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Stable isotopes as trophic tracers: combining field sampling and manipulative labelling of food resources for macrobenthos

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ABSTRACT: We combined 3 different approaches to determine the relative importance of microphytobenthos production as food for intertidal macrobenthic animals: (1) the natural abundance of stable-isotope ratios of carbon and nitrogen, (2) an *in situ* deliberate tracer addition of ^{13}C-bicarbonate, which was transferred through the benthic food chain after its incorporation by benthic algae, and (3) a dual labelling experiment in a flume, where pelagic and benthic algae were labelled with ^{15}N and ^{13}C, respectively. The results of the 3 approaches confirmed the high importance of microphytobenthos as a food source for (surface) deposit feeders. Despite the clearly demonstrated resuspension of benthic algae at high current velocities, suspension feeders appeared to depend almost exclusively on pelagic algae (and possibly detrital carbon) as a food source. Based on the results of the experiments, we determined an approximate degree of dependence on microphytobenthos for different species of intertidal macrobenthos. The macrobenthic biomass at 5 study locations, when weighted by these coefficients, correlated very well with measured productivity of the microphytobenthos.

KEY WORDS: Food web · Microphytobenthos · Deposit feeding · Suspension feeding · Westerschelde-flume

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INTRODUCTION

Estuarine tidal flats are sites of intensive biological activity. Substantial primary production by benthic algae usually occurs, typically in the range of 100 g C m^{-2} yr^{-1} (Underwood & Kromkamp 1999). The biomass produced by these algae may be shunted directly into the local benthic food web, but can also be resuspended and exported from the place of production and deposited elsewhere (Lucas et al. 2000). de Jonge & van Beusekom (1992) asserted that resuspended benthic diatoms comprise half of the food of benthic suspension feeders in the Ems estuary. Their estimate was based on indirect evidence, using the proportion of benthic diatoms in the suspended matter. Kamermans (1994) analysed species composition of algae in the water, at the sediment surface and in the stomachs of different bivalves. She concluded that both suspension-feeding and deposit-feeding species were similarly dependent on algae in the water column.

Stable isotopes are a classical way to trace food sources of aquatic animals (Peterson & Fry 1987). The isotopic signature of a heterotroph is a function of the food composition, the signatures of the different food sources and the fractionation during food processing. Fractionation during heterotrophic consumption is small for carbon and sulphur, but higher (~3.5‰) for nitrogen isotopes (Minigawa & Wada 1984). Therefore, stable-isotope ratios of nitrogen may be used to reconstruct food webs and estimate the trophic level of consumers (Cabana & Rasmussen 1994), but this approach

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is critically dependent on the homogeneity of the food source. A proper characterisation of the baseline isotopic signatures, which may differ considerably between sub-habitats of the same system, is essential (Vander Zanden & Rasmussen 1999).

In general, benthic plants (including benthic algae) have a different isotopic signature than pelagic plants (France 1995), and therefore stable isotopes can be used to trace the relative importance of benthic and pelagic algae in the food of intertidal macrobenthic animals. Currin et al. (1995) reviewed $\delta^{13}C$ values of benthic microalgae and calculated an average $\delta^{13}C$ of –14.9‰ (n = 18, SE = 3.1). For marine phytoplankton they calculated an average of –21.1‰ (n = 10, SE = 2.4), but along estuarine gradients values tend to become depleted towards the freshwater end-member (Middelburg & Nieuwenhuize 1998). Stribling & Cornwell (1997) reported a value of –14.85‰ for benthic microalgae. Créach et al. (1997) reported average values of –14.4‰ on an intertidal mudflat, and –20.1‰ in the drainage channels. Riera et al. (1999) also found a value of –14.4‰ (±0.2) in a salt marsh in France. A consistent within-site difference between benthic and planktonic primary producers was demonstrated by France (1995) over a wide variety of freshwater and marine habitats. This author attributed the difference to a larger diffusion resistance for DIC uptake in benthic environments. Hence, the plants are less able to discriminate between isotopes and will be heavier in carbon. However, other factors may also contribute to the difference (Currin et al. 1995, Newell et al. 1995, Stribling & Cornwell 1997): (1) the source of DIC for benthic microalgae may be a mixture of atmospheric carbon, water-column DIC and the products of benthic mineralisation, and (2) carbon-dioxide limitation may lead to a more exhausted substrate stock and less discrimination. Most explanations of the difference between benthic and pelagic algal signatures have in common that they can be expected to vary with season, with productivity level of the microalgae, with current velocity above the bed, or other factors. Consequently, one could expect seasonal differences in the benthic microalgal $\delta^{13}C$ (Schwinghamer et al. 1983) and one should be cautious in calculating back from the differences between macrobenthic $\delta^{13}C$ observed during a particular field campaign and composition of the diet at that moment. Body-tissue composition integrates over a longer period, during which isotopic composition of the food may have been different. Riera & Richard (1996) and Créach et al. (1997) used natural stable-isotope signatures to estimate the contribution of microphytobenthos to the diet of benthic animals.

