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Organic Geochemistry 37 (2006) 1371–1382

Organic Geochemistry

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Stable carbon isotopic composition of *Mytilus edulis* shells: relation to metabolism, salinity, $\delta^{13}C_{DIC}$ and phytoplankton

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Received 25 August 2005; accepted 6 March 2006
Available online 9 June 2006

Abstract

Bivalve shells can potentially record the carbon isotopic signature of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) in estuarine waters, thereby providing information about past estuarine biogeochemical cycles. However, the fluid from which these animals calcify is a 'pool' of metabolic CO_2 and external dissolved inorganic carbon (DIC). The incorporation of respired ^{13}C-depleted carbon into the skeletons of aquatic invertebrates is well documented, and may affect the $\delta^{13}C$ record of the skeleton. 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 $\delta^{13}C$ value of bivalve shells. In this study, it is assessed if the $\delta^{13}C$ values of *Mytilus edulis* shells can be used as a proxy of $\delta^{13}C_{DIC}$ as well as providing an indication of salinity. First, the $\delta^{13}C$ values of respired CO_2 ($\delta^{13}C_R$) were considered using the $\delta^{13}C$ values of soft tissues as a proxy for $\delta^{13}C_R$. Along the strong biogeochemical gradient of the Scheldt estuary (The Netherlands–Belgium), $\delta^{13}C_R$ was linearly related to $\delta^{13}C_{DIC}$ ($r^2 = 0.87$), 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 $\delta^{13}C$ varied differently than $\delta^{13}C_{DIC}$ and $\delta^{13}C_{POC}$, most likely due to lipid content of the tissue. All shells contained less than 10% metabolic carbon, but ranged from near zero to 10%, thus excluding the use of $\delta^{13}C$ in these shells as a robust $\delta^{13}C_{DIC}$ or salinity proxy. As an example, an error in salinity of about 5 would have been made at one site. Nevertheless, large changes in $\delta^{13}C_{DIC}$ (>2‰) can be determined using *M. edulis* shell $\delta^{13}C$.

1. Introduction

The stable carbon isotopic composition of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) in estuarine waters is a valuable tool for tracing the biogeochemical cycling of carbon (Mook and Tan, 1991;

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doi:10.1016/j.orggeochem.2006.03.008

Hellings et al., 1999; Bouillon et al., 2003). Having past records of $\delta^{13}C_{DIC}$ would not only be useful to determine past biogeochemical processing, but would also give insight into anthropogenic pollution (both atmospheric and riverine) (Hellings et al., 2001) and upwelling (Killingley and Berger, 1979). Bivalve shell geochemistry has long been known to reflect the environmental conditions under which the bivalve grew (Epstein et al., 1953; Mook and Vogel, 1968; Mook, 1971; Dettman et al., 2004). Originally, bivalve shell carbonate $\delta^{13}C$ was believed to track $\delta^{13}C_{DIC}$ (Mook and Vogel, 1968; Killingley and Berger, 1979; Arthur et al., 1983). However, more recently it has been proposed that the carbonate skeleton is synthesized from both DIC and organically derived CO_2 from internal respiration (Dillaman and Ford, 1982; Swart, 1983; Tanaka et al., 1986; McConnaughey et al., 1997; Furla et al., 2000; Lorrain et al., 2004; and others), which both affect the skeletal stable carbon isotopic signature ($\delta^{13}C_S$). 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 $\delta^{13}C_S$ equilibrium with $\delta^{13}C_{DIC}$ in marine settings) (McConnaughey et al., 1997; Kennedy et al., 2001; Lorrain et al., 2004; Gillikin et al., 2005a), but may reach as high as 35% (Gillikin, 2005). Therefore, it is of interest to have a better understanding of what controls the $\delta^{13}C$ value of respired CO_2. As there has been much work on the isotope geochemistry of *Mytilus edulis* shells (Epstein et al., 1953; Mook and Vogel, 1968; Mook, 1971; Tanaka et al., 1986; Vander Putten et al., 2000), this species is the ideal candidate for this study.

The $\delta^{13}C$ value of respired CO_2 ($\delta^{13}C_R$) can be roughly estimated from the tissue $\delta^{13}C$ value. At the pH of bivalve body fluids (7–8; Crenshaw, 1972), more than 90% of CO_2 hydrates and ionizes to produce HCO_3^-, which should be at most 1‰ enriched in ^{13}C compared to the respiring tissue (McConnaughey et al., 1997). Yet, considering other processes affecting the $\delta^{13}C_R$, it has been estimated to be 0.5‰ heavier than the tissues on average, but this difference can generally be ignored (McConnaughey et al., 1997). However, a recent study on a zooxanthellate scleractinian coral suggested that $\delta^{13}C_R$ might not always follow tissue $\delta^{13}C$ (Swart et al., 2005). The amount of respired CO_2 in the skeleton can be approximated using the equation of McConnaughey et al. (1997):

$$M(\delta^{13}C_R) + (1 - M) * \delta^{13}C_{DIC} = \delta^{13}C_S - \varepsilon_{cl-b} \quad (1)$$

