Experimental shift of diet and DIC stable carbon isotopes: influence on shell δ13C values in the Manila clam Ruditapes philippinarum

Céline Poulain, Anne Lorrain, Remy Mas, David Paul Gillikin, F. Dehairs, Rene Robert, Yves-Marie Paulet

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Poulain C.[a], Lorrain A.[a], Mas R.[b], Gillikin D.P.[c], Dehairs F.[b], Robert R.[d], Paulet Y-M.[a]

[a] LEMAR, UMR CNRS/UBO/IRD 6539, Institut Universitaire Européen de la Mer, Place Nicolas Copernic, 29280 Plouzané, France

[b] Earth System Sciences Research Group and Dept. of Analytical and Environmental Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium

[c] Department of Earth Science and Geography, Vassar College, Poughkeepsie, New York 12604, USA

[d] IFREMER, UMR Laboratoire de Physiologie des Invertébrés, Station Expérimentale d'Argenton, Presqu'ile du vivier, 29840 Argenton, France

Corresponding Author:
Céline Poulain
LEMAR, UMR CNRS/UBO/IRD 6539, Institut Universitaire Européen de la Mer,
Technopole Brest-Iroise, Place Nicolas Copernic, 29280 Plouzané, France
Email: celine.poulain@univ-brest.fr
Phone number : + 33 2 98 49 88 38
Fax number : + 33 2 98 49 86 45

Abstract

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

Keywords: $\delta^{13}C_{shell}$, DIC, salinity, metabolic carbon, *Ruditapes philippinarum*, estuarine water, proxy

1 Introduction

Bivalves record environmental information in the form of chemical or structural messages archived in their calcareous exoskeleton during growth. For this reason, they are widely used for paleo-climatic and paleo-oceanographic reconstructions (e.g. Davenport, 1938, Weidman et al., 1994 , Tripati et al., 2001, Dutton et al., 2002 , Schöne et al., 2003, Lazareth et al., 2006, Ivany et al., 2008, Goewert and Surge, 2008, Wanamaker et al., 2008). Epstein et al. (1953) showed that oxygen isotope composition of mollusk shells ($\delta^{18}O_{shell}$) reflect the temperature and oxygen isotope composition of the water ($\delta^{18}O_w$) in which they were formed. This allows the use of shell carbonate $\delta^{18}O$ as a temperature proxy at least in cases where $\delta^{18}O_w$ remains constant and is known (e.g. Weidman et al., 1994, Chauvaud et al., 2005, Andrus and Rich, 2008). However, $\delta^{18}O_w$ is often strongly dependant on salinity (Epstein and Mayeda, 1953, Craig and Gordon, 1965, Mook and Tan, 1991, Dettman et al., 2004). Therefore, the estimation of $\delta^{18}O_w$ is difficult without an independent salinity proxy in environments displaying large salinity variations such as estuaries. In well flushed estuaries with short residence times, stable carbon isotope composition of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) is well correlated with salinity year round, especially when salinity is higher than 25 (Mook, 1971, Surge et al., 2001, Fry, 2002, Gillikin et al., 2006). As carbon precipitating during shell mineralization is known to originate mainly from dissolved inorganic carbon (DIC) of seawater (Mook and Vogel, 1968, McConnaughey et al., 1997 , Gillikin et al., 2006, McConnaughey and Gillikin, 2008, Owen et al., 2008), the carbon isotope composition of bivalve shells ($\delta^{13}C_{shell}$) living in estuaries may possibly be used for reconstructing past variations of salinity. A paleo-salinity proxy would also be useful for correcting paleo-temperature based on $\delta^{18}O$ of estuarine bivalve shells (see Gillikin et al., 2006).

The carbon isotope composition of bivalve shell carbonates is, however, not only affected by $\delta^{13}C_{DIC}$, but also by the physiology of the bivalve (Dillaman and Ford, 1982, Tanaka et al., 1986, McConnaughey et al., 1997, Lorrain et al., 2004, Gillikin et al., 2006, 2007, 2009, McConnaughey and Gillikin, 2008). Previous studies have shown that isotopically light metabolic carbon, derived from food, is incorporated into shell carbonate (e.g. Tanaka et al., 1986, McConnaughey et al., 1997, Wanamaker et al., 2007, Owen et al., 2008, Gillikin et al., 2006, 2007, 2009). However, the processes of metabolic carbon incorporation into the shell remain poorly known and the estimation of metabolic carbon contribution to the shell appears highly variable between studies. McConnaughey et al. (1997), Lorrain et al. (2004) and

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

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

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

2 Material and methods

2.1 Biological material

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

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

2.2 Experimental conditions

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

Water flowing into the 25 L tanks was supplemented continuously during the entire experiment with cultured microalgae (50% *Isochrysis affinis galbana* (Tahitian strain *T.iso*) and 50% *Chaetoceros calcitrans*) depleted in ^{13}C ($\delta^{13}C$ = - 58 ‰; 1 σ = 4 ‰). ^{13}C depleted microalgae were obtained by bubbling ^{13}C-depleted industrial CO_2 into the culture medium (see Paulet et al., 2006 for elaboration). Algae were cultured at a salinity of 35. The experiment was performed over 64 days, during which temperature (20°C) and photoperiod (12/12) were kept constant.