Stable carbon and nitrogen isotopes can also be used in field and laboratory labelling experiments to trace different food sources and test specific hypotheses. This has been extensively used in pelagic studies, but application of the labelling technique to benthic systems has been limited (e.g. Blair et al. 1996, Levin et al. 1999, Moodley et al. 2000) and focused on the fate of sedimenting phytodetritus in sediments without *in situ* primary production.

Herman et al. (1999) have argued that food availability is an important factor limiting estuarine macrobenthic biomass. Over a range of estuarine systems, a good predictive relation exists between primary productivity and macrobenthic biomass at the scale of the system. As microphytobenthic production is a significant term in the primary production of many estuaries (Heip et al. 1995), it could substantially influence the macrobenthic biomass. However, as the importance of microphytobenthos as a food source is probably different for different species or trophic groups of macrobenthos, one could also expect differences in macrobenthic community structure according to the productivity and fate of microphytobenthos.

In the present study we use (1) natural stable-isotope signatures, as well as (2) field and (3) tracer-addition experiments in a laboratory flume to investigate the relative importance of microphytobenthic production as food for different estuarine macrobenthic species. Each approach has its advantages and disadvantages, but together they provide strong constraints on food sources for macrobenthos. Based on this information, we attempt to relate the trophic structure of macrobenthic assemblages to the microphytobenthic production and turnover.

MATERIAL AND METHODS

Study site. This investigation is part of the ECOFLAT project, aiming at identification and quantification of the major carbon and nutrient flows as a function of physical forcing on a small tidal flat (the Molenplaat) in the turbid, nutrient-rich and heterotrophic Westerschelde estuary. In the framework of this study, measurements of microphytobenthos have concerned primary production (Barranguet et al. 1998, Barranguet & Kromkamp 2000, in this issue), resuspension/sedimentation (Lucas & Holligan 1999, Lucas et al. 2000, Widdows et al. 2000), *in situ* production and transfer along the food chain (Middelburg et al. 2000). These studies have been accompanied by studies of the species structure, biomass and feeding relations of micro-, meio-, and macrobenthos (Hamels et al. 1998, this study).

All measurements were performed at the Molenplaat (51° 26′ N, 3° 57′ E) (Fig. 1). Salinity in this region of the estuary varies around 20 to 25. Most of the tidal flat is located between –1 and +1 m relative to mean tidal level. Mean tidal range is approximately 5 m. The

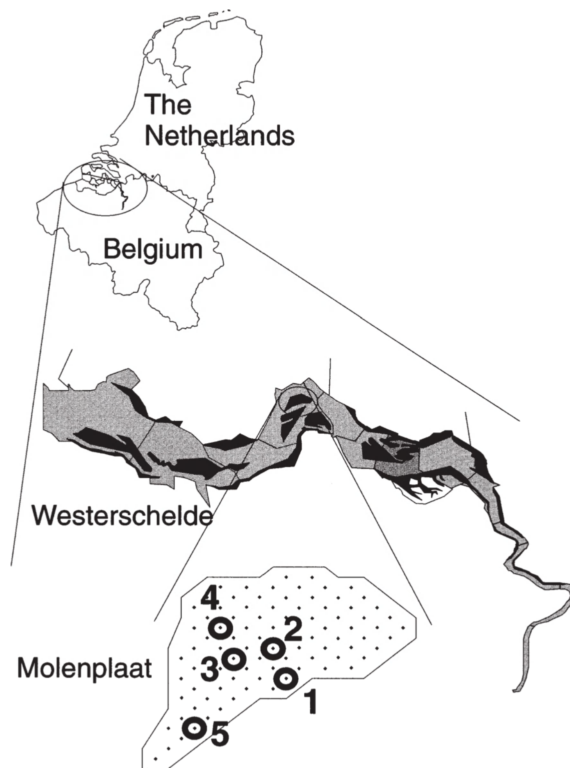

Fig. 1. Map of study area in the Westerschelde estuary showing the 5 sites sampled

period of emersion varies between 4.5 h (Stn 1), 7 h (Stns 2, 3, 4) and 8 h (Stn 5) per tidal cycle.