where M is the percent metabolic CO_2 contribution and ε_{cl-b} is the enrichment factor between calcite and bicarbonate (1.0 ± 0.2‰ in Romanek et al., 1992). Other factors may also play a role in determining the $\delta^{13}C$ value of the internal DIC pool. For example, the enzyme carbonic anhydrase (CA), which catalyses the reaction of bicarbonate to CO_2 to facilitate the diffusion of DIC through membranes (Paneth and O'Leary, 1985), may add or remove carbon species from this pool. Activity of CA is known to change with salinity in some bivalves, but is tied to osmoregulation (Henry and Saintsing, 1983). Since *M. edulis* does not osmoregulate (Newell, 1989), salinity should not affect CA activity in these organisms. Nevertheless, CA activity itself has been shown to be inhibited by 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 $\delta^{13}C$ in the calcifying fluid.

Considering that many bivalves incorporate only a small amount of respired CO_2, their skeletons should be able to trace large changes in $\delta^{13}C_{DIC}$, as was found by Mook and Vogel (1968) and Mook (1971) for *M. edulis* in the Scheldt estuary (The Netherlands). This is also true if the offset is constant as was found in a freshwater mussel (Kaandorp et al., 2003). Such shell data could then be useful for determining the $\delta^{13}C_{DIC}$ and the salinity where the animals grew. Considering that shell $\delta^{13}C$ values are not dependent on temperature (i.e., the calcite–bicarbonate enrichment factor is independent of temperature between 10 and 40 °C; Romanek et al., 1992), this would also provide a valuable addition to the interpretation of shell $\delta^{18}O$ profiles, which are dependent on both temperature and salinity, or more precisely, the $\delta^{18}O$ value of the water (see Gillikin et al., 2005a, for more discussion). Unfortunately, unlike other biogenic carbonates, many minor elements (e.g., Sr and Mg) in bivalves cannot be used to obtain reliable paleoenvironmental information (Stecher et al., 1996; Vander Putten et al., 2000; Gillikin et al., 2005b; Lorrain et al., 2005). Therefore, $\delta^{13}C_S$ may provide an alternative to estimate salinity and thus allow a better estimation of the $\delta^{18}O$ value of the water. To evaluate this potential proxy, the $\delta^{13}C$ values of *M. edulis* shells and mantle tissues, DIC, and particulate organic carbon (POC) were measured

across a salinity gradient and over 1 year in the Scheldt estuary.

2. Materials and methods

Mussels (*M. edulis*) were collected from the intertidal zone along the salinity gradient of the Scheldt estuary (Westerschelde) from Knokke (KN), Hoofdplaat (HF), Griete (GR) and Ossenisse (OS; the most upstream occurrence of wild *Mytilus* populations) (Fig. 1) on various dates (see Baeyens et al., 1998 for a general description of the Scheldt estuary). Mussel tissues were sampled on 17 March ($n = 3$), 3 May ($n = 7$), and 29 September 2002 ($n = 13$) from KN; on 17 March ($n = 5$), 3 May ($n = 7$), 28 July ($n = 9$), and 29 September 2002 ($n = 16$) from HF; and on 23 March 2002 from GR ($n = 7$) and OS ($n = 12$). One shell was sampled from KN on 20 February 2003 (shell KN1) and one on 29 September 2002 (shell KN1); from HF on 9 December 2002; from GR on 21 April 2003; and from OS on 9 December 2002 (shell OS1) and 21 April 2003 (shell OS2). It is well known that bivalve shell growth slows in colder weather (e.g., Gillikin et al., 2005a,b); therefore, even the shells sampled after the last water sampling date (November 2002) will mostly correspond to the water sampling period. However, it should be kept in mind that water was sampled over the full year (monthly and bi-weekly in the spring), whereas shell growth probably is highest in spring. Mussels at HF and GR were transplanted from Wemmeldinge (WD; Fig. 1) (see Gillikin et al., 2006, for a more detailed description).

Mantle tissues were collected using a scalpel and were stored frozen (−20 °C). After thawing, tissues were dried in an oven at 60 °C for 24 h, homogenized with a mortar and pestle, and about 1 mg was placed into a silver cup. Two to three drops of 5% HCl were added to decarbonate the sample and the cups were allowed to dry in an oven overnight, after which they were folded closed. Tissue $\delta^{13}C$ was measured on an Elemental Analyzer (Flash 1112 EA ThermoFinnigan) coupled via a CONFLO III to a ThermoFinnigan Delta^{plus}XL isotope ratio mass spectrometer (IRMS). Using this same instrument and method, Verheyden et al. (2004) report a long-term analytical precision for $\delta^{13}C$ of 0.08‰ on 214 analyses of the IAEA-CH-6 standard (1σ).