During the first 35 days, salinity of the three tanks was maintained constant at 35. From days 35 to 64, one tank was kept at 35, while salinity was modified to 28 and 20 in the other two tanks. Salinities of 20 and 28 were obtained by mixing seawater and tap water within the 300 L buffer tanks, and salinity was checked and adjusted using a conductivity meter (WTW, LF 197-S). Salinity was recorded every five minutes in the three tanks using an autonomous data logger (YSI-600 OMS) from day 7 (t_7) to the end of the experiment (t_{64}).

2.3 Sampling

2.3.1 Biological sampling

Soft tissue sampling

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

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

CI = (Soft Tissues Dry Weight / Shell Dry Weight) × 100.

Calcein marking and shell sampling

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

2.3.2 Food and DIC sampling

In order to determine the carbon isotope composition of the diet during the experiment, 15 mL of the algal mix was filtered on a precombusted Whatman GF/F filter every week. Following Lorrain et al. (2003), filters were immediately dried for 12h at 60°C and stored until analysis. When the clams were collected, water was sampled in the field and 200 mL was filtered on a precombusted Whatman GF/F filter to determine the carbon isotope composition of particulate organic matter ($\delta^{13}C_{POM}$) using the method described above. The suspended POM pool is a mixture of different sources of carbon (e.g. phytoplankton, microphytobenthos, resuspended sediment, terrestrial carbon, marine micro-algae detritus). However, $\delta^{13}C_{POM}$ may be used as a proxy for $\delta^{13}C_{phytoplankton}$ (see Gillikin et al., 2006). Following Gillikin and Bouillon (2007), water from the field and water within the 25 L clam tanks were sampled at t_0 and weekly respectively using 12 mL glass vials and poisoned with 20 µL of saturated mercuric chloride ($HgCl_2$) solution until analysis of the DIC isotope composition.

2.4 Shell preparation and calcium carbonate sampling

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

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

2.5 Isotopic analyses

Freeze-dried tissues were ground to a homogeneous fine powder. 500 µg of the powder was packed into 5×9 mm precombusted silver cups. Carbon stable isotope analysis was performed at the Earth System Sciences laboratory (Vrije Universiteit Brussel) using an Elemental Analyzer (Flash 1112 EA Thermo Finnigan) coupled via a CONFLO III to a Thermo Delta V

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

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

The $\delta^{13}C$ of the total DIC was analyzed according to the protocol of Gillikin and Bouillon, (2007). Briefly, 9.5 mL of water sample were acidified with 200 µL of pure orthophosphoric acid in a 12 mL helium-flushed headspace vial, followed by overnight equilibration and injection of 500 µL headspace into the carrier gas stream of the continuous flow EA-IRMS (same instrument used for tissues and diet). The Miyajima et al. (1995) algorithm was used to correct for the partitioning of CO_2 between headspace and water phase and to calculate the $\delta^{13}C_{DIC}$. Along with the headspace injections a CO_2 house standard was injected (n = 28, 1 σ = 0.2 ‰). Based on this and an average reproducibility of DIC sample measurements of 0.11 ‰, a precision of the $\delta^{13}C_{DIC}$ better than 0.2 ‰ (1 σ) can be expected (also see Gillikin and Bouillon, 2007).