Five sites were selected for intensive measurements on the flat, based on sedimentology and composition of the fauna in a preliminary survey. Stn 2 has the finest sediment, Stns 4 and especially 5 are more dynamic and sandier, and Stns 1 and 3 are intermediate (Table 1).

In situ labelling experiments were performed at Stns 2 and 4. The flume experiment was done with sediment from Stn 4. Natural stable-isotope data came from Stns 2 and 4, macrofauna biomass and species composition from all sites.

Benthic species composition and biomass. At all 5 stations, macrofauna species composition, density and biomass were determined from 10 replicate cores (11 cm diameter) per station. Cores were taken to approximately 30 cm depth and sliced in the following sections: 0–2, 2–4, 4–9, 9–14, 14–19, 19–24 and >24 cm. Slices were wet-sieved in the field over a 1 mm sieve, and the remaining material was fixed with buffered formaldehyde (final concentration 8%). Only depth-integrated biomass values will be presented here. All macrofauna was picked out and sorted to species. Bivalves were measured to the nearest mm, and ash-free dry weight (AFDW) was determined from a length-AFDW regression established per species for the study area and the year and season of sampling. For other species the blotted wet weight was determined, and AFDW was calculated from a species-specific conversion factor determined for the study area. AFDW for these conversion relations was determined as the difference between the dry weight (4 h at 110°C) and the ash weight (2 h at 500°C).

Natural stable-isotope signatures of macrofauna. Prior to the *in situ* labelling experiments, macrofauna was collected by wet-sieving sediment recovered from the experimental sites. Animals were picked live from

Table 1. Characteristics of the sampling stations on the Molenplaat. Bottom shear stress is the maximum during a tidal cycle (derived from hydrodynamic modelling: van de Koppel et al. in press). Benthic primary production is based on oxygen microelectrode measurements (Hamels et al. 1998). Sediment grain size and organic carbon data are from 9 June 1996

Depth range (cm)	% mud <63 µm	Median grain size (µm)	Bottom shear stress (Pa)	Organic carbon (wt %)	Benthic primary production (mg C m^{-2} h^{-1})
Stn 1					
0–1	24	137	0.43	0.29	225
0–25	11	171		0.18	
Stn 2					
0–1	43	77	0.36	0.64	105
0–25	30	94		0.41	
Stn 3					
0–1	14	160	0.58	0.20	152
0–25	10	168		0.22	
Stn 4					
0–1	4	170	1.15	0.06	131
0–25	5	167		0.07	
Stn 5					
0–1	5	166	3.37	0.04	15
0–25	4	174		0.05	

the sieve residues, rinsed twice in filtered seawater, collected by species in glass vials and freeze-dried. Soft parts of bivalve molluscs were removed from their shells after dipping the animals into boiling filtered seawater. The freeze-dried tissues were homogenized in a mortar. For *Hydrobia ulvae*, which could not be removed from its shell, the homogenate was treated with 2 N HCl to dissolve inorganic carbonates prior to analysis of stable carbon-isotope ratios. At least 5, but for most species many more, individuals were combined into 1 sample for stable-isotope determinations in order to obtain a representative sample. During the procedure, which was also used for the labelling experiments, gut contents of the animals were not separated from the tissue. The reported stable-isotope ratios refer to the animals plus their gut contents.

***In situ* labelling experiments.** Microphytobenthos was labelled *in situ* by spraying the sediment surface in two 0.25 m^2 plots with $NaH^{13}CO_3$ (final concentration 1 g ^{13}C m^{-2}) at the beginning of low tide. The plots were lined with a stainless steel frame pushed 8 cm into the sediment and level with the surface of the sediment. Full details of the experiment are given by Middelburg et al. (2000). During exposure of the flat, the labelled DIC was incorporated into the algae, and in the following days its retention in the sediment and its transfer along the microbial food chain were recorded. At the end of the experiment (after 4 d at Stn 2; after 3 d at Stn 4), macrobenthos was sampled from the experimental plots following the same procedures as for natural stable-isotope signatures. All experiments took place between 6 and 15 June 1997, in very bright, warm, summer weather.