Shells were sectioned along the axis of major growth and samples were drilled from the calcite layer along the growth–time axis every 300 μm using a Merchantek MicroMill and 300 μm drill bit. Although *M. edulis* has both calcite and aragonite shell layers, the aragonite layer is not suitable for time resolved sampling (see Vander Putten et al., 2000). Carbonate powders were reacted in a Kiel

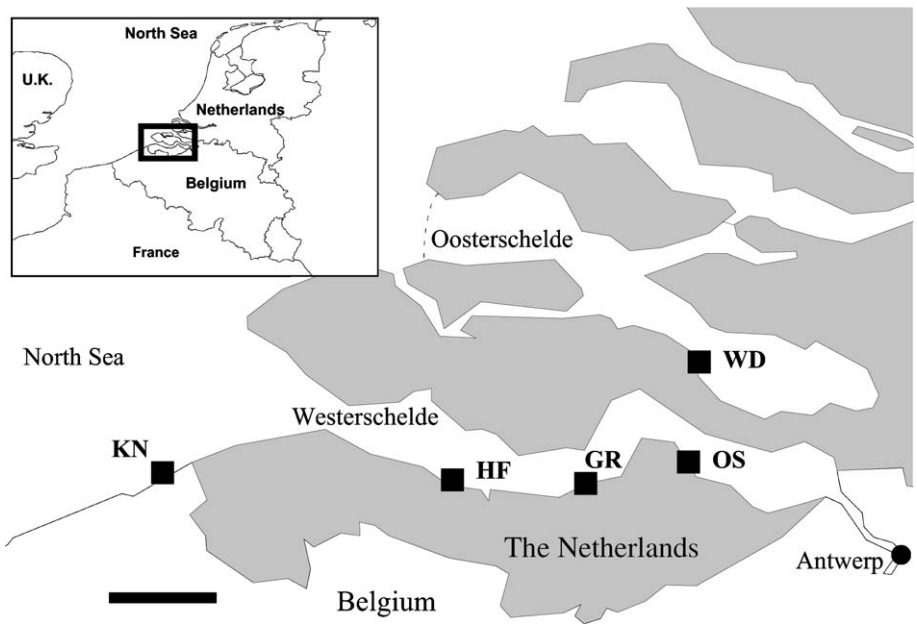

Fig. 1. Map of the Westerschelde estuary (referred to as the Scheldt estuary in the text). The four study sites are indicated Knokke (KN), Hoofdplaat (HF), Griete (GR), and Ossenisse (OS). Scale bar = 10 km. Wemmeldinge (WD) is also shown.

III coupled to a ThermoFinnigan Delta^{plus}XL dual inlet IRMS with a long-term $\delta^{13}C$ precision of 0.039‰ on the NBS-19 standard ($\delta^{13}C = +1.95$‰, $n = 292$) and 0.068‰ on the NBS-18 standard ($\delta^{13}C = -5.04$‰, $n = 22$). More details regarding the treatment of these shells can be found in Gillikin et al. (2006).

Near-shore water was sampled during high tide at least monthly from November 2001 to November 2002 for salinity, chlorophyll *a* concentrations (Chl *a*), $\delta^{13}C_{DIC}$, $\delta^{13}C_{POC}$, and suspended particulate matter (SPM). Water temperature was monitored hourly at each site using an Onset TidBit data logger (from November 2001 to May 2003) (Fig. 2). Salinity was measured in situ with a WTW multiline P4 multimeter. Chlorophyll *a* concentrations were determined by filtering 200–500 ml of seawater through Whatman GF-F filters in the field. Filters were wrapped in aluminum foil and placed in liquid nitrogen; three replicate filters per site were taken on each sampling date. In the laboratory, samples were transferred to a −85 °C freezer until analysis at NIOO-CEME, Yerseke, NL, using reverse-phase HPLC (Gieskes et al., 1988) with a reproducibility of 2.7% (or 0.3 μg/l; 1σ) for Chl *a* (based on an in-house standard, $n = 7$). The $\delta^{13}C_{DIC}$ was determined by acidifying 5 ml of water in an 8 ml helium-flushed headspace vial, followed by overnight equilibration, and subsequently injecting 400 μl of the headspace into the carrier gas stream of the continuous flow EA-IRMS. Precision of $\delta^{13}C_{DIC}$ was better than 0.2‰ based on replicate measurements; data were corrected using calibrated CO_2 gas according to Miyajima et al. (1995) (see Gillikin et al., 2005a; Gillikin, 2005). To approximate the $\delta^{13}C$ value of phytoplankton, 20‰ was subtracted from the $\delta^{13}C_{DIC}$ values ($\delta^{13}C_{DIC\text{-}20}$; see Section 4). The $\delta^{13}C_{POC}$ was measured following Lorrain et al. (2003). Briefly, 200–500 ml of seawater were filtered through Whatman GF-F filters, which were dried at 50 °C, weighed, fumed in HCl vapors, wrapped in silver cups and analyzed on the EA-IRMS described above. Concentrations of SPM are based on the dry weights of these filters.