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

2.6 Data analysis

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

3 Results

3.1 Animal growth and condition during the experiment

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

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

3.2 Salinity and $\delta^{13}C_{DIC}$

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

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

3.3 $\delta^{13}C_{tissues}$

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

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

3.4 $\delta^{13}C_{shell}$

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

4 Discussion

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

In our study, there was no significant effect of salinity on the clams physiology as indicated by the incorporation of carbon into the tissues, the condition index and shell growth rates. These observations are in agreement with the results of Kim et al. (2001), who showed that reasonable changes in salinity (35 to 20) have a small effect on activity rhythm and oxygen 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 $\delta^{13}C_{shell}$ in just 7 days (Fig. 5). Since the difference
311 between field and laboratory $\delta^{13}C_{DIC}$ was less than 1 ‰, the change in $\delta^{13}C_{shell}$ was most likely
312 due to the diet $\delta^{13}C$ 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 $\delta^{13}C_{DIC}$ (as we mixed waters with different
315 $\delta^{13}C_{DIC}$ values), which was followed by significant changes of $\delta^{13}C_{shell}$ 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 ($\delta^{13}C_{phytoplankton}$ shift at t_0 and $\delta^{13}C_{DIC}$ 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 $\delta^{13}C_{shell}$ 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, $\delta^{13}C_{DIC}$ 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}), $\delta^{13}C_{DIC}$ fluctuations are not explained by salinity variations. Two other hypotheses may
335 be considered. (1) Clam respiration and calcification will modify the CO_2 fluxes and then
336 $\delta^{13}C_{DIC}$, however, aquaria water was continuously renewed at a rate of 25% h^{-1} (see Materials
337 and methods), which would greatly reduce or even eliminate this effect on $\delta^{13}C_{DIC}$.
338 Nevertheless, future studies should consider this and analyze pH, total DIC, pCO_2 and even

O_2 levels as all these parameters have been proposed to influence shell $\delta^{13}C$ (see McConnaughey and Gillikin, 2008 for a review); (2) Variable quantities of food supplied to the clams is a more probable cause of these $\delta^{13}C_{DIC}$ fluctuations. In order to obtain the appropriate phytoplankton concentration within the clam tanks, the flow of algae culture supplied was adjusted daily as a function of algae culture concentration and the number of clams in the tank. The $\delta^{13}C_{DIC}$ value in this algae culture was -22 ‰ during this experiment. Therefore, when variable quantities of algae solutions are supplied to the clams, corresponding variable ^{13}C-depleted DIC in the culture medium might induce differential $\delta^{13}C_{DIC}$ values within the clam tanks. Despite the relative instability of $\delta^{13}C_{DIC}$, $\delta^{13}C_{shell}$ followed the same variations as $\delta^{13}C_{DIC}$. There was a positive linear relationship between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{shell}$ during the experiment with a high correlation coefficient value ($r^2 = 0.77$, $p < 0.0001$, n = 83, Fig. 6). It seems that, in this species, $\delta^{13}C_{shell}$ may be a promising proxy of large scale variations in $\delta^{13}C_{DIC}$ and therefore of salinity in estuarine water.

In order to estimate the percentage of metabolic carbon in the shell carbonate (C_M), previous studies used the classical two end-member mixing equation proposed by McConnaughey et al. (1997): $C_M = 100 \times (\delta^{13}C_{shell} - \varepsilon_{ar\text{-}b} - \delta^{13}C_{DIC}) / (\delta^{13}C_R - \delta^{13}C_{DIC})$ where $\varepsilon_{ar\text{-}b}$ is the enrichment factor between bicarbonate and aragonite (+ 2.7 ‰; Romanek et al., 1992) and $\delta^{13}C_R$ is the carbon isotope composition of respired carbon which has never been measured in bivalves. According to Tanaka et al. (1986) and McConnaughey et al. (1997), $\delta^{13}C_{tissues}$ values provide a good approximation of $\delta^{13}C_R$. Nevertheless, there is no consensus on the tissue used to estimate $\delta^{13}C_R$; consequently we performed this estimation with all potential $\delta^{13}C_R$ values ($\delta^{13}C$ of muscle, digestive gland, mantle, total soft tissues and cultured phytoplankton). There was no significant difference between C_M values calculated for the three salinity conditions with all potential $\delta^{13}C_R$ values and at any sampling date (Kruskal-Wallis, $p > 0.05$). The similarity between C_M values at the three salinities seems to reject a strong impact of carbonic anhydrase (CA) activity on shell $\delta^{13}C$. Low salinity could result in decreased carbonic anhydrase (CA) activity in the animal, as has been described for some bivalves (Henry and Saintsing, 1983). In addition, CA activity has also been shown to be inhibited by Cl^- ions (Pocker and Tanaka, 1978) and therefore by salinity levels. Gillikin et al. (2006) hypothesized that CA, which catalyses the reaction of bicarbonate to CO_2, thereby facilitating diffusion of DIC through membranes (Paneth and O'Leary, 1985), may add or remove carbon species from the internal calcifying fluids. A reduction in CA activity due to low salinity could cause a reduction in environmental DIC entering the animal, resulting in a larger relative