Dual labelling experiment in an annular flume. The Plymouth Marine Laboratory annular flume was used to determine the relative importance of pelagic and benthic algae in the diet of different species of macrobenthos. The flume has a 64 cm outer and 44 cm inner diameter, creating a 10 cm annular channel. It has a total bed area of 0.17 m^2 and a volume of 60 l. Free-stream current velocities are created by a rotating annular drive plate without paddles situated 20 cm above the sediment and range from 1 to 50 cm s^{-1}. A detailed description of the flume is given by Widdows et al. (1998a,b). Sediment from Stn 4 was cored to 10 cm depth using quadrant box cores (4 cores forming an annulus) designed to fit the flume precisely. The cores were placed in the flume and the boxes removed without disturbing the sediment surface. The surface of the sediment was sprayed with $NaH^{13}CO_3$ (1 g ^{13}C m^{-2}) and left air-exposed in tempered sunlight. After 4 h incubation, the excess ^{13}C not incorporated into the sediment and benthos was flushed out by filling the flume with seawater (without disturbance to the sediment surface) and draining immediately. The flume was then filled with 36 l seawater sampled from a nearby gully, which had been pre-incubated in ambient light for 3 d with addition of 100 µM $^{15}NH_4^+$ in order to label the pelagic algae. The incubation took place in clear polycarbonate 15 l jars, with stirring produced by aeration from aquarium airstones.

After addition of the water, the current velocity was changed in 5 min steps to simulate the tidal current velocities measured at Stn 4 (Widdows et al. 2000). At regular intervals, the suspended matter in the flume was sampled through a sampling port in the sidewall. Suspended particulate matter (SPM) was filtered on pre-dried pre-weighted Whatman GF/F filters. These filters were oven-dried and the SPM determined from weight difference. The filter was analysed for stable carbon and nitrogen isotopes.

Separate samples of suspended material were taken to determine photosynthetic pigments and to count diatom species. Chlorophyll *a* and fucoxanthin were analysed by HPLC according to Lucas & Holligan (1999). Cell counts were carried out in sedimentation chambers, also described in Lucas & Holligan (1999) and in Lucas et al. (2000).

After completion of the tidal cycle simulation (t = 270 min), 3 sediment cores were sampled from the flume and sliced vertically (0–1, 1–10, 10–30, 30–60, 60–90 mm). The system was then left overnight operating at low (2 cm s^{-1}) current velocity. The following morning the water was drained and the sediment sampled again with vertically sliced cores. Macrofauna was sampled live from the sieved flume sediment.

Analytical techniques. Freeze-dried sediment, animal or filter samples were analysed for organic carbon and nitrogen using a Carlo Erba elemental analyser following an *in situ* acidification procedure (Nieuwenhuize et al. 1994). The carbon and nitrogen isotopic composition of the samples was determined using a Fisons CN analyser coupled on line via a Finnigan conflo 2 interface, to a Finnigan Delta S mass spectrometer. The carbon and nitrogen isotope ratios are expressed in the delta notation $\delta^{13}C$ and $\delta^{15}N$. Reproducibility of the measurements is ~0.15‰.

Incorporation of ^{13}C label was calculated as excess (above background) ^{13}C uptake in either mg ^{13}C m^{-2} or µg ^{13}C cm^{-3}. This is the product of excess ^{13}C (E) and organic carbon or biomass per unit sediment surface or sediment volume. A dry density of 2.5 g cm^{-3} for sediment was assumed. E is the difference between the fraction ^{13}C of the control ($F_{control}$) and the sample (F_{sample}): $E = F_{sample} - F_{control}$, where $F = {}^{13}C/({}^{13}C + {}^{12}C) = R/(R+1)$. The carbon isotope ratio (R) was derived from the measured $\delta^{13}C$ values as: $R = (\delta^{13}C/1000 + 1) \cdot R_{VPDB}$, with $R_{VPDB} = 0.0112372$ the carbon isotope ratio of the Vienna PDB, the reference material with respect to which $\delta^{13}C$ is expressed.

