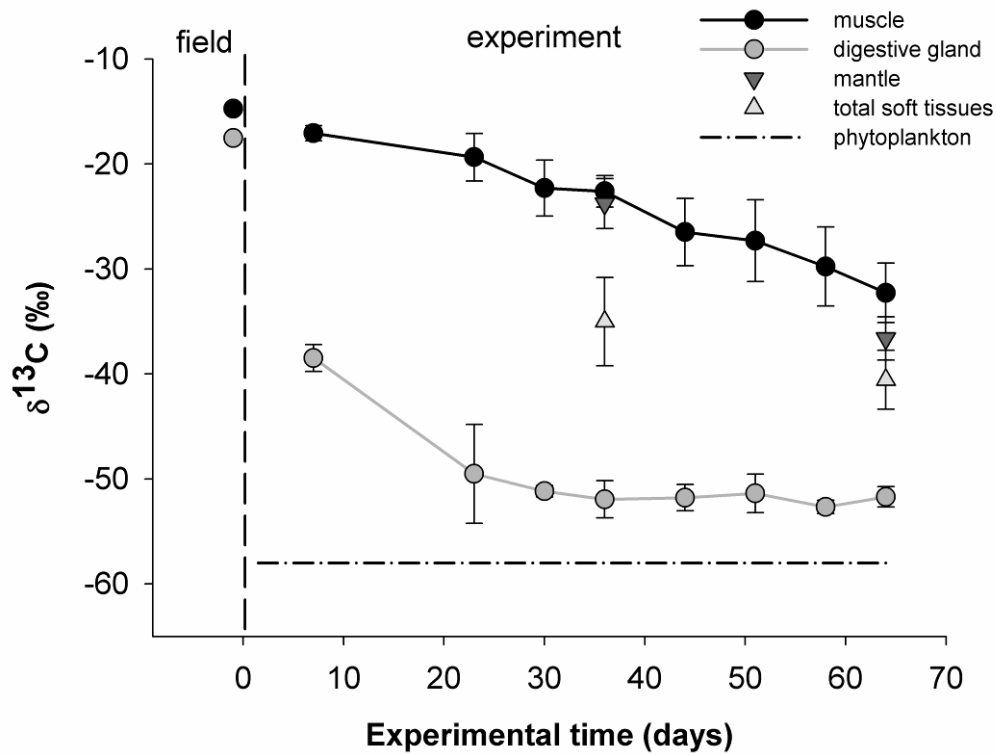
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626 Fig. 3. A: DIC stable carbon isotope values ( $\delta^{13}\text{C}_{\text{DIC}}$ ) during the experiment. Three salinity  
 627 conditions (20, 28 and 35) were tested from days 35 to 64. B:  $\delta^{13}\text{C}_{\text{DIC}}$  as a function of salinity  
 628 during the experiment. Error bars represent the analytical precision ( $1 \sigma$ ).

