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## **Geochemistry of Marine Bivalve Shells: the potential for paleoenvironmental reconstruction**

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## Chapter 6

### The link between salinity, phytoplankton, and $\delta^{13}\text{C}$ in *Mytilus edulis*

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#### Foreword

In the previous Chapter, it was demonstrated that the amount of metabolic carbon incorporation into aragonite shells of *Mercenaria mercenaria* changed dramatically through the life of the animal. This chapter focuses on a calcitic bivalve with a much shorter lifespan. With no strong age trend, it is possible that large changes in  $\delta^{13}\text{C}_{\text{DIC}}$ , common along many estuaries, would be recorded in the shells and potentially be useful as a salinity indicator.

*Chapter 6 is now in print:*

Gillikin, D. P., A. Lorrain, S. Bouillon, P. Willenz and F. Dehairs, 2006. Stable carbon isotopic composition of *Mytilus edulis* shells: relation to metabolism, salinity,  $\delta^{13}\text{C}_{\text{DIC}}$  and phytoplankton. *Organic Geochemistry* 37: 1371-1382.

**Abstract**

The incorporation of respired  $^{13}\text{C}$  depleted carbon into the skeletons of aquatic invertebrates is well documented. The fluid from which these animals calcify is a 'pool' of metabolic  $\text{CO}_2$  and external dissolved inorganic carbon (DIC). Typically, less than 10 % of the carbon in the skeleton is metabolic in origin, although higher amounts have been reported. If this small offset is more or less constant, large biogeochemical gradients in estuaries may be recorded in the  $\delta^{13}\text{C}$  value of bivalve shells. In this study, it is assessed if the  $\delta^{13}\text{C}$  values of *Mytilus edulis* shells can be used as a proxy of  $\delta^{13}\text{C}_{\text{DIC}}$  and provide an indication of salinity. First the  $\delta^{13}\text{C}$  values of respired  $\text{CO}_2$  ( $\delta^{13}\text{C}_{\text{R}}$ ) was considered using the  $\delta^{13}\text{C}$  values of tissues as a proxy for  $\delta^{13}\text{C}_{\text{R}}$ . Along the strong biogeochemical gradient of the Scheldt estuary,  $\delta^{13}\text{C}_{\text{R}}$  was linearly related to  $\delta^{13}\text{C}_{\text{DIC}}$  ( $R^2 = 0.87$ ), which in turn was linearly related to salinity ( $R^2 = 0.94$ ). The mussels were highly selective, assimilating most of their carbon from phytoplankton out of the total particulate organic carbon (POC) pool. However, on a seasonal basis, tissue  $\delta^{13}\text{C}$  varies differently than  $\delta^{13}\text{C}_{\text{DIC}}$  and  $\delta^{13}\text{C}_{\text{POC}}$ , most likely due to lipid content of the tissue. All shells contained less than 10 % metabolic C, but ranged from near zero to 10 %, thus excluding the use of  $\delta^{13}\text{C}$  in these shells as a robust  $\delta^{13}\text{C}_{\text{DIC}}$  or salinity proxy. As an example, an error in salinity of about 5 would have been made at one site. Nevertheless, large changes in  $\delta^{13}\text{C}_{\text{DIC}}$  ( $>2\text{‰}$ ) can be determined using *M. edulis* shell  $\delta^{13}\text{C}$ . Preliminary hemolymph  $\delta^{13}\text{C}$  data are presented and suggest that salinity affects the  $\delta^{13}\text{C}$  of the internal DIC pool of bivalves independently from the external  $\delta^{13}\text{C}_{\text{DIC}}$ .

## 1. INTRODUCTION

The incorporation of respired  $^{13}\text{C}$  depleted carbon into the skeletons of aquatic invertebrates is well documented (Tanaka et al., 1986; McConnaughey et al., 1997; Lorrain et al., 2004a; Swart et al., 2005). The fluid from which these animals calcify is a ‘pool’ of metabolic  $\text{CO}_2$  and external dissolved inorganic carbon (DIC), which both affect the skeletal stable carbon isotopic signature ( $\delta^{13}\text{C}_\text{S}$ ). The amount of respired carbon ending up in the skeleton is species specific, with most aquatic animals incorporating less than 10 % (or  $< 2$  ‰ offset from  $\delta^{13}\text{C}_\text{S}$  equilibrium with  $\delta^{13}\text{C}_\text{DIC}$  in marine settings) (McConnaughey et al., 1997; Kennedy et al., 2001; Lorrain et al., 2004a; Chapter 4), but may be as high as 35 % (Chapter 5). Therefore it is of interest to have a better understanding of what controls the  $\delta^{13}\text{C}$  value of respired  $\text{CO}_2$ .

The  $\delta^{13}\text{C}$  value of respired  $\text{CO}_2$  ( $\delta^{13}\text{C}_\text{R}$ ) can be roughly estimated from the tissue  $\delta^{13}\text{C}$  value. At the pH of *M. edulis* body fluids (7 – 8; Crenshaw, 1972), more than 90 % of  $\text{CO}_2$  hydrates and ionizes to produce  $\text{HCO}_3^-$ , which should be at most 1 ‰ enriched in  $^{13}\text{C}$  compared to the respiring tissue (McConnaughey et al., 1997). Yet, due to other processes affecting the  $\delta^{13}\text{C}_\text{R}$  (e.g., the type of material being respired such as lipids) it can roughly be considered to be 0.5 ‰ heavier than the tissues (McConnaughey et al., 1997). However, a recent study on a zooxanthellate scleractinian coral suggested that  $\delta^{13}\text{C}_\text{R}$  might not always follow tissue  $\delta^{13}\text{C}$  (Swart et al., 2005). The amount of respired  $\text{CO}_2$  in the skeleton can be approximated using the equation of McConnaughey et al. (1997) (see also previous chapter):

$$M(\delta^{13}\text{C}_\text{R}) + (1 - M) * \delta^{13}\text{C}_\text{DIC} = \delta^{13}\text{C}_\text{S} - \varepsilon_{\text{cl-b}} \quad (1)$$