Incorporation of ^{15}N label was calculated analogously. In the calculations, R_{VPDB} is replaced by R_{AIR} = 0.0036765, the nitrogen isotope ratio of atmospheric nitrogen.

RESULTS

Benthic species composition and biomass

Species composition and biomass distribution of the macrobenthos at the 5 stations is given in Table 2. Highest biomass was observed at Stn 3, where the assemblage was composed of a mixture of deposit feeders (*Arenicola marina*, *Heteromastus filiformis*), suspension feeders (*Cerastoderma edule*, *Mya arenaria*, *Scrobicularia plana*), surface grazers and interface feeders (*Hydrobia ulvae*, *Macoma balthica*, *Pygospio elegans*). Dominance shifted toward suspension feeders at the most muddy station (2) and towards deposit feeders at Stn 1. Stn 4 was generally poorer in biomass, dominated by *A. marina*, *C. edule*, *M. balthica* and *H. ulvae*. Stn 5 was poor in biomass and diversity, with the assemblage mainly dominated by mobile species. The last column of Table 2 summarises the relative dependence of the different species on microphytobenthos, as derived from our experiments (see 'Discussion').

Natural stable-isotope signatures of macrofauna

In Fig. 2, the stable carbon and nitrogen isotope ratios are plotted for the different species sampled at

Table 2. Macrofauna biomass at 5 stations on the Molenplaat. Values are average biomass per species (mg ash-free dry wt m^{-2}) over 4 sampling campaigns (June 1996, September 1996, June 1997, September 1997). +: presence of species with average biomass <1 mg ash-free dry wt m^{-2}; MPB coeff.: relative dependence of species on microphytobenthos food (see first subsection of 'Results'; –: no MPB coeff. determined)

Species	Station 1	Station 2	Station 3	Station 4	Station 5	MPB coeff.
Anaitides sp.	5	1	3			–
Arenicola marina	22527	1160	12398	4411		0.81
Bathyporeia pilosa		+	3	113	1013	1
Bathyporeia sarsi				98		1
Capitella capitata	421	5	34	26	+	–
Cerastoderma edule	8	6756	7018	3572	71	0.17
Corophium arenarium	3	2	1	3	1	–
Corophium volutator	+	1	27	+	+	–
Crangon crangon	15	144	+	27		–
Ensis sp.	13	+	+			–
Eteone sp.	89	50	191	24	34	0.73
Eurydice pulchra	+				117	–
Harmothoe sp.	14					–
Haustorius arenarius					17	–
Heteromastus filiformis	7476	6798	5261	788	18	0.53
Hydrobia ulvae	49	717	1468	1830	31	0.96
Lanice conchilega	12					–
Macoma balthica	3995	6823	12424	8734	374	0.49
Mya arenaria	2538	6100	8008		1	0.24
Mytilus edulis	+	+	+	+	+	–
Nemertea			36	22	59	–
Nephtys cirrosa				5		0.49
Nephtys hombergii	258	+	98	463		0.49
Nereis diversicolor	1030	+	6	142		0.86
Nereis succinea	171	2861	1092	173		0.86
Oligochaeta	5	2	3	1	+	–
Polydora ligni	5	613	165			–
Pygospio elegans	197	848	1619	124	47	0.62
Scoloplos armiger	24	+	3	72	218	0.77
Scolelepis squamata				44	212	–
Scrobicularia plana	2277	345	362			–
Tharyx marioni	1025	49	62		+	0.62
Total	42160	33279	50287	20674	2216	