373 contribution of metabolic DIC and more negative $\delta^{13}C$ 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 O_2 and CO_2 levels on shell $\delta^{13}C$ (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 $\delta^{13}C_{tissues}$ values pooled from all
384 salinities as there was no salinity effect on C_M). The estimations using $\delta^{13}C_{mantle}$ and $\delta^{13}C_{total\ soft\ tissues}$ resulted in intermediate values that ranged between the estimations made using
386 muscle and digestive gland $\delta^{13}C$ 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 $\delta^{13}C$, 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}C depleted porewater plays little to no role in biomineralization (see McConnaughey
393 and Gillikin, 2008). Indeed, incorporation of ^{13}C depleted porewater in the field would lead to
394 more negative shell $\delta^{13}C$ 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 $\delta^{13}C_{muscle}$ as $\delta^{13}C_R$ than using $\delta^{13}C_{digestive\ gland}$ (50 to 20 % using muscle; 21 to 12 % using digestive gland). Interestingly, the estimation
399 using $\delta^{13}C_{phytoplankton}$ (which is lower than $\delta^{13}C_{tissues}$, especially at the beginning of the
400 experiment) results in an even more constant estimation of C_M over time than using $\delta^{13}C_{digestive\ gland}$, 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 $\delta^{13}C_R$ is close to $\delta^{13}C_{phytoplankton}$ during this experiment
406 illustrates that metabolic CO_2 originates from very reactive tissues or directly from food

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

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

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

Conclusion

This study experimentally highlights the incorporation of two carbon sources into shell carbonate: dissolved inorganic carbon from seawater and respired carbon derived from food. The incorporation time of both carbon sources into the shell is very short (less than 7 days). These short incorporation times indicate that proxies based on $\delta^{13}C_{shell}$ may be used with high temporal resolution. Our experimental results also demonstrate that (1) the physiology of this euryhaline clam was not affected by the salinity treatments ranging between 20 and 35 during this experiment; (2) salinity does not seem to affect carbon cycling within the clam; and (3) $\delta^{13}C_{shell}$ is highly correlated to $\delta^{13}C_{DIC}$. Consequently, $\delta^{13}C_{shell}$ of *R. philippinarum* is a

promising proxy of large scale variations in $\delta^{13}C_{DIC}$ and therefore of salinity in estuarine water.

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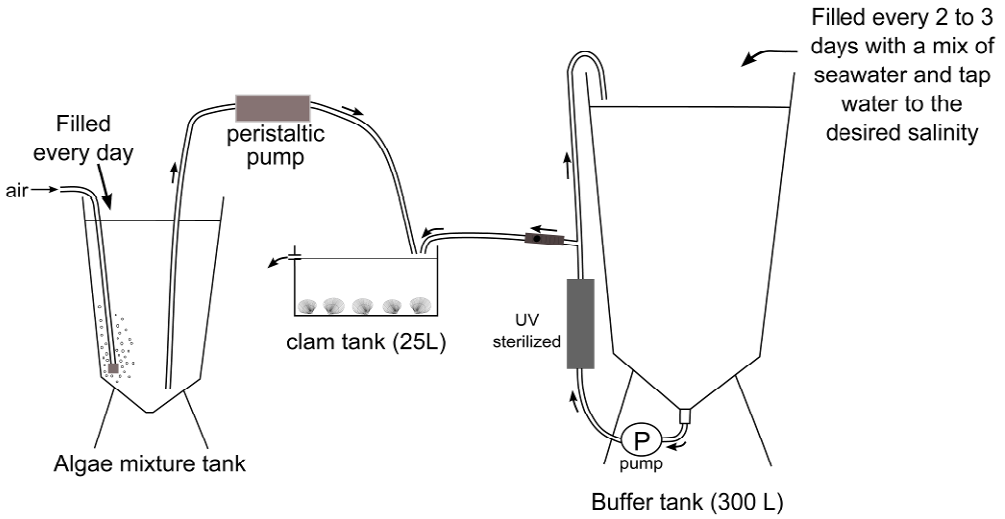
609 **Figures**
610