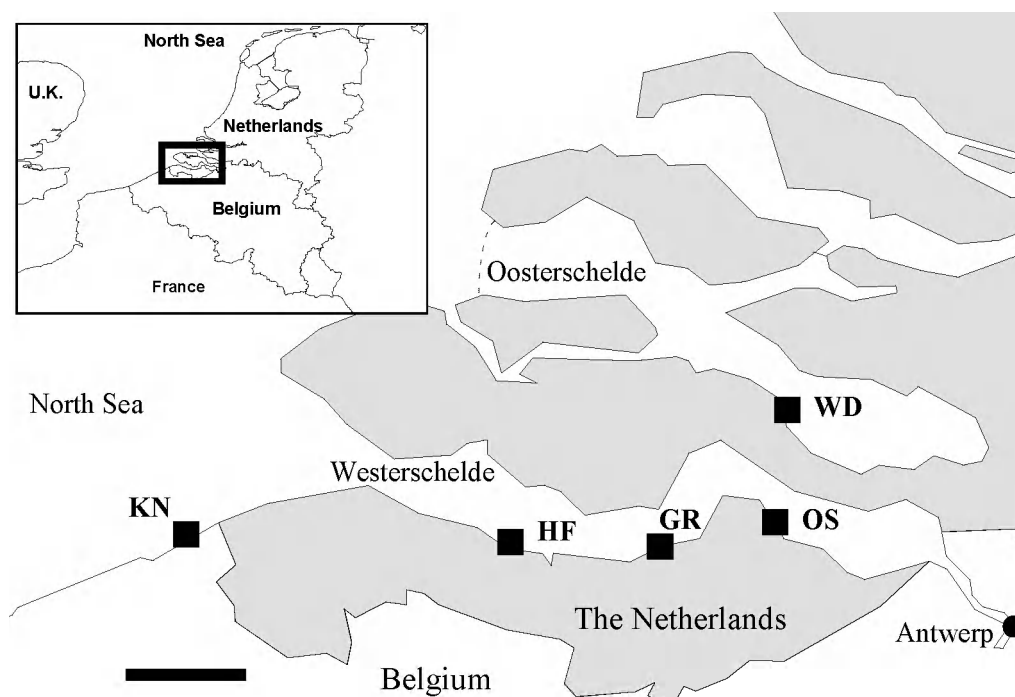
where  $M$  is the percent metabolic  $\text{CO}_2$  contribution and  $\varepsilon_{\text{cl-b}}$  is the enrichment factor between calcite and bicarbonate ( $1.0 \pm 0.2$  ‰ in Romanek et al., 1992). Other factors may also play a role in determining the  $\delta^{13}\text{C}$  value of the internal DIC pool. For example, the enzyme carbonic anhydrase, which catalyses the reaction of bicarbonate to  $\text{CO}_2$ , which can more easily diffuse through membranes (Paneth and O’Leary, 1985), may add or remove carbon species from this pool (see 3.2.2.1 of Chapter 1).

Considering that many bivalves incorporate only a small amount of respired CO<sub>2</sub>, their skeletons should be able to trace large changes in  $\delta^{13}\text{C}_{\text{DIC}}$ , as was found by Mook and Vogel (1968) and Mook (1971) for *M. edulis* in the Schelde estuary. This is also true if the offset is constant as was found in a freshwater mussel (Kaandorp et al., 2003). Such data could then be useful for roughly determining the salinity where the animals grew, which could be a valuable addition to the interpretation of shell  $\delta^{18}\text{O}$  profiles (see Chapter 4). Therefore, the  $\delta^{13}\text{C}$  values of *M. edulis* shells and mantle tissues, DIC, and particulate organic carbon (POC) were measured across a salinity gradient and over one year. Additionally, a preliminary experiment on the  $\delta^{13}\text{C}$  of hemolymph was conducted in the laboratory at different  $\delta^{13}\text{C}_{\text{DIC}}$  values and a different salinity.

## 2. MATERIALS AND METHODS

### 2.1 Field data collection

Mussels were collected from the intertidal zone of the Schelde estuary from Knokke (KN) and Hooftplaat (HF) on 17 March 2002 and from Griete (GR) and Ossenisse (OS) on 23 March 2002 (Fig. 1). In addition, mussels were also sampled from HF on 3 May, 28 July, and 29 September and from KN on 3 May and 28 July 2002. Mantle tissues were collected using a scalpel and stored frozen until preparation. During preparation, tissues were dried in an oven at 60 °C for 24 hours, homogenized with a mortar and pestle, and ~1 mg material was placed into a silver cup. 2 - 3 drops of 5 % HCl was added and the cups were allowed to dry in an oven overnight after which they were folded closed. Tissue  $\delta^{13}\text{C}$  was measured on an Element Analyzer (Flash 1112 Series EA ThermoFinnigan) coupled via a CONFLO III to an IRMS (Delta<sup>plus</sup>XL, ThermoFinnigan). Using this same instrument and method, Verheyden et al. (2004) report a long term analytical precision for  $\delta^{13}\text{C}$  of 0.08 ‰ on 214 analyses of the IAEA-CH-6 standard (1 $\sigma$ ). Shells were sectioned along the axis of major growth and samples were drilled from the calcite layer along the growth-time axis. Carbonate powders were reacted in a Kiel III coupled to a ThermoFinnigan Delta<sup>plus</sup>XL dual inlet IRMS with a long-term  $\delta^{13}\text{C}_{\text{S}}$  precision of 0.039 ‰ (see Chapter 2 for more details).



**Figure 1.** Map of the Westerschelde estuary. The four study sites are indicated Knokke (KN), Hooftplaat (HF), Griete (GR) and Ossenisse (OS). Scale bar = 10 km.

Near-shore water samples were collected at least monthly from Nov. 2001 to Oct. 2002 for chlorophyll *a* concentrations (Chl *a*),  $\delta^{13}\text{C}_{\text{DIC}}$ ,  $\delta^{13}\text{C}_{\text{POC}}$  and suspended particulate matter (SPM). Chlorophyll *a* was measured using standard protocols (see Chapter 2). The  $\delta^{13}\text{C}_{\text{DIC}}$  was determined by acidifying 5 ml of water in an 8 ml helium flushed headspace vial, overnight equilibration, and subsequently injecting 400  $\mu\text{l}$  of the headspace into the carrier gas stream of the continuous flow IRMS. Precision of  $\delta^{13}\text{C}_{\text{DIC}}$  was better than 0.2 ‰ based on replicate measurements; data were corrected using calibrated  $\text{CO}_2$  gas according to Miyajima et al. (1995) (see Chapter 2). The  $\delta^{13}\text{C}_{\text{POC}}$  was measured following Lorrain et al. (2003) (see Chapter 2 for more details) and SPM is based on the dry weights of these filters.