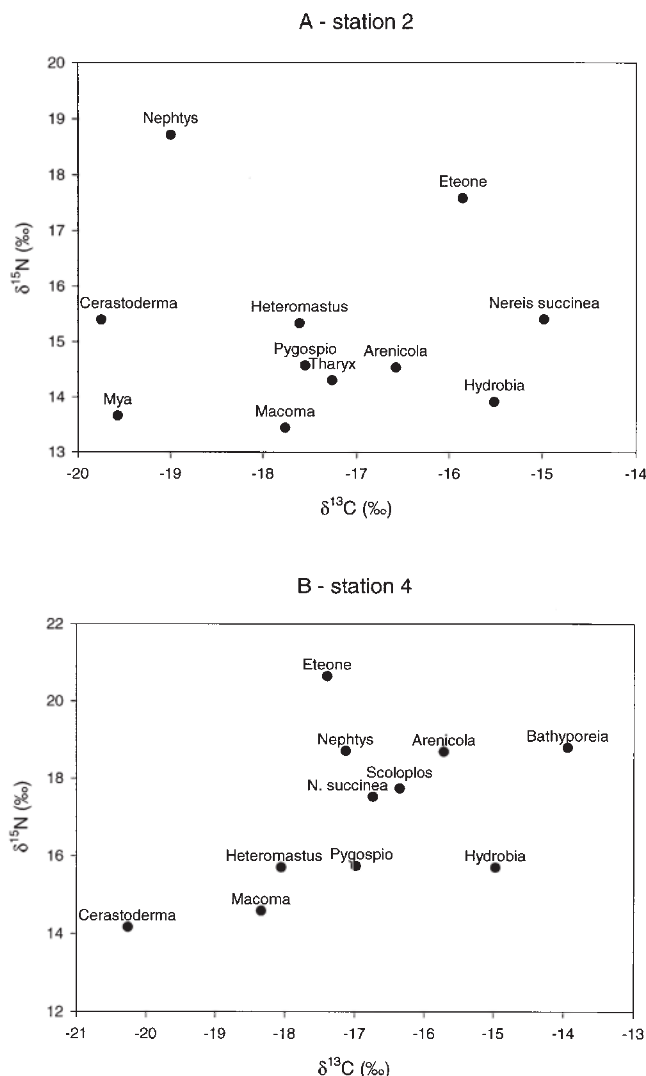

Fig. 2. Natural stable-isotope signatures of macrobenthic species sampled at Stns 2 and 4 in June 1997 on the Molenplaat tidal flat

Stns 2 and 4. The suspension-feeding bivalves *Cerastoderma edule* and *Mya arenaria* have relatively low $\delta^{13}C$, whereas the surface deposit-feeding (grazing) species *Hydrobia ulvae* and *Bathyporeia* sp. have the highest $\delta^{13}C$. Other species, such as the facultative surface deposit/suspension feeders *Macoma balthica* and *Pygospio elegans*, have intermediate values. This is also true for the deep deposit-feeding *Heteromastus filiformis* and for *Arenicola marina*, although the latter is heavier in carbon. Predatory species, e.g. *Eteone* sp. and *Nephtys hombergii* are intermediate (and variable) in their $\delta^{13}C$, but have high $\delta^{15}N$, as would be expected from the increase in the latter variable with increasing trophic level.

Previous measurements (Middelburg & Nieuwenhuize 1998) have shown that in this part of the Westerschelde estuary pelagic algae have a $\delta^{13}C$ between −22 and −20‰, typical for marine phytoplankton. Specific markers for benthic algae at Stn 2 (Middelburg et al. 2000) allowed the estimation of the $\delta^{13}C$ of these benthic algae at around −15‰. The gradient of the macrofauna species suggests that the relative importance of benthic algae in their diet increases from left to right on the $\delta^{13}C$ axis. In contrast to Stn 2, at Stn 4 this increase in $\delta^{13}C$ seems to be correlated with an increase in $\delta^{15}N$. Unfortunately, we have no independent estimate of the $\delta^{15}N$ of benthic algae at the 2 stations.

The $\delta^{13}C$ of the bulk organic matter in the top 10 cm of the sediment at Stn 2 was −23.0‰. For the upper 1 mm it was −22.3‰. At Stn 4, the bulk organic matter in the sediment was slightly heavier (average −21.8‰ in the top 10 cm); this was especially true for the superficial 1 mm (−19.3‰). The bulk organic carbon in the sediment was consistently more depleted than all the macrobenthic species sampled.

In situ labelling experiments

For all species examined, uptake of label after its incorporation into benthic algae could be qualitatively demonstrated (Fig. 3). It is expressed as $\Delta\delta^{13}C$, the difference in $\delta^{13}C$ of animals before and after labelling. For all species sampled at both locations, labelling was stronger at Stn 4 than at Stn 2, consistent with the stronger specific labelling of the organic matter at Stn 4, where label incorporation per unit area was the same as at Stn 2, but where organic matter content and biomass of the algae, measured as chlorophyll *a*, were 8 to 10 times less in the top 1 mm (Middelburg et al. 2000).