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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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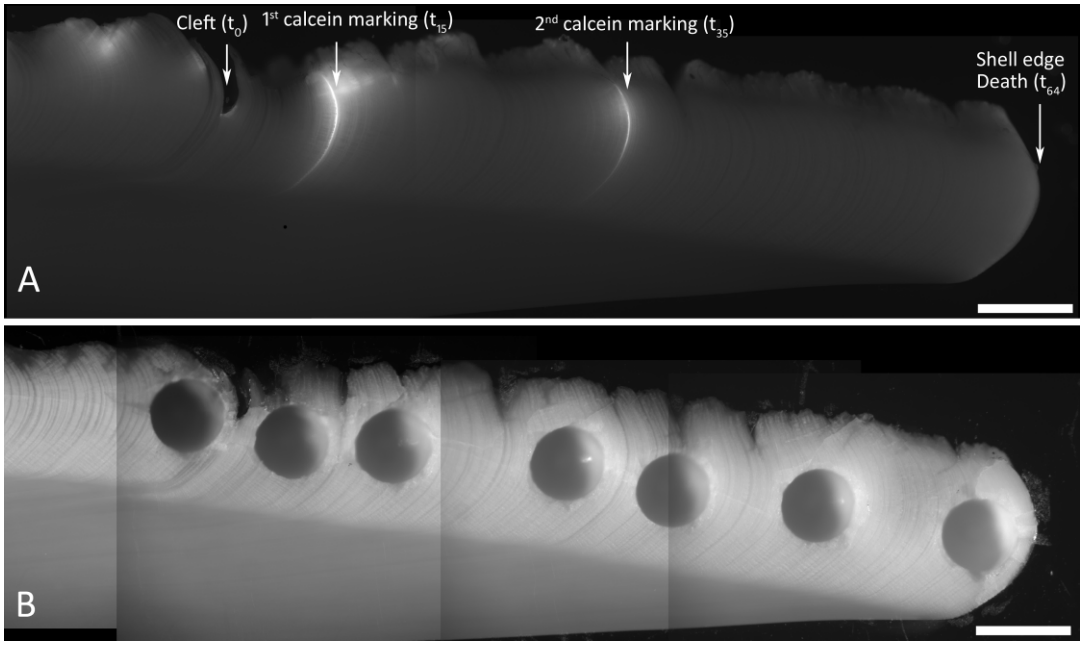
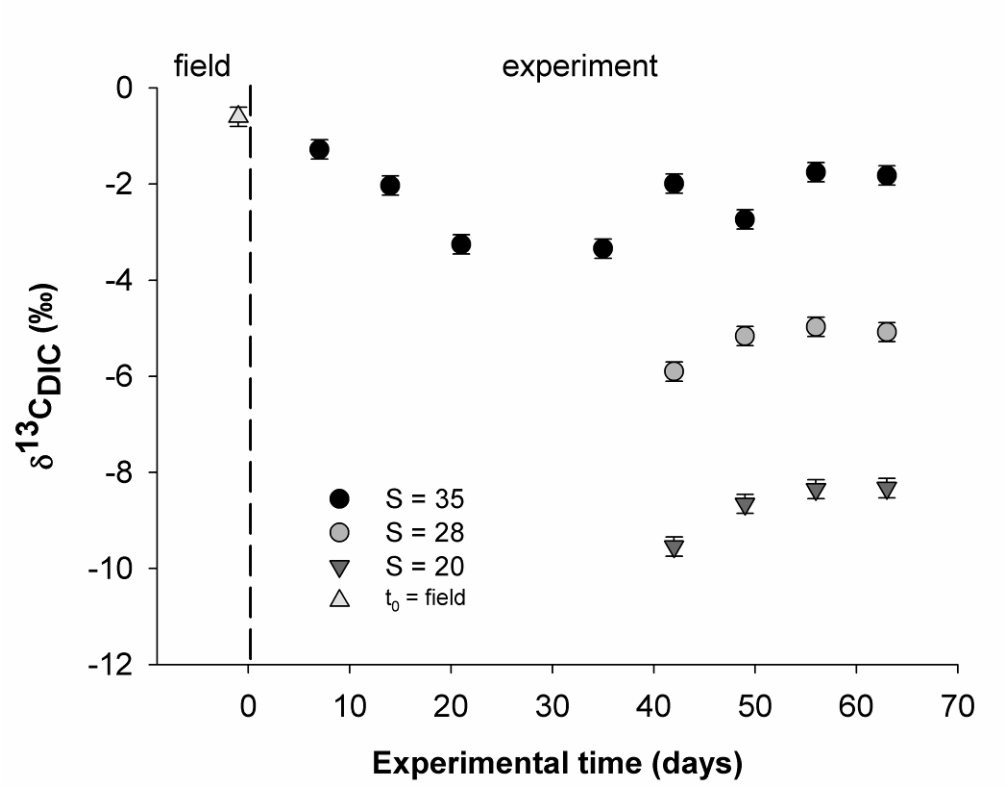


616

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 18th 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 µm diameter; 350 µm depth) made in the middle of the outer
622 layer. Scale bar = 500 µm.