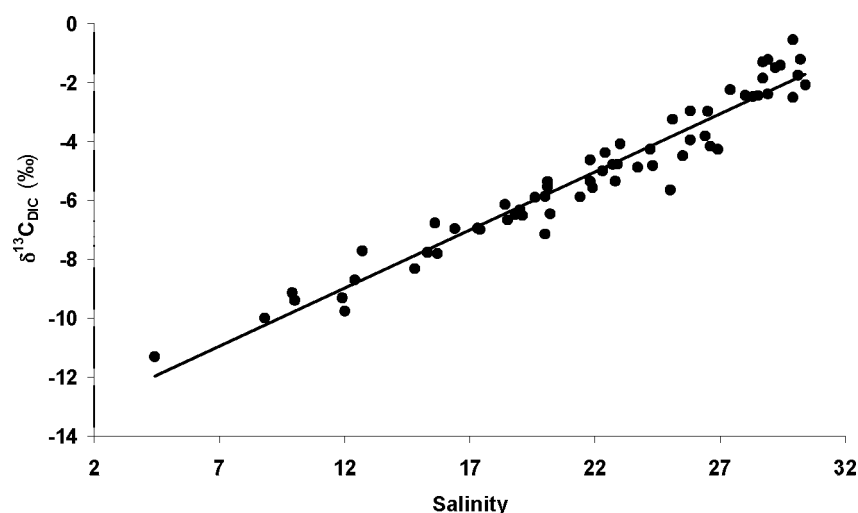
## 2.2 Laboratory experiment

Mussels were held in four tanks for more than one month and were fed yeast. Three tanks had the same salinity (35) and varying  $\delta^{13}\text{C}_{\text{DIC}}$  values, while one tank had a salinity of 19. The  $\delta^{13}\text{C}_{\text{DIC}}$  of the water in the tanks was measured as described above. Two to five mussels per tank were sampled. Hemolymph samples were drawn from the adductor muscle of the mussels with a sterile needle and syringe, injected into He

flushed 2 ml headspace vials, and were analyzed similar to water  $\delta^{13}\text{C}_{\text{DIC}}$ , except 1000  $\mu\text{l}$  was injected into the IRMS.

### 3. RESULTS

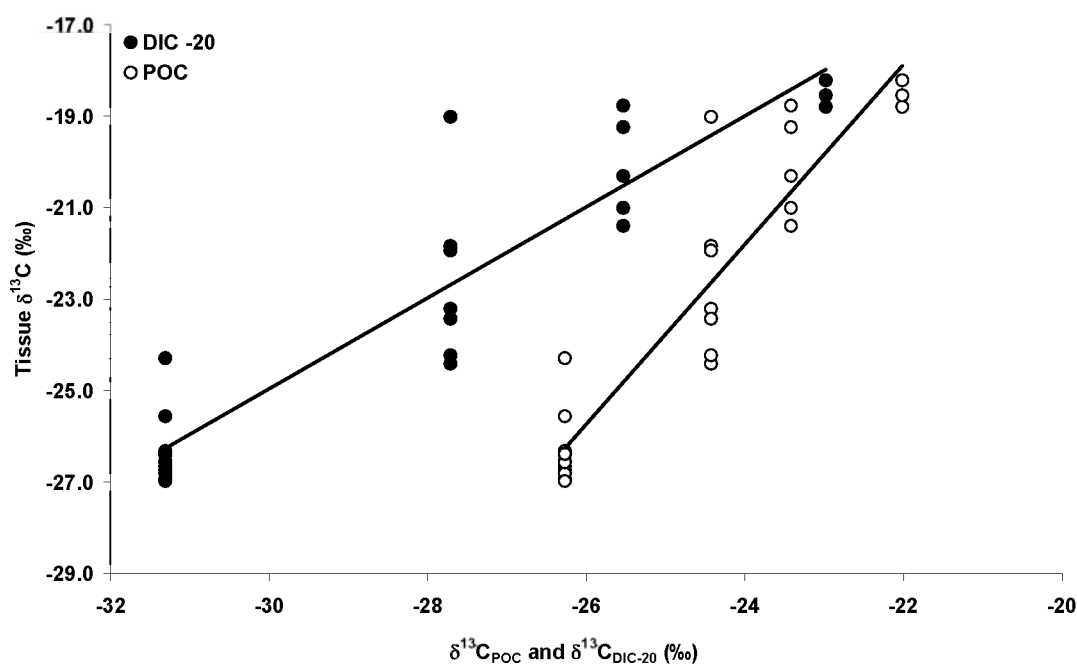
The  $\delta^{13}\text{C}_{\text{DIC}}$  was strongly related to salinity with the linear relationship:  $\delta^{13}\text{C}_{\text{DIC}} = \text{Salinity} * 0.39 (\pm 0.03) - 13.71 (\pm 0.57)$  ( $R^2 = 0.94$ ,  $p < 0.0001$ ,  $n = 63$ ; for the salinity range of  $\sim 5$  to 30) (Fig. 2). To approximate the  $\delta^{13}\text{C}$  value of phytoplankton, 20 ‰ was subtracted from the  $\delta^{13}\text{C}_{\text{DIC}}$  values (see discussion). There were strong linear relationships between mantle tissue and both  $\delta^{13}\text{C}_{\text{POC}}$  and  $\delta^{13}\text{C}_{\text{DIC}} - 20$  ‰ ( $\delta^{13}\text{C}_{\text{DIC}-20}$ ; Fig. 3) for samples collected in March. The slope between mantle tissue and  $\delta^{13}\text{C}_{\text{DIC}-20}$  was not significantly different from one ( $p < 0.0001$ ). Mantle tissue varied considerably throughout the year at HF and KN with a 2 to 3 ‰ decrease between March and September (Fig. 4). In both sites, mantle tissue was least negative in March, just before the phytoplankton bloom, but was more similar to the  $\delta^{13}\text{C}$  of potential food sources in May, July and September.



**Figure 2.**  $\delta^{13}\text{C}_{\text{DIC}}$  versus salinity from samples taken over one year along the Schelde estuary with the relationship:  $\delta^{13}\text{C}_{\text{DIC}} = \text{Salinity} * 0.39 (\pm 0.03) - 13.71 (\pm 0.57)$  ( $R^2 = 0.94$ ,  $p < 0.0001$ ,  $n = 63$ ; for the salinity range of  $\sim 5$  to 30).