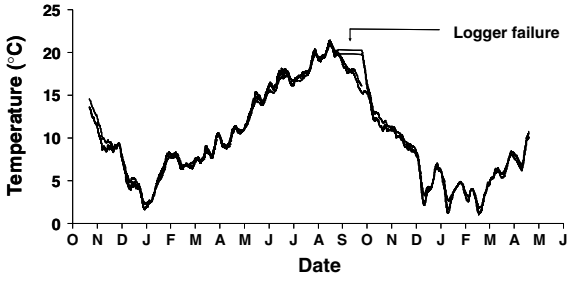

Fig. 2. Water temperature recorded hourly using Onset TidBit dataloggers at all four sites. The weekly running average is shown from October 2001 to May 2003. The loggers failed at GR and HF for about a month as is indicated on the graph.

3. Results

The strong salinity gradient of the Scheldt is obvious from the data presented in Fig. 3. There is a significant positive linear relationship between $\delta^{13}C_{DIC}$ and salinity, with $\delta^{13}C_{DIC} = \text{Salinity} * 0.39(\pm 0.03) - 13.71(\pm 0.57)$ ($r^2 = 0.94$, $p < 0.0001$, $n = 63$; for the salinity range of ~5–30) (Fig. 4).

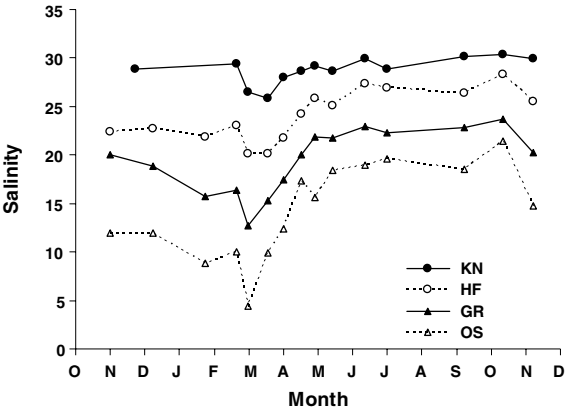

Fig. 3. Salinity at the four Scheldt sites measured over 1 year (November 2001 to November 2002).

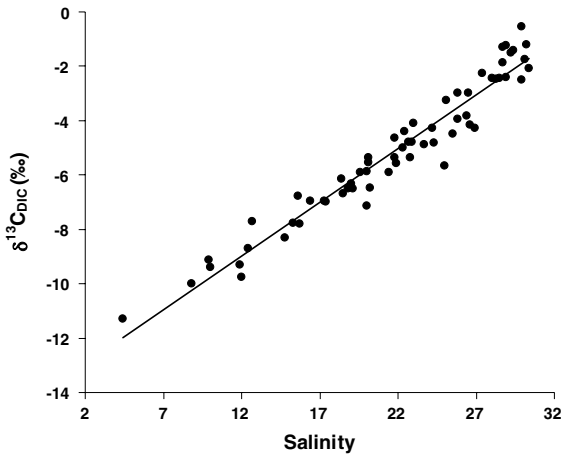

Fig. 4. $\delta^{13}C_{DIC}$ versus salinity from samples taken over 1 year at the four sites along the Scheldt estuary ($r^2 = 0.94$, $p < 0.0001$).

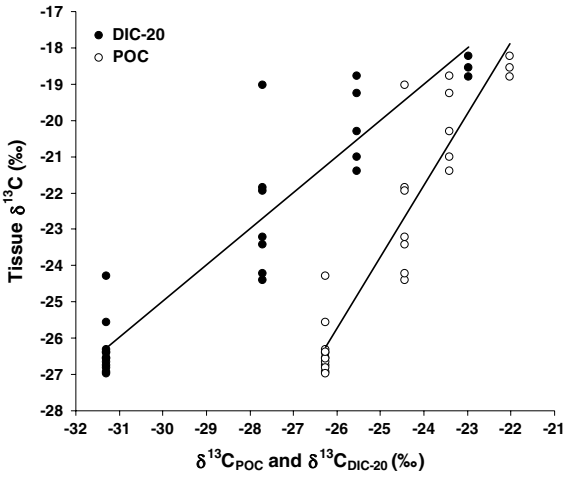

Fig. 5. Linear regressions between mantle tissue $\delta^{13}C$ and both $\delta^{13}C_{POC}$ (open symbols) and $\delta^{13}C_{DIC\text{-}20}$ (solid symbols) (in ‰) from mussels collected at all four sites in March 2002. $n = 27$ for each.

There were strong linear relationships between mantle tissue and both $\delta^{13}C_{POC}$ and $\delta^{13}C_{DIC-20}$ for samples collected from all four sites in March 2002 (Fig. 5). The relationships are: Tissue $\delta^{13}C = 0.99(\pm0.16) * \delta^{13}C_{DIC\text{-}20} + 4.89(\pm4.48)$ ($r^2 = 0.87$, $n = 27$, $p < 0.0001$) and Tissue $\delta^{13}C = 1.97(\pm 0.31) * \delta^{13}C_{POC} + 25.39(\pm7.87)$ ($r^2 = 0.87$, $n = 27$, $p < 0.0001$). $\delta^{13}C_{POC}$ and $\delta^{13}C_{DIC}$ were also significantly correlated ($\delta^{13}C_{POC} = 0.42(\pm0.09) * \delta^{13}C_{DIC} - 21.0(\pm0.5)$; $r^2 = 0.61$, $n = 59$, $p < 0.0001$). The slope of the relationship between mantle tissue and $\delta^{13}C_{DIC\text{-}20}$ was not significantly different from one ($p < 0.0001$). SPM was generally high at all four sites (range = 13–550 mg/l, mean = 86 mg/l).