For several macrofaunal species, specific uptake was relatively small, with $\Delta\delta^{13}C < 10$‰ (*Cerastoderma edule*, *Heteromastus filiformis*, *Mya arenaria*, *Arenicola marina*, *Nephtys hombergi*). *Hydrobia ulvae* and *Scoloplos armiger* also displayed only moderate uptake at Stn 4. At both stations, by far the strongest labelling was found for *Pygospio elegans*, *Nereis succinea* and *Macoma balthica*. For the latter species at Stn 4, samples of different size classes were taken, and showed strong size-dependence of label uptake. Animals with a shell length <3, 3 to 10 and >10 mm showed average $\Delta\delta^{13}C$ of 300, 120 and 10‰, respectively. The species sampled represent the majority of the biomass of the macrofauna at these stations. Based on the species-specific labelling and the biomass distribution at the time of sampling, one can calculate that macrofauna consumption accounted for 4.4 and 8 mg ^{13}C m^{-2} at Stns 2 and 4, respectively. This is 4.3 and 6.8% of the total label incorporated during the labelling phase at Stns 2 and 4, respectively (Middelburg et al. 2000).

Dual labelling experiment

The labelled DIC sprayed on the sediment was successfully incorporated into the benthic algae. The total uptake of ^{13}C after 4 h was approximately 150 mg ^{13}C m^{-2}; an average of 62 mg ^{13}C m^{-2} was found in the top 1 mm at the end of incorporation and before the flume was filled with water. The depth distribution after incorporation (approximately 40% in the top 1 mm) was the same as in the *in situ* labelling experiment at Stn 4 and was related to the depth of the euphotic zone of approximately 2.6 mm (Middelburg et al. 2000). The rate of incorporation (35 mg ^{13}C m^{-2} h^{-1}) was lower than in the field experiment (60 mg ^{13}C m^{-2} h^{-1}; Middelburg et al. 2000). The difference can be attributed to the difference in light intensity between the fully exposed field site, and the more sheltered and shadowed position of the sediment surface in the flume. The labelled material was mixed into the sediment column during the simulated tidal cycle. The depth profiles and total inventory of ^{13}C sampled in the sediment at the end of the tidal cycle (t = 270 min) and at the end of the experiment (t = end) after remaining in the flume overnight were very consistent (Fig. 4). A near-exponential decrease in the concentration was observed, with the labelled material penetrating through the total depth of the sediment. Spatial variability was greatest in the top 1 mm.

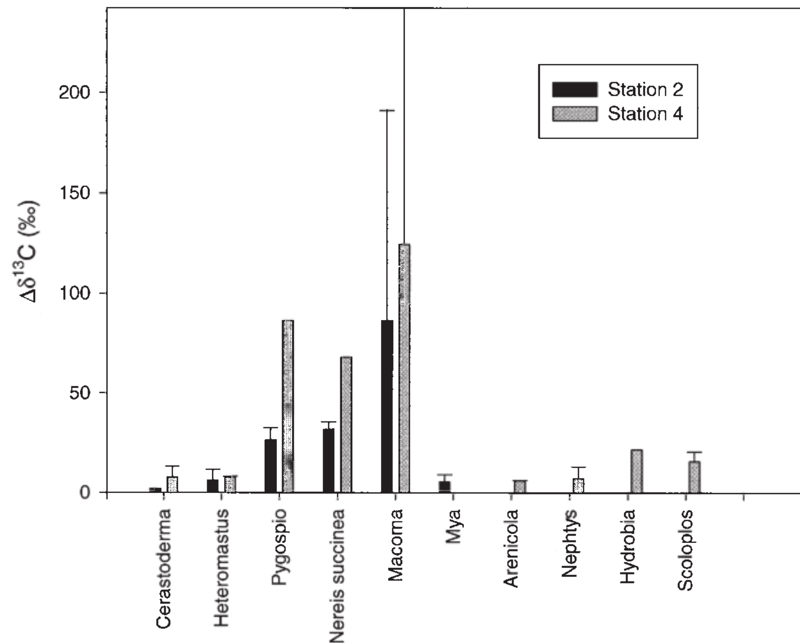

Fig. 3. $\Delta\delta^{13}C$ in different macrobenthic species at Stns 2 and 4. $\Delta\delta^{13}C$ is difference in $\delta^{13}C$ between macrofauna sampled at end of *in situ* labelling experiment and background $\delta^{13}C$ measured for same species and station before the experiment. Error bars are standard deviations of 2 to 3 replicate determinations. Full specific names as in Table 2