Using the  $\epsilon_{\text{cl-b}}$  from Romanek et al. (1992), shells from KN were on average not in equilibrium with  $\delta^{13}\text{C}_{\text{DIC}}$ , but the three other sites were (Fig. 5). Although the regression using the mean of all four sites is significant ( $p = 0.039$ ,  $R^2 = 0.94$ ),

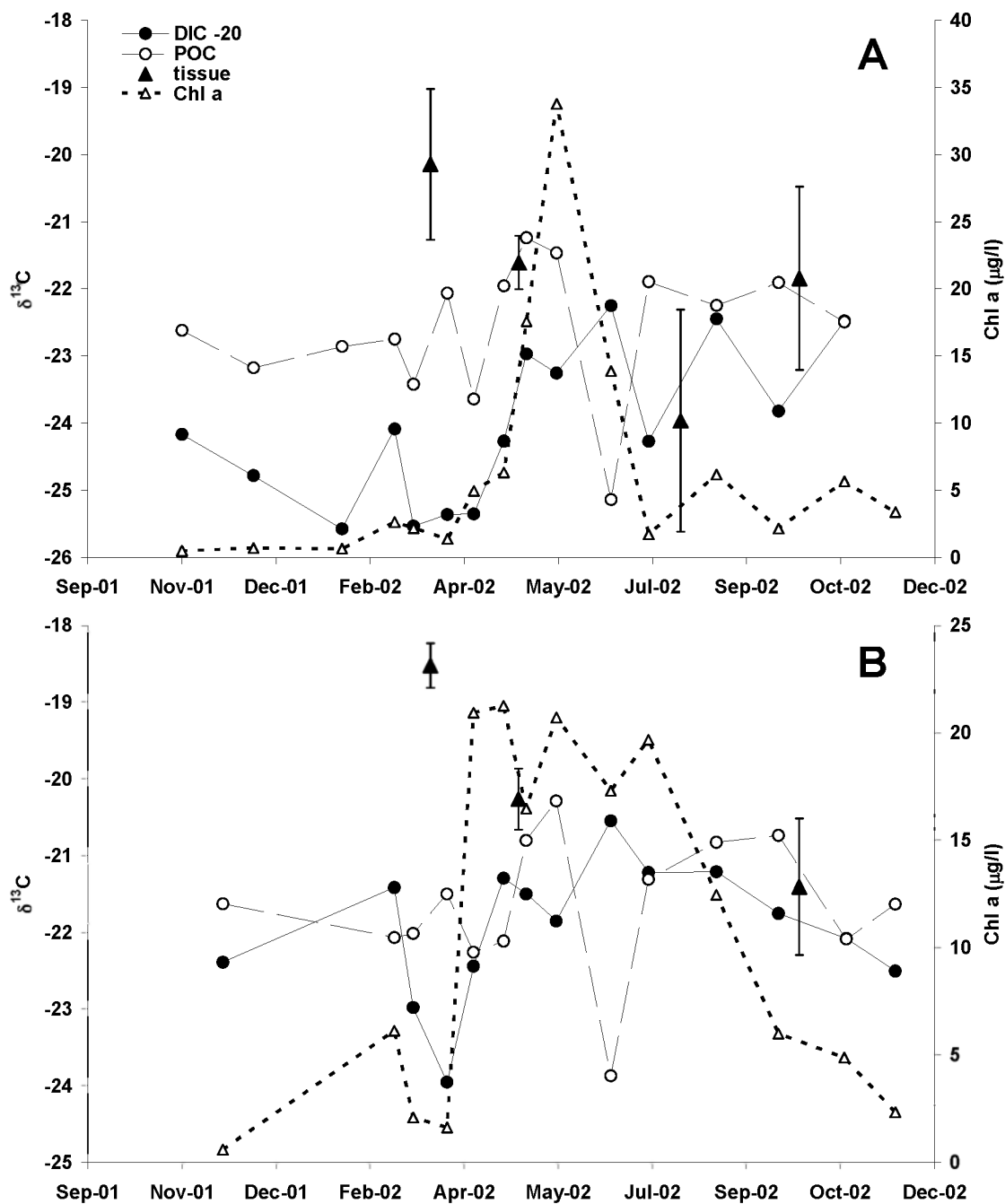
removing KN results in a regression line which perfectly bisects the three means (Fig. 5). The intercept of this latter model is  $1.65 (\pm 0.001)$ , which is close to the expected equilibrium value with  $\delta^{13}\text{C}_{\text{DIC}} = 0 \text{ ‰}$  (i.e.,  $+1.0 \pm 0.2 \text{ ‰}$ , Romanek et al., 1992). High-resolution  $\delta^{13}\text{C}_\text{s}$  profiles can be found in Figure 7 of Chapter 10, and show that in general,  $\delta^{13}\text{C}_\text{s}$  is more negative in spring.



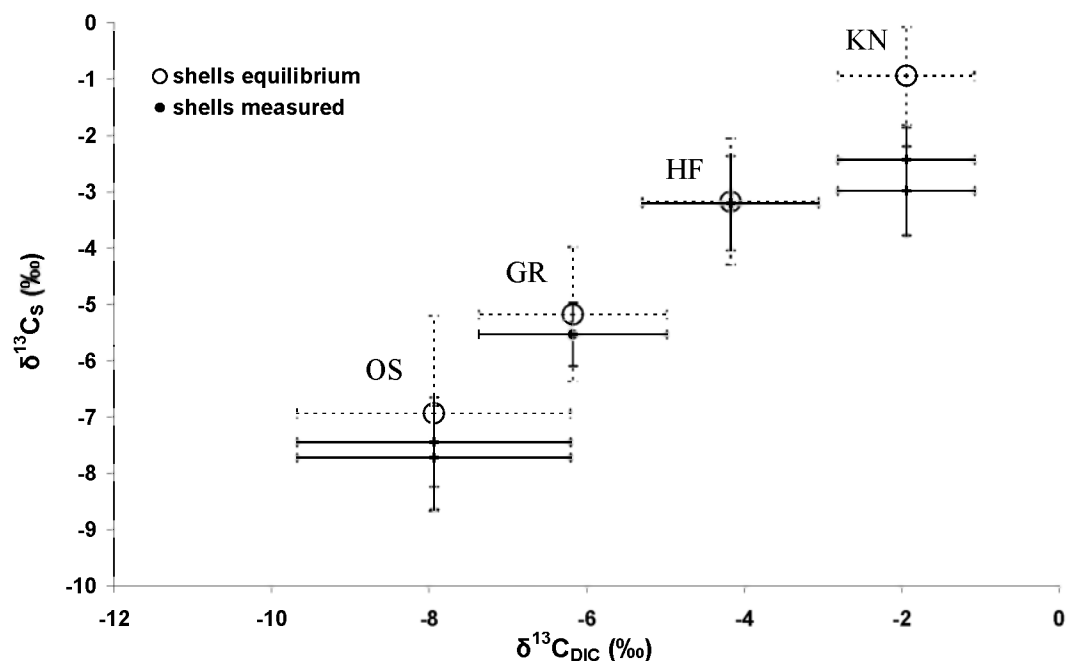
**Figure 3.** Linear regressions between mantle tissue  $\delta^{13}\text{C}$  and both  $\delta^{13}\text{C}_{\text{POC}}$  and  $\delta^{13}\text{C}_{\text{DIC}} - 20 \text{ ‰}$  from mussels collected in March 2002.  $N = 27$  for all. The relationships are: Tissue  $\delta^{13}\text{C} = 0.99 (\pm 0.16) * \delta^{13}\text{C}_{\text{DIC-20}} + 4.89 (\pm 4.48)$  ( $R^2 = 0.87$ ,  $n = 27$ ,  $p > 0.0001$ ), and Tissue  $\delta^{13}\text{C} = 1.97 (\pm 0.31) * \delta^{13}\text{C}_{\text{POC}} + 25.39 (\pm 7.87)$  ( $R^2 = 0.87$ ,  $n = 27$ ,  $p > 0.0001$ ).