Mantle tissue $\delta^{13}C$ varied considerably throughout the year at HF and KN, with a 2–3‰ decrease between March and September 2002 (Fig. 6). At both sites, mantle tissue was least negative in March, just before the phytoplankton bloom (as indicated by the Chl *a* data), but was more similar to the $\delta^{13}C$ of potential food sources (i.e., $\delta^{13}C_{DIC\text{-}20}$) in May, July, and September.

High resolution $\delta^{13}C_S$ data are provided in Fig. 7 and are discussed in more detail in Gillikin et al. (2006). The average annual shell $\delta^{13}C$ is compared with the average annual $\delta^{13}C_{DIC}$ at each site in Fig. 8 along with the predicted equilibrium calcite based on the ε_{cl-b} from Romanek et al. (1992). Average shell and DIC $\delta^{13}C$ are presented in Table 1 along with average salinity and metabolic C contribution to the shell. With the exception of the two shells from KN, shells were on average in equilibrium with $\delta^{13}C_{DIC}$ (Fig. 8).

4. Discussion

4.1. Metabolic carbon sources

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., 2004). Therefore, similar to Fry (2002), an average value of 20‰ is used in this study. From Fig. 5 it is clear that *M. edulis* is a highly selective feeder, as the slope between the expected $\delta^{13}C$ of phytoplankton (i.e., $\delta^{13}C_{DIC\text{-}20}$) and tissues is not significantly different from one, whereas the slope between $\delta^{13}C$ of tissues and $\delta^{13}C_{POC}$ was far from one (slope = 2.0 ± 0.3). The suspended POC pool is a mixture of different sources of carbon, each with an often distinct $\delta^{13}C$ value, such as phytoplankton, terrestrial carbon (in general, ~−27‰ from C3 plants and ~−14‰ from C4 plants; Mook and Tan, 1991), resuspended sediments (Scheldt: ~−19 to −24‰; Middelburg and Nieuwenhuize, 1998; Herman et al., 2000), marine macro-algae detritus (Scheldt: green algae ~−17‰, brown algae ~−25‰; Gillikin unpublished data), microphytobenthos (Scheldt: ~−15‰; Middelburg et al., 2000; Herman et al., 2000), and other components from which the mussels must select. As our samples were taken near the shore, there was probably a large amount of suspended sediments, which is indicated by the high SPM content. Particle selection can occur both at the gills (pre-ingestive) and in the gut (post-ingestive) (reviewed in Ward and Shumway, 2004), but using $\delta^{13}C$ as a tracer deals only with assimilated carbon. Moreover, using the selectivity equation from Bouillon et al. (2004),

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

where Δ is the overall estuarine gradient in tissue, POC and DIC $\delta^{13}C$ values (assumes that selectivity is similar at all stations, see Bouillon et al., 2004), suggests that the mussels are ~90% selective, which further illustrates that they assimilate their carbon primarily from phytoplankton, which in turn