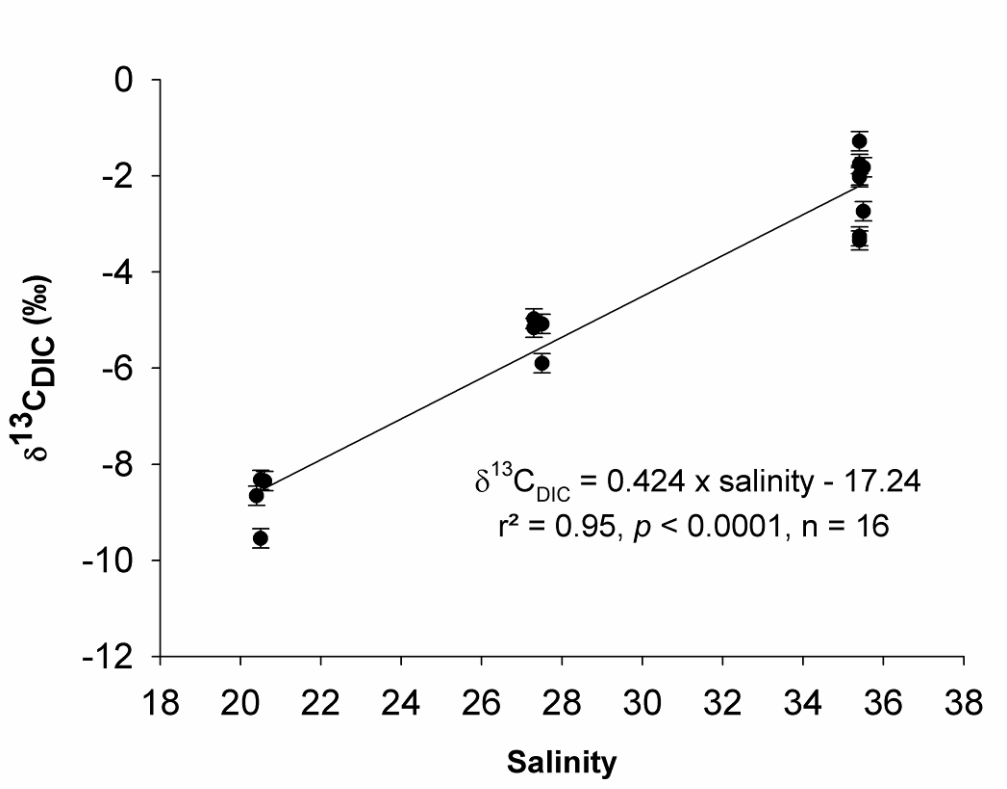
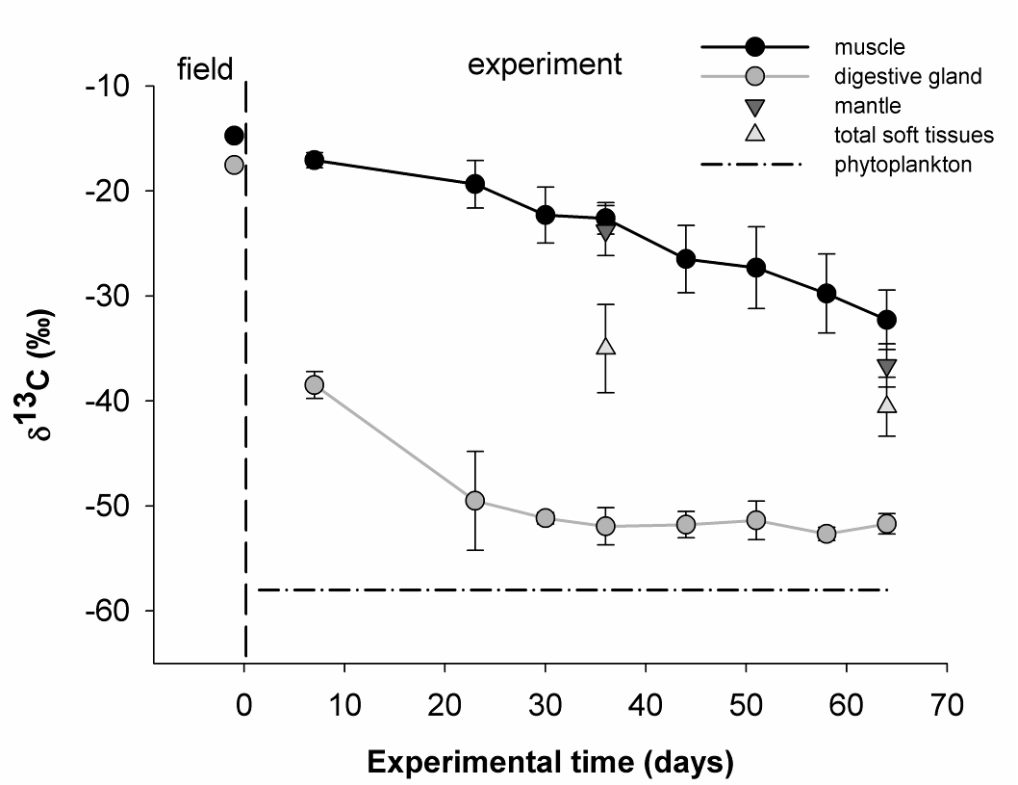