Hemolymph  $\delta^{13}\text{C}$  values were linear with  $\delta^{13}\text{C}_{\text{DIC}}$  between the three tanks with a salinity of 35, while hemolymph  $\delta^{13}\text{C}$  values were more negative in the lower salinity tank despite  $\delta^{13}\text{C}_{\text{DIC}}$  being in the same range (Fig. 6). However, these data should be regarded with caution. Although preliminary attempts to determine if there was an effect of using small headspace vials (2 ml; usually, 8 – 20 ml vials are used) suggested that there was no effect, later attempts could not reproduce this. No satisfactory explanation could be found for this. Nevertheless, the fact that there was no effect of vial size during the period that these samples were measured, and the reproducibility between mussels from the same tank, indicates that the more negative hemolymph  $\delta^{13}\text{C}$  values in the lower salinity tank is probably not an artifact of measurement.

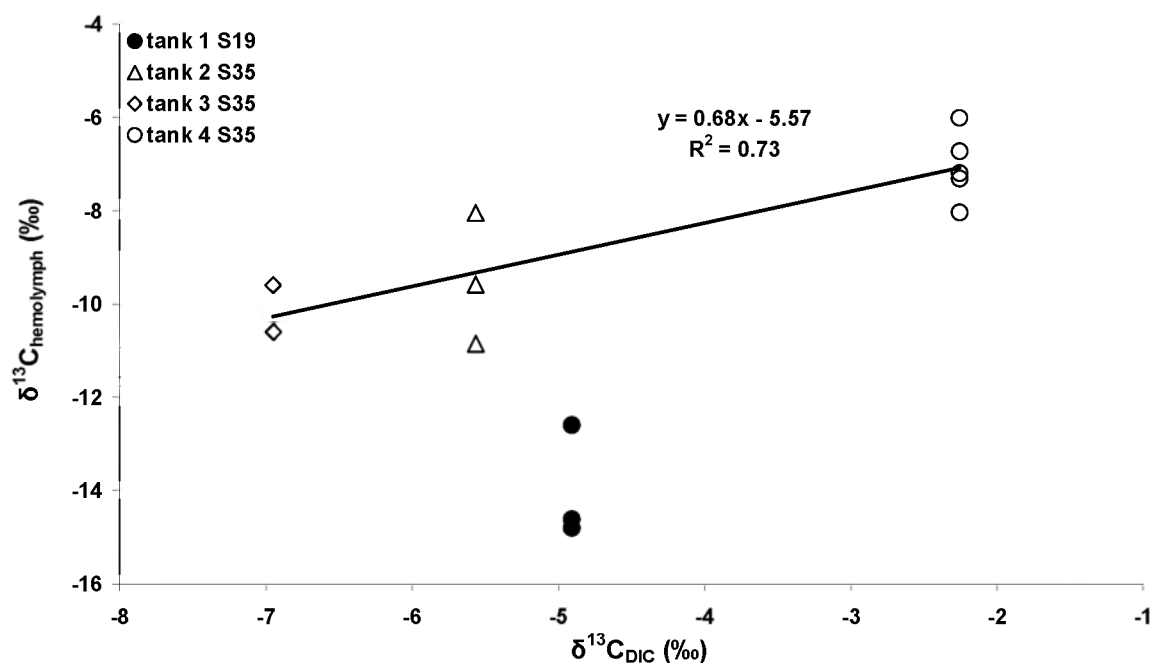




**Figure 4.** Seasonal samples of mantle tissue  $\delta^{13}\text{C}$ ,  $\delta^{13}\text{C}_{\text{POC}}$ ,  $\delta^{13}\text{C}_{\text{DIC-20}}$ , and chlorophyll *a* taken from Hooftplaat (A) and Knokke (B). Error bars represent standard deviations.



**Figure 5.** Mean  $\delta^{13}\text{C}_{\text{s}}$  and  $\delta^{13}\text{C}_{\text{DIC}}$  averaged over the full year for the four sites (noted above data points, see Fig. 1 for description of site codes). High-resolution profiles can be found in Figure 7 of Chapter 10. Also plotted are the expected shell values based on the fractionation factor between  $\delta^{13}\text{C}_{\text{DIC}}$  and calcite (+1.0 ‰; Romanek et al., 1992). Error bars represent standard deviations.



**Figure 6.** Hemolymph  $\delta^{13}\text{C}$  from laboratory held mussels. Mussels were held in four tanks, three with a salinity of 35 (Tanks 2, 3 and 4) and one with a salinity of 19 (Tank 1).