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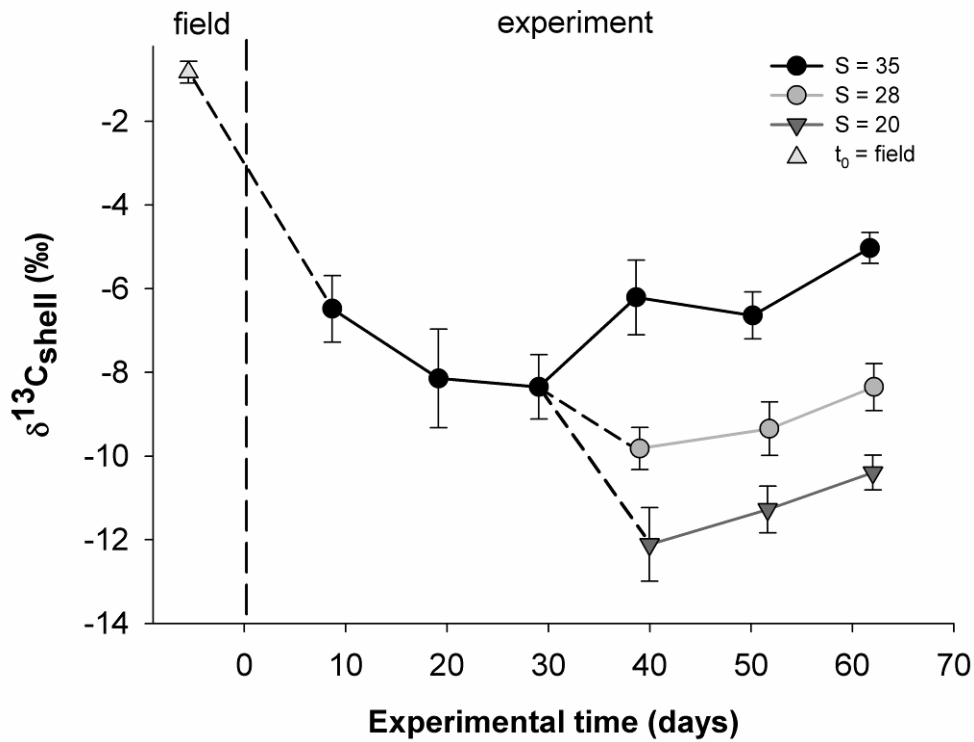


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631 Fig. 4. Evolution of stable carbon isotope values ( $\delta^{13}\text{C}$ ) of Manila clam tissues (muscle,  
 632 digestive gland, mantle and total soft tissues) and phytoplankton during the experiment.

633 Values are expressed as means and error bars represent standard deviations,  $n = 5$  during the  
 634 first 35 days;  $n = 15$  from day 35 to 64 because the results for the three different salinities  
 635 were pooled.

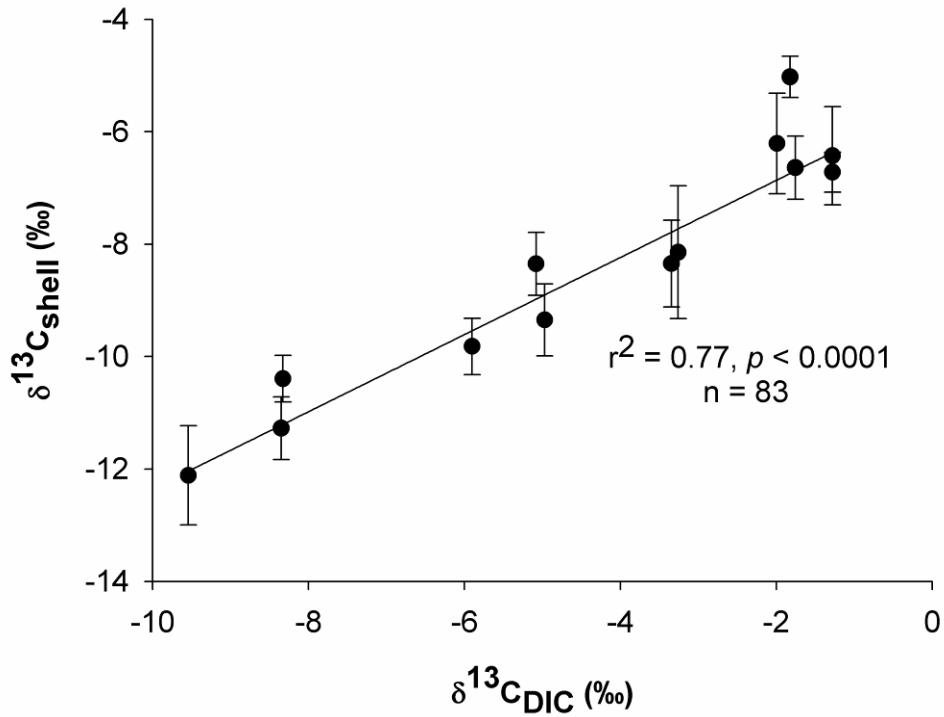
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638 Fig. 5. Evolution of stable carbon isotope values ( $\delta^{13}\text{C}_{\text{shell}}$ ) in *R. philippinarum* shells during  
 639 the experiment for the three salinity conditions (20, 28 and 35). Values are expressed as  
 640 means and error bars represent standard deviations,  $n = 15$  during the first 35 days;  $n = 5$  from  
 641 day 35 to 64 for each point.