Fig. 3. A: DIC stable carbon isotope values ($\delta^{13}C_{DIC}$) during the experiment. Three salinity conditions (20, 28 and 35) were tested from days 35 to 64. B: $\delta^{13}C_{DIC}$ as a function of salinity during the experiment. Error bars represent the analytical precision (1 σ).



630

631 Fig. 4. Evolution of stable carbon isotope values ($\delta^{13}C$) 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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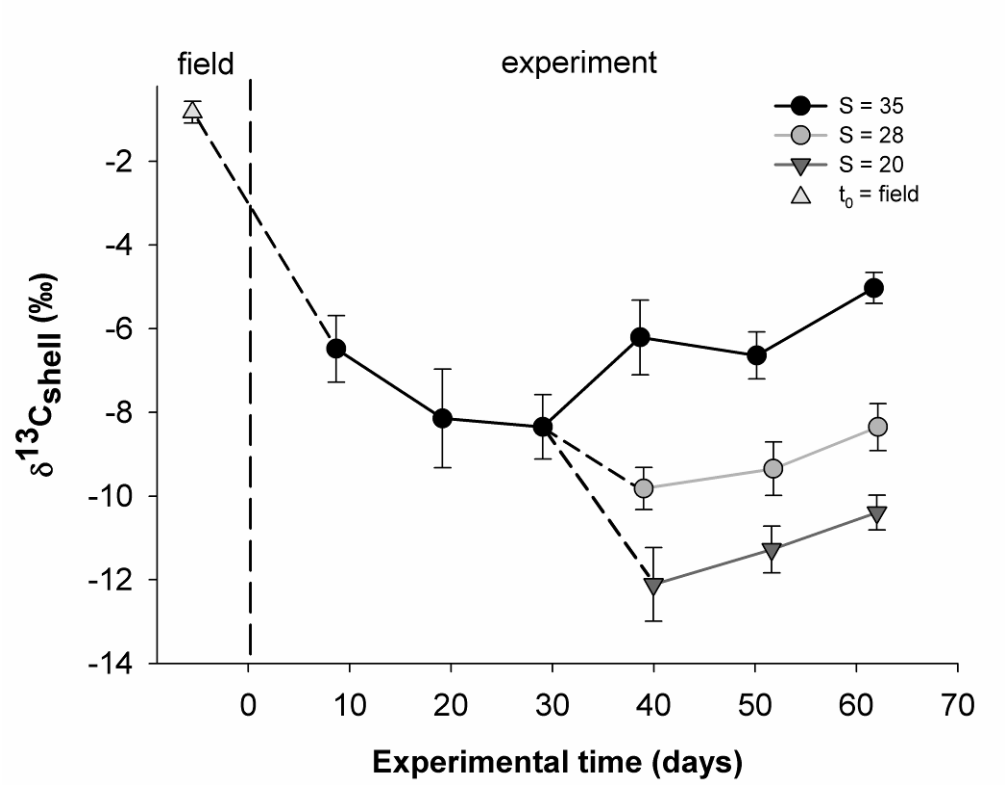

637

638 Fig. 5. Evolution of stable carbon isotope values ($\delta^{13}C_{shell}$) 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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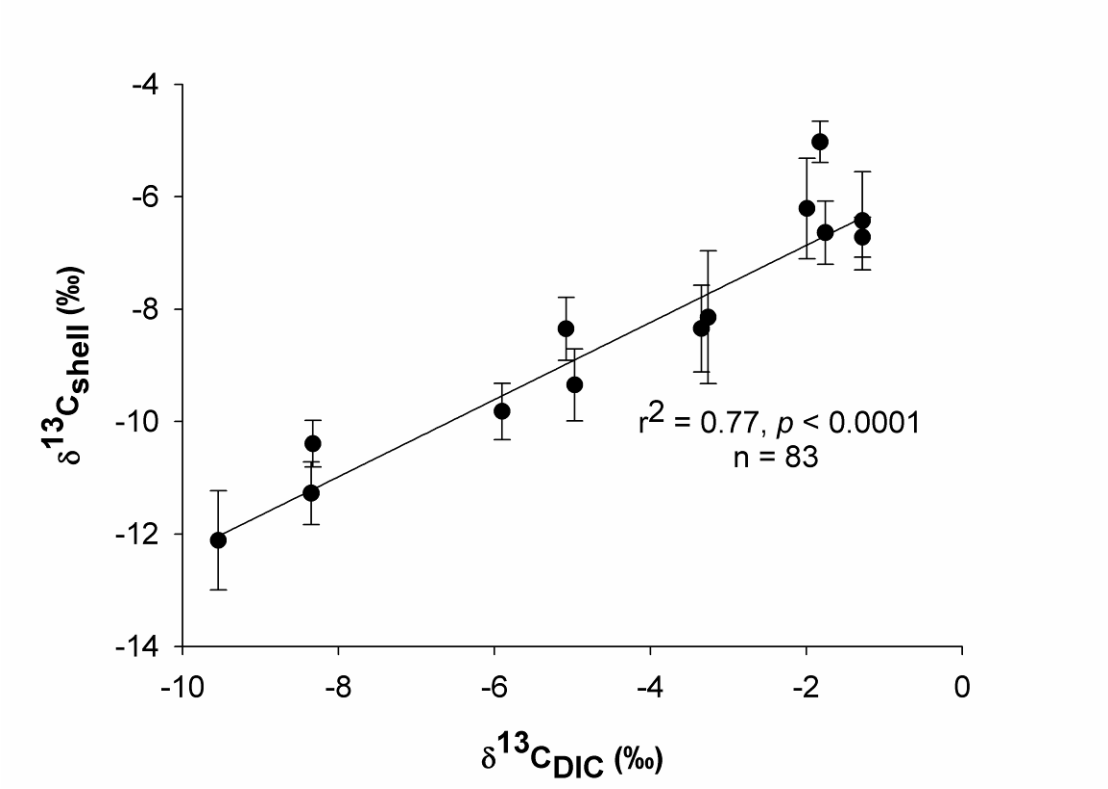

644

645 Fig. 6. $\delta^{13}C_{shell}$ vs $\delta^{13}C_{DIC}$ 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).

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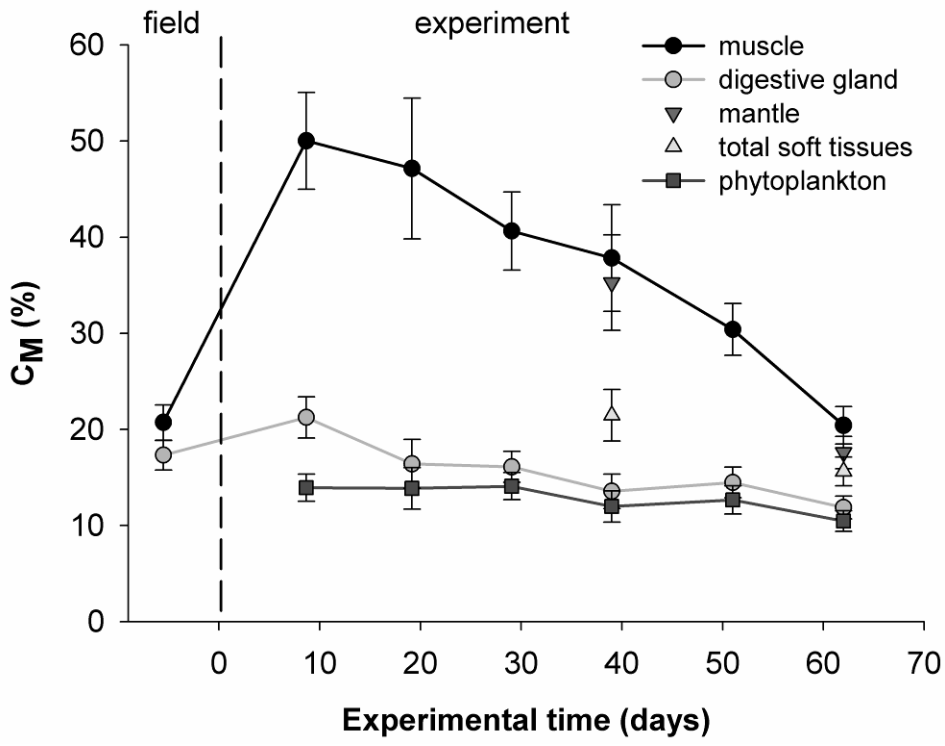

Fig. 7. Estimates of the proportions of metabolic carbon (C_M) incorporated into shell carbonates using the equation of McConnaughey et al. (1997). Muscle, digestive gland, mantle, total soft tissues and phytoplankton carbon isotope composition were used to approximate $\delta^{13}C_R$. Values are expressed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