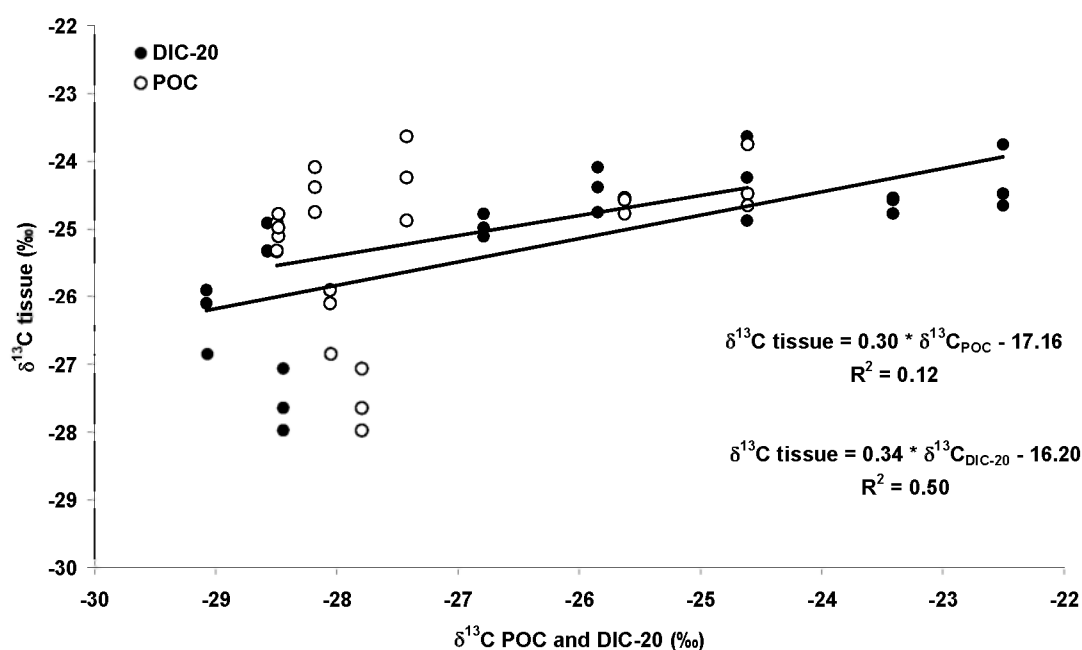
## 4. DISCUSSION

Although it is well established that the carbon isotope fractionation between phytoplankton and DIC is variable (Rau et al., 1992; Hinga et al., 1994; Boschker et al., 2005), a value between 18 and 22 ‰ is often used as an estimate (Cai et al., 1988; Hellings et al., 1999; Fry, 2002; Bouillon et al., 2004b). Therefore, similar to Fry (2002), the average value of 20 ‰ is used. From Fig. 3 it is clear that *M. edulis* is highly selective as the slope between the expected  $\delta^{13}\text{C}$  of phytoplankton and tissues is not significantly different from one, whereas the slope between  $\delta^{13}\text{C}$  of tissues and  $\delta^{13}\text{C}_{\text{POC}}$  was 2.0 ( $\pm 0.3$ ).  $\delta^{13}\text{C}_{\text{POC}}$  and  $\delta^{13}\text{C}_{\text{DIC}}$  were also significantly correlated ( $\delta^{13}\text{C}_{\text{POC}} = 0.42 (\pm 0.09) * \delta^{13}\text{C}_{\text{DIC}} - 21.0 (\pm 0.5)$ ;  $R^2 = 0.61$ ,  $n = 59$ ,  $p < 0.0001$ ). The POC is a mixture of different sources of carbon, each with their own  $\delta^{13}\text{C}$  values, such as phytoplankton, terrestrial carbon (in general,  $\sim -26$  ‰ from C3 plants and  $\sim -14$  ‰ from C4 plants; Mook and Tan, 1991), resuspended sediments (Schelde:  $\sim -19$  to  $-24$  ‰; Middelburg and Nieuwenhuize, 1998; Herman et al., 2000), marine macro-algae detritus (Schelde: green algae  $\sim -17$  ‰, brown algae  $\sim -25$  ‰; Gillikin unpublished data), microphytobenthos (Schelde:  $\sim -15$  ‰; Middelburg et al., 2000; Herman et al., 2000), and other substances from which the mussels must select from. As these samples were taken near the shore, there was probably a large amount of suspended sediments, which is indicated by the high SPM content (range = 13 to 550 mg/l, mean = 86 mg/l). Selection can occur both at the gills (pre-ingestive) and in the gut (post-ingestive) (reviewed in Ward and Shumway, 2004), but using  $\delta^{13}\text{C}$  as a tracer deals only with assimilated carbon. Moreover, using the selectivity equation from Bouillon et al. (2004b),

$$\text{Selectivity} = (\Delta\delta^{13}\text{C}_{\text{tissue}} - \Delta\delta^{13}\text{C}_{\text{POC}} / \Delta\delta^{13}\text{C}_{\text{DIC}} - \Delta\delta^{13}\text{C}_{\text{POC}}) * 100 [\%] \quad (2)$$

where  $\Delta$  is the overall estuarine gradient in tissue, POC and DIC  $\delta^{13}\text{C}$  values (assumes that selectivity is similar at all stations, see Bouillon et al., 2004b), suggests that they are  $\sim 90$  % selective, which further illustrates that they primarily assimilate their carbon from phytoplankton, which in turn obtains its carbon from the DIC. It is generally accepted that the  $\delta^{13}\text{C}$  value of an organism reflects the  $\delta^{13}\text{C}$  value of its diet with little (1 ‰) or no change (DeNiro and Epstein, 1978; Fry and Sherr, 1984).

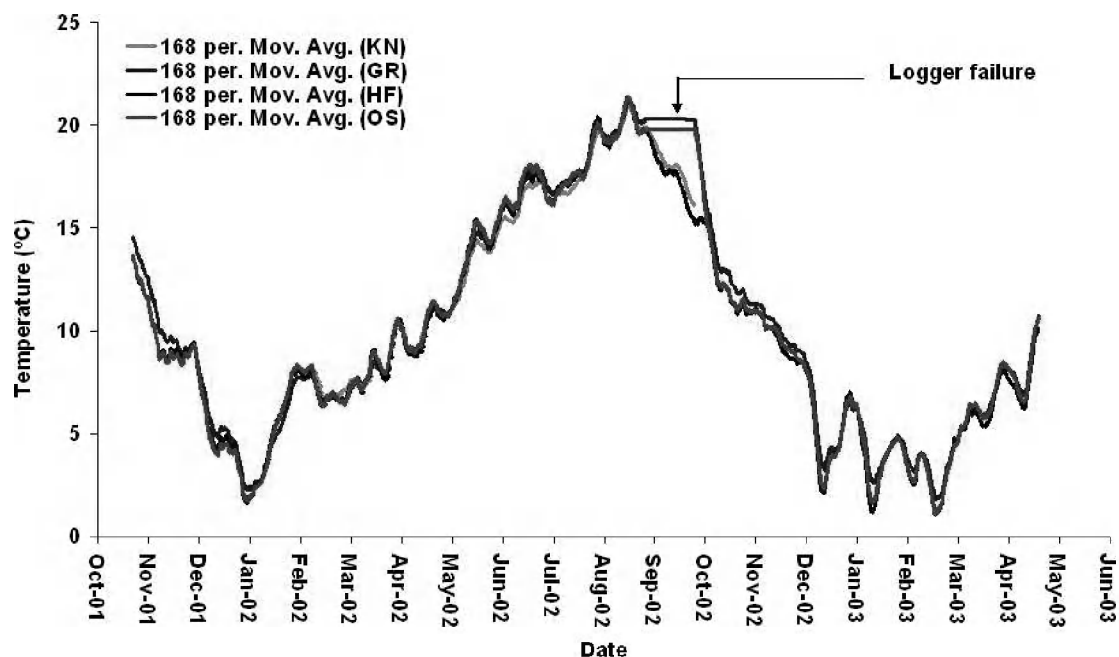
Therefore, the intercept between tissue  $\delta^{13}\text{C}$  and  $\delta^{13}\text{C}_{\text{DIC-20}}$  should be +1. However, it should be kept in mind that the 20 ‰ fractionation used here is a rough estimate. The intercept of  $4.89 \pm 4.48$  ‰ in Fig. 3 can therefore be explained by an error in the phytoplankton fractionation as well as the individual variation in tissue  $\delta^{13}\text{C}$ . Moreover, errors in this simplified model can arise from the mussels feeding on other food items. Mussels have been shown to feed on dissolved organic carbon (DOC) (Roditi et al., 2000), their own and other bivalve larvae (Lehane and Davenport, 2004), zooplankton (Lehane and Davenport, 2002; Wong et al., 2003), and macroalgae detritus (Levinton et al., 2002); all with different  $\delta^{13}\text{C}$  values (see above). Nevertheless, as a first approximation,  $\delta^{13}\text{C}_\text{R}$  values should roughly follow DIC, as has been noticed in other bivalves (e.g., Fry, 2002). However, Swart et al. (2005) found that  $\delta^{13}\text{C}_\text{R}$  from a coral significantly deviated from the  $\delta^{13}\text{C}$  of tissues (both positive and negative deviations of up to 3 ‰), which they attributed to different compounds (e.g., lipids) being respired at different times of the year.