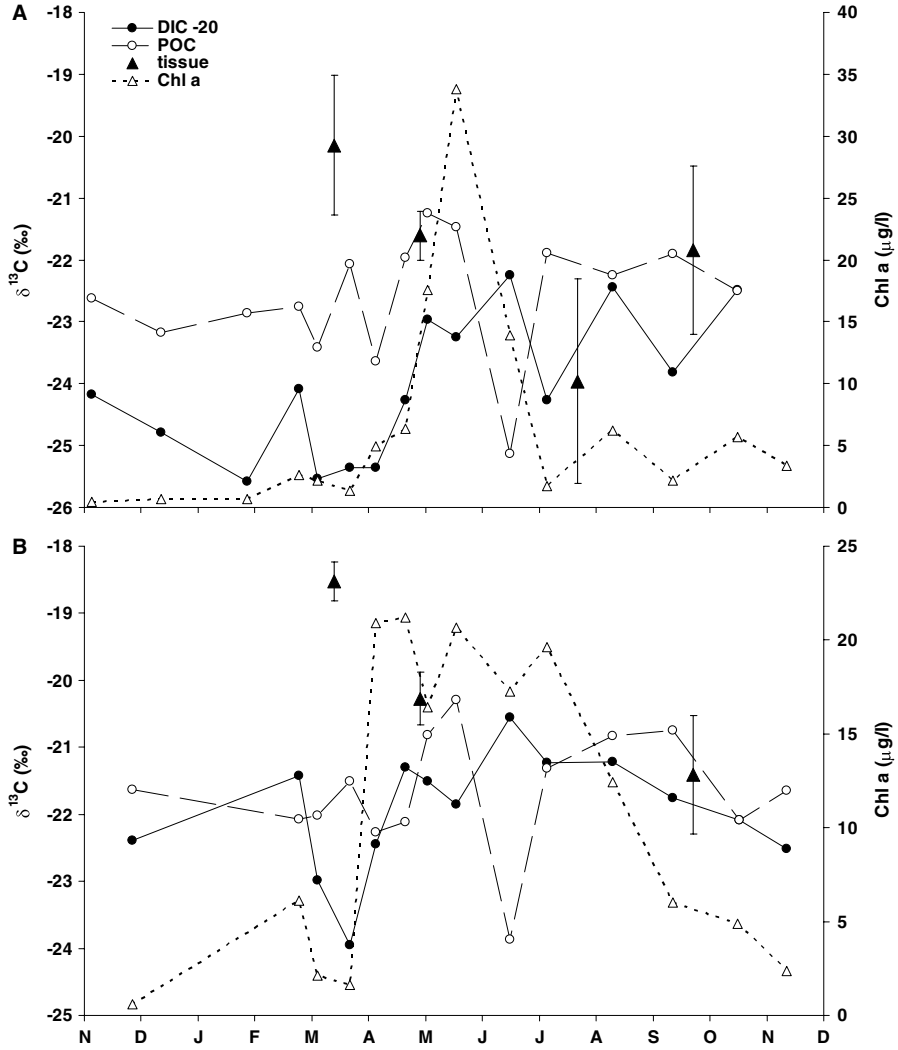

Fig. 6. Temporal variations in mantle tissue $\delta^{13}C$, $\delta^{13}C_{POC}$, $\delta^{13}C_{DIC\text{-}20}$ (in ‰), and chlorophyll *a* for Hoofdplaat (A) and Knokke (B) for the period November 2001 to November 2002. Error bars represent standard deviations.

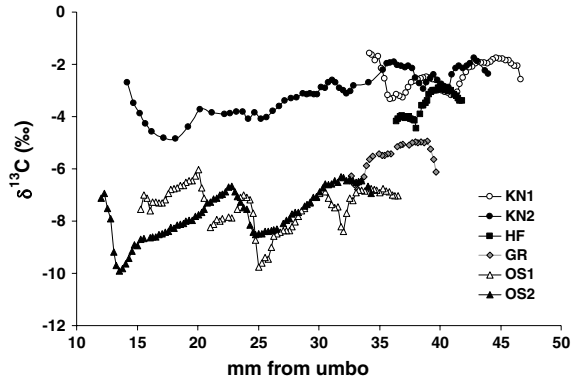

Fig. 7. High resolution $\delta^{13}C$ shell data from the six shells plotted versus distance from the umbo (growth direction is from left to right). See Fig. 1 for site codes.

obtains its carbon from the DIC pool. It is generally accepted that the $\delta^{13}C$ value of an organism reflects the $\delta^{13}C$ value of its diet, with little ($\Delta\delta^{13}C = +1‰$) or no change (DeNiro and Epstein, 1978; Fry and Sherr, 1984). However, extreme values are not uncommon with some $\Delta\delta^{13}C$ values being greater than +3‰ (Post, 2002; McCutchan et al., 2003). Therefore, the intercept of the regression between tissue $\delta^{13}C$ and phytoplankton $\delta^{13}C$ ($\delta^{13}C_{DIC\text{-}20}$) should be +1. Nevertheless, it should be kept in mind that the 20‰ fractionation used in this paper is a rough estimate. The intercept of $4.89 \pm 4.48‰$ in our dataset (Fig. 5) can therefore be explained by an extreme fractionation factor between mussel tissue and phytoplankton, an error in the phyto-

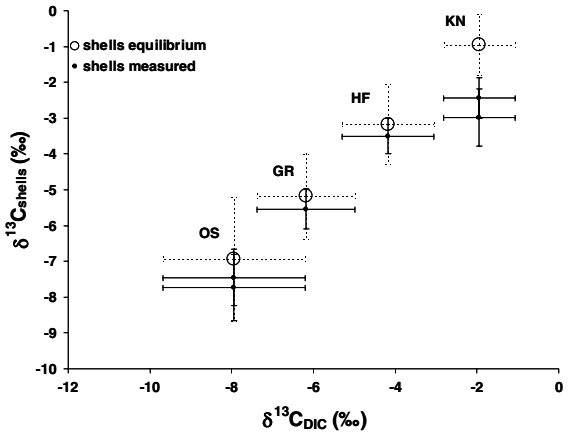

Fig. 8. Mean $\delta^{13}C_s$ and $\delta^{13}C_{DIC}$ (in ‰) averaged over the full year for the four sites (noted above data points, see Fig. 1 for description of site codes). Also plotted are the expected shell values based on the fractionation factor between $\delta^{13}C_{DIC}$ and calcite (+1.0‰; Romanek et al., 1992). Error bars represent standard deviations.

plankton fractionation used, and/or by individual variation in tissue $\delta^{13}C$. Moreover, errors in this simplified model can arise from the mussels feeding on food items other than phytoplankton. 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 $\delta^{13}C$ values (see above). Nevertheless, as a first approximation, $\delta^{13}C_R$ values should roughly mirror $\delta^{13}C$ values, which in turn should be linearly correlated with $\delta^{13}C_{DIC}$ values, as has been noticed in other bivalves (e.g., Fry, 2002). However, Swart et al. (2005) found that $\delta^{13}C_R$ from a coral deviated significantly from the $\delta^{13}C$ of tissues (both positive and negative deviations of up to 3‰), which they attributed to different compounds (e.g., lipids) being respired at various times of the year.