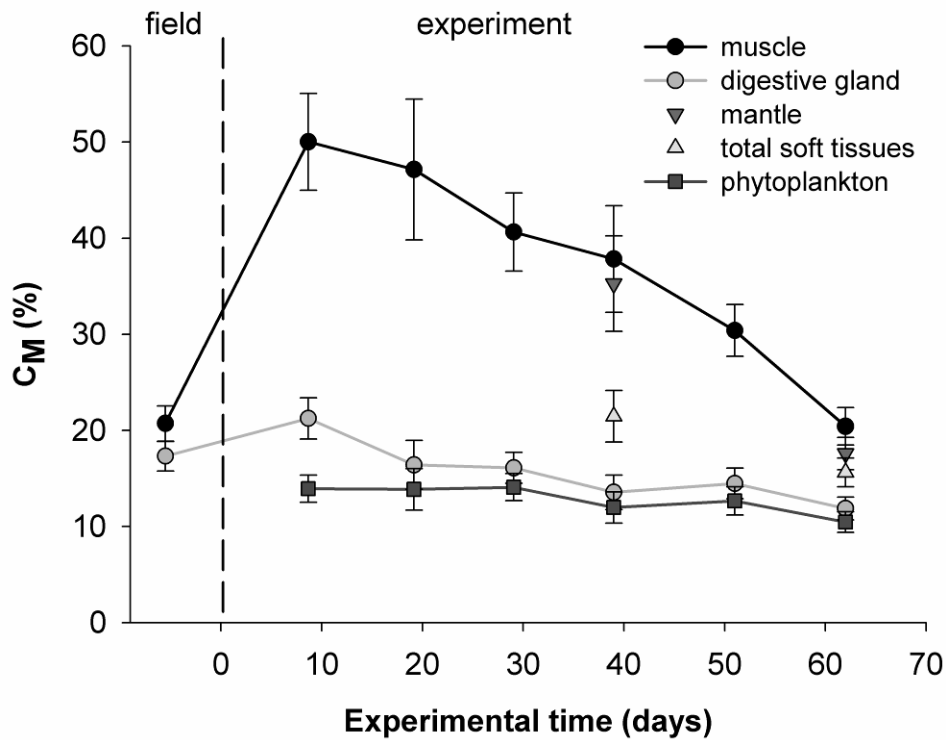
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645 Fig. 6.  $\delta^{13}\text{C}_{\text{shell}}$  vs  $\delta^{13}\text{C}_{\text{DIC}}$  in clam tanks for samples taken over the experiment. Error bars  
 646 represents standard deviations. The solid line shows the linear least squares regression  
 647 calculated with all data obtained during this experiment ( $r^2 = 0.77$ ,  $p < 0.0001$ ,  $n = 83$ ).

648



650

651 Fig. 7. Estimates of the proportions of metabolic carbon ( $C_M$ ) incorporated into shell  
 652 carbonates using the equation of McConnaughey et al. (1997). Muscle, digestive gland,  
 653 mantle, total soft tissues and phytoplankton carbon isotope composition were used to  
 654 approximate  $\delta^{13}C_R$ . Values are expressed as means and error bars represent standard  
 655 deviations. Different patterns are observed depending on the tissue used for the approximation  
 656 of  $\delta^{13}C_R$ .