**Figure 7.** Ribbed mussel (*Geukensia demissa*) bulk tissue  $\delta^{13}\text{C}$  versus both  $\delta^{13}\text{C}_{\text{POC}}$  and  $\delta^{13}\text{C}_{\text{DIC-20}}$  from mussels collected in August 2004 from along a salinity gradient in a North Carolina salt marsh (N = 24). Data from Gillikin and Bouillon (unpublished).

Although it might seem obvious that the  $\delta^{13}\text{C}$  of filter feeders that eat phytoplankton, which in turn assimilate their carbon from DIC, would all be related to the  $\delta^{13}\text{C}_{\text{DIC}}$ , as is the case with many bivalves (e.g., Fry, 2002), the relationship does not always hold true. For example, despite the linear relationship between salinity and  $\delta^{13}\text{C}_{\text{DIC}}$  in a

North Carolina salt marsh creek ( $\delta^{13}\text{C}_{\text{DIC}} = 0.44 * \text{Salinity} - 14.45$ ,  $R^2 = 0.97$ ,  $n = 15$ , for the salinity range of 1 – 29; Gillikin and Bouillon, unpublished data), tissues collected from the ribbed mussel, *Geukensia demissa*, did not follow either  $\delta^{13}\text{C}_{\text{POC}}$  or  $\delta^{13}\text{C}_{\text{DIC-20}}$  (Fig. 7). Consequently, no correlation between shell  $\delta^{13}\text{C}$  and  $\delta^{13}\text{C}_{\text{DIC}}$  was noticed in these specimens (data not shown).



**Figure 8.** Water temperature recorded hourly using Onset TidBit dataloggers at all four sites. The weekly running average is shown. The loggers failed at two sites for about a month as is indicated on the graph.

From Fig. 4 it is evident that the relationship found in March (Fig. 3) does not necessarily hold true for the whole year. This could be contributed to changing food sources, such as resuspended benthic algae, or variable fractionation between phytoplankton and DIC throughout the year. Indeed, Boschker et al. (2005) found that DIC – diatom fractionation varied from about 16 ‰ to 24 ‰ along this same estuary. Other factors such as temperature and phytoplankton growth rate can also influence the fractionation between phytoplankton and DIC (see Savoye et al., 2003). However, a possible explanation is changing lipid concentration in *M. edulis* tissues. In *M. edulis*, the mantle contains much of the gonad (Morton, 1992); and in this region, *M. edulis* spawning peaks when temperatures exceed approximately 10 °C (Hummel et al., 1989). At all four sites this occurs in mid-March (Fig. 8), approximately at the same time as the tissue samples were collected. In March the mussels have probably just spawned and therefore the tissues have a low lipid content (see de Zwaan and

Mathieu, 1992). Lipids are known to have significantly more negative  $\delta^{13}\text{C}$  than other biochemical components (Abelson and Hoering, 1961; Tieszen et al., 1983; Focken and Becker, 1998; Lorrain et al., 2002), and the mantle exhibits a sharp drop in lipid content just after spawning (de Zwaan and Mathieu, 1992), thus explaining the more positive tissue  $\delta^{13}\text{C}$  values in this month. After the phytoplankton bloom, which begins in April or May, the lipid content of the tissues is restored, thus lowering the  $\delta^{13}\text{C}$  value. Indeed, Lorrain et al. (2002) found that  $\delta^{13}\text{C}$  of scallop tissues were highest in spring when lipids were low and decreased as lipids increased toward late summer. However in shells, the spawning period is reflected by more negative  $\delta^{13}\text{C}$  values (Fig. 7 of Chapter 10) although the  $\delta^{13}\text{C}_{\text{DIC}}$  is generally becoming more positive (Fig. 4). This could possibly be explained by high metabolic rates just after spawning, to restore the energy lost during the spawn. Vander Putten et al. (2000) also described these patterns in  $\delta^{13}\text{C}_s$  in *M. edulis* from the Schelde as being a result of increased respiration associated with periods of high food availability.

Despite the variability in tissue  $\delta^{13}\text{C}$  throughout the year, the mean shell values roughly match equilibrium values for three of the four sites (Fig. 5). The differences between measured and predicted values vary between sites (Table 1), with salinity apparently having little to do with disequilibrium. According to the hemolymph  $\delta^{13}\text{C}$  data from the laboratory experiment, salinity affects the internal DIC pool  $\delta^{13}\text{C}$  value (Fig. 6), which then would be expected to affect  $\delta^{13}\text{C}_s$ . The laboratory hemolymph data show that at a lower salinity, hemolymph  $\delta^{13}\text{C}$  is more negative, while in opposition, shells from the highest salinity field site (KN) were more negative than equilibrium predicts (Fig. 5). Nevertheless, all shells generally fall within the 10 % metabolic C incorporation suggested to be typical for aquatic marine invertebrates by McConnaughey et al. (1997) (Table 1).