It is evident that the relationship between tissue $\delta^{13}C$ and $\delta^{13}C_{DIC}$ found in March (Fig. 5) does not necessarily hold true for the whole year (Fig. 6). This could be attributed 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 more likely explanation is changing lipid levels in *M. edulis* tissues. In this species, 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. 2), approximately at the same time as the tissue samples were collected. As a result of spawning, the tissues would have a lower lipid content (see de Zwaan and Mathieu, 1992). Since lipids have a lighter $\delta^{13}C$ signal than other biochemical components (Abelson and Hoering, 1961; Tieszen et al., 1983; Focken and Becker, 1998) and since the mantle exhibits a sharp drop in lipid content just after spawning (de Zwaan and Mathieu, 1992), the more positive tissue $\delta^{13}C$ values observed for March can be explained. After the phytoplankton bloom, which begins in April or May, tissue lipid reserves would be restored, thus lowering the $\delta^{13}C$ value. Indeed, Lorrain et al. (2002) found that $\delta^{13}C$ of scallop tissues were highest in spring when lipids were low, and decreased as lipids accumulated toward late summer. In the shells, however,

Table 1
Average shell and water $\delta^{13}C$ data, predicted minus measured $\delta^{13}C_S$ (pred–meas, in ‰) and percent metabolic C incorporation (%M) in *M. edulis* shells at each site

Shell	KN1	KN2	HF	GR	OS1	OS2
Average $\delta^{13}C_S$ (‰)	−2.98	−2.43	−3.50	−5.54	−7.45	−7.73
SD $\delta^{13}C_S$ (‰)	0.79	0.57	0.49	0.56	0.80	0.92
$n\delta^{13}C_S$	68	57	35	26	80	86
$\delta^{13}C_{DIC}$ (‰)	−1.9	−1.9	−4.0	−6.2	−7.9	−7.9
SD $\delta^{13}C_{DIC}$ (‰)	0.9	0.9	1.2	1.2	1.7	1.7
$n\delta^{13}C_{DIC}$	15	15	15	15	16	16
Salinity	29	29	25	20	14	14
Pred–meas (‰)	2.04	1.49	0.33	0.36	0.51	0.79
%M	10.9	8.0	1.8	2.3	3.0	4.7

%M was calculated using average data and the equation of McConnaughey et al. (1997, see text).

the spawning period is reflected by more negative $\delta^{13}C_S$ values (see Gillikin et al., 2006), although the $\delta^{13}C_{DIC}$ is generally becoming more positive (Fig. 6). This could be explained by higher metabolic rates just after spawning, as energy lost during spawning is restored. Vander Putten et al. (2000) also described these patterns in $\delta^{13}C_S$ in *M. edulis* from the Scheldt as being a result of increased respiration associated with periods of higher food availability.

4.2. Shell carbon isotopic signature

There are several hypotheses that try to explain disequilibrium isotopic fractionation (or vital effects) in biological carbonates. The two leading hypotheses are the "kinetic" model (McConnaughey, 1989a,b) and the "carbonate" model (Adkins et al., 2003), which each lead to disequilibrium of both $\delta^{13}C$ and $\delta^{18}O$ (reviewed in Shanahan et al., 2005). As bivalves are known to precipitate in oxygen isotopic equilibrium (Epstein et al., 1953; Chauvaud et al., 2005), these effects do not seem to be acting on bivalve carbonates.

In order to compare the $\delta^{13}C_{DIC}$ with shell $\delta^{13}C$, the shell data must be assigned calendar dates. Typically, the $\delta^{18}O$ signal in the shell can be used to date the samples, based on the marked winter–summer temperature contrast (e.g., Klein et al., 1996; Gillikin et al., 2005a); however, this was difficult with these samples due to the large salinity influence (more precisely, the $\delta^{18}O$ of the water) on the $\delta^{18}O$ signal in shells from upstream sites (data presented in Gillikin et al., 2006). The $\delta^{18}O$ signal from the KN shells had a clear periodicity indicating that a full year was sampled. Shells from HF and GR were stained with calcein (October 01; see Gillikin et al., 2006) and cover a full year, but growth seems reduced. Shells from OS were each sampled along 2 cm of growth (~15–35 mm from umbo), so represent at least 1 year of growth. Therefore, the average annual shell $\delta^{13}C$ was compared with the average annual $\delta^{13}C_{DIC}$ at each site (Fig. 8).

Despite the variability in tissue $\delta^{13}C$ throughout the year, the mean shell values closely match equilibrium values ($\delta^{13}C_{DIC} + 1‰$; Romanek et al., 1992) for three of the four sites (Fig. 8). The differences between measured and predicted values vary between sites (Table 1), with salinity apparently having little to do with disequilibrium as would be expected if the enzyme CA was responsible for changing the $\delta^{13}C$ value of the internal DIC pool (see Section 1). 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).

Although $\delta^{13}C_R$ does not seem to largely affect the $\delta^{13}C_S$ (~<10% incorporation of metabolic CO_2 into the shell), the variability in the percent incorporated is enough to preclude its use as a robust $\delta^{13}C_{DIC}$ proxy, and hence a salinity proxy. For example, if the $\delta^{13}C_S$ values of the seaward KN shells were used to predict $\delta^{13}C_{DIC}$ and salinity, one would conclude that this shell came from a site similar to HF (Fig. 8), even though the difference in salinity between these sites is typically around 5. From Fig. 8, it may seem that mussel shells from the same environment could be used to determine $\delta^{13}C_{DIC}$, but another study has shown that *Mercenaria mercenaria* shells collected from similar environments had very different metabolic contributions to their shells (Gillikin, 2005), suggesting this might not generally be the case. The reason why the KN shells were farther from equilibrium than the others could be linked to 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 (e.g., barnacles (Buschbaum and Saier, 2001) which are more abundant at the KN site), exposure to predators (Frandsen and Dolmer, 2002), and pollution (Wang et al., 2005).

An alternative explanation for the higher metabolic C incorporation in shells at the KN site may be the differences in the ambient CO_2/O_2 ratios. McConnaughey et al. (1997) describe a simple respiratory gas exchange model, where the inward flux of environmental CO_2 dilutes the CO_2 produced internally by respiration (see also Shanahan et al., 2005). In this model, the ambient CO_2/O_2 ratios and blood O_2/ambient O_2 ratios control the amount of respired CO_2 in the tissues and precipitating carbonates of the bivalve. With higher ambient CO_2/O_2 ratios there is more flushing of CO_2 produced internally by respiration. The Scheldt estuary is known to have particularly high pCO_2 values (Frankignoulle et al., 1998). The pCO_2 and pO_2 data collected in the Scheldt estuary in July 2000 by Frankignoulle and Borges (2001) indeed show that the upstream sites have higher ambient CO_2/O_2

ratios (ranging from 0.11 at HF to 0.22 at OS) as compared to a site with salinity similar to the KN site (~0.06). However, if this was the main factor controlling the amount of metabolic C incorporation into the shells, then a steady increase would be expected from low salinity (OS) to high salinity (KN), which was not observed in our data (Table 1).

The difference between the results presented here and those from earlier studies for the same species and estuary (i.e., Mook and Vogel, 1968; Mook, 1971), which state that $\delta^{13}C_S$ is a good proxy of $\delta^{13}C_{DIC}$, can be explained by three main considerations. First, these earlier authors analyzed mixtures of aragonite and calcite from the shells, which differ greatly in equilibrium $\delta^{13}C$ values with HCO_3^- (i.e., +1‰ for calcite and +2.7‰ for aragonite; Romanek et al., 1992). Second, they roasted their samples and found significant differences between roasted and non-roasted δ-values, while Vander Putten et al. (2000) found no difference in calcite due to roasting samples from this same species, indicating a possible isotopic alteration in these earlier studies. Finally, these earlier studies did not consider metabolic effects, and perhaps did not sample populations with markedly different metabolic rates.

In conclusion, although $\delta^{13}C_R$ values can closely follow $\delta^{13}C_{DIC}$ values, and although 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 $\delta^{13}C_{DIC}$ and salinity determinations based on $\delta^{13}C_S$. The data presented here could not be used to differentiate between sites with a salinity difference of 5, which in terms of $\delta^{18}O$ paleothermometry would correspond to about 4 °C at these sites (Gillikin, 2005). Thus, $\delta^{13}C_S$ is not a robust proxy of environmental conditions in *M. edulis* calcite, but may be useful for assessing metabolic differences between different populations, and can nevertheless be used as an indicator of large $\delta^{13}C_{DIC}$ (and salinity) differences. It remains possible that samples from within the estuary proper, or samples from the same site, may have similar metabolic contributions to the shell $\delta^{13}C$ and therefore could provide a better indication of changes in $\delta^{13}C_{DIC}$ through time; however, more samples from the same site are needed to test this hypothesis.

Acknowledgements

We are much indebted to V. Mubiana for assistance with mussel collection and setting up the field experiment. A. Van de Maele and M. Korntheuer both assisted with keeping the Kiel III operational; and A. Borges kindly provided pCO_2 and O_2 data from the Scheldt. Constructive criticism, which greatly improved this manuscript, was given by T. McConnaughey, M.D. Delafontaine, M.E. Böttcher (guest editor), and A. Verheyden. S.B. is funded by a postdoctoral mandate of the FWO-Flanders. Funding was provided by the Belgian Federal Science Policy Office, Brussels, Belgium (CALMARS, contract: EV/03/04B) and the ESF Paleosalt project funded by the FWO-Flanders (contract: G.0642.05).

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