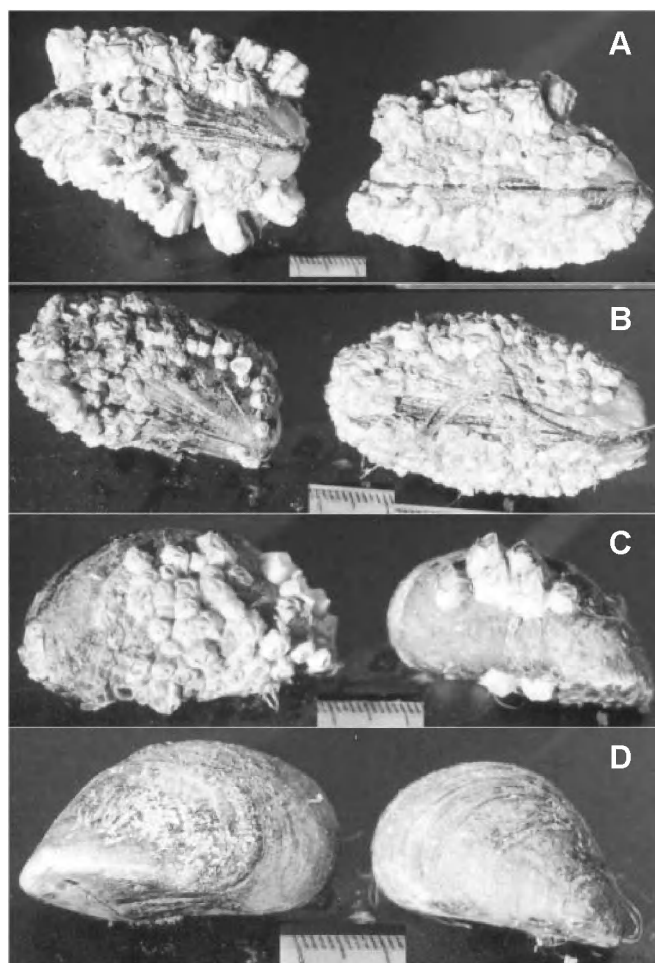
**Table 1.** Predicted minus measured  $\delta^{13}\text{C}_s$  (pred – meas, in ‰) and percent metabolic C incorporation (%M) in the shells at each site. %M calculated using average data and the equation of McConnaughey et al. (1997, see eq. 1).

	KN1	KN2	HF	GR	OS1	OS2
Salinity*	29	29	25	20	14	14
pred – meas	2.04	1.49	0.03	0.36	0.51	0.79
%M	10.9	8.0	0.2	2.3	3.0	4.7

\*Annual mean.

Although  $\delta^{13}\text{C}_\text{R}$  does not seem to largely affect the  $\delta^{13}\text{C}_\text{S}$  ( $\sim < 10\%$  incorporation of metabolic  $\text{CO}_2$  into the shell), the variability in the percent incorporated is enough to preclude its use as a robust  $\delta^{13}\text{C}_\text{DIC}$  proxy, and hence a salinity proxy. For example, if the  $\delta^{13}\text{C}_\text{S}$  values of the KN shell were used to predict  $\delta^{13}\text{C}_\text{DIC}$  and salinity, one would conclude that this shell came from a site similar to HF (Fig. 5), even though the salinity difference between these sites is typically around 5. From Fig. 5, it may seem that mussel shells from the same environment could be used to determine  $\delta^{13}\text{C}_\text{DIC}$ , but *Mercenaria mercenaria* shells collected from similar environments had very different metabolic contributions to their shells (Chapter 5), suggesting this might result in large errors. These data do not provide an explanation why the KN shells were farther from equilibrium than the others, but it could be the result of higher metabolic rates caused by the stronger wave action at this site, which increases water flow and thus food availability. Moderate wave action has been shown to increase growth rates and condition values in *Mytilus* (Steffani and Branch, 2003), which would lead to higher metabolic rates. There are also other possibilities which can increase metabolic rate, such as epibiont cover (such as barnacles (Buschbaum and Saier, 2001), which there are more of at the KN site, see Fig. 9), exposure to predators (Frandsen and Dolmer, 2002), and pollution (Wang et al., in press).

The difference between the results presented here and those from earlier studies on the same species and estuary (i.e., Mook and Vogel, 1968; Mook, 1971), who state that  $\delta^{13}\text{C}_\text{S}$  is a good proxy of  $\delta^{13}\text{C}_\text{DIC}$ , can be caused by many factors. First, these earlier studies did not separate aragonite and calcite from the shells, which greatly differ in equilibrium  $\delta^{13}\text{C}$  values with  $\text{HCO}_3^-$  (i.e.,  $+1\text{‰}$  for calcite and  $+2.7\text{‰}$  for aragonite; Romanek et al., 1992). Second, they roasted their samples and found significant differences between roasted and non-roasted  $\delta$ -values, while Vander Putten et al. (2000) found no difference in calcite from this same species, indicating a possible isotopic alteration in these earlier studies. Finally, these earlier works did not consider metabolic effects and perhaps did not sample populations with markedly different metabolic rates.



**Figure 9.** Barnacle cover on caged mussels along the Schelde Estuary from Knokke (A), Hooftplaat (B), Griete (C), and Ossenisse (D). Scales are in mm.

The salinity effect on the  $\delta^{13}\text{C}$  of the internal DIC pool is an interesting finding, but difficult to explain. It could be the result of increased metabolism in the osmotically stressed mussels, but *M. edulis* is an osmoconformer (Newell, 1989), so this does not seem probable. An alternative hypothesis is that carbonic anhydrase (CA) is being affected by the change in salinity. The enzyme CA is responsible for ion exchange at the gills, and has been shown to correlate with growth in bivalves and to be involved in respiration and acid-base regulation (Duvail et al., 1998). Activity of CA is known to change with salinity in some bivalves, but again, is tied to osmoregulation (Henry and Saintsing, 1983), so does not apply to *M. edulis* since they do not osmoregulate. Therefore, salinity should not affect CA activity in *M. edulis*, but CA activity has been shown to be inhibited by  $\text{Cl}^-$  ions (Pocker and Tanaka, 1978). A reduction in CA activity could cause a reduction in environmental DIC entering the animal, resulting in a larger ratio of metabolic DIC and more negative  $\delta^{13}\text{C}$  in the hemolymph.



In conclusion, although  $\delta^{13}\text{C}_\text{R}$  values can closely follow  $\delta^{13}\text{C}_\text{DIC}$  values and the percentage of metabolic C incorporated into the shells of *M. edulis* is low, the variability in metabolic C incorporation is too high to allow confident salinity determinations based on  $\delta^{13}\text{C}_\text{S}$ . The example presented here could not differentiate between two sites with a salinity difference of 5, which in terms of  $\delta^{18}\text{O}$  paelothermometry would correspond to about 4 °C (see Chapter 1). Thus  $\delta^{13}\text{C}_\text{S}$  is not a robust proxy of environmental conditions in *M. edulis* calcite, but may be useful for determining metabolic differences between different populations and can still be used as an indicator of large  $\delta^{13}\text{C}_\text{DIC}$  (and salinity) differences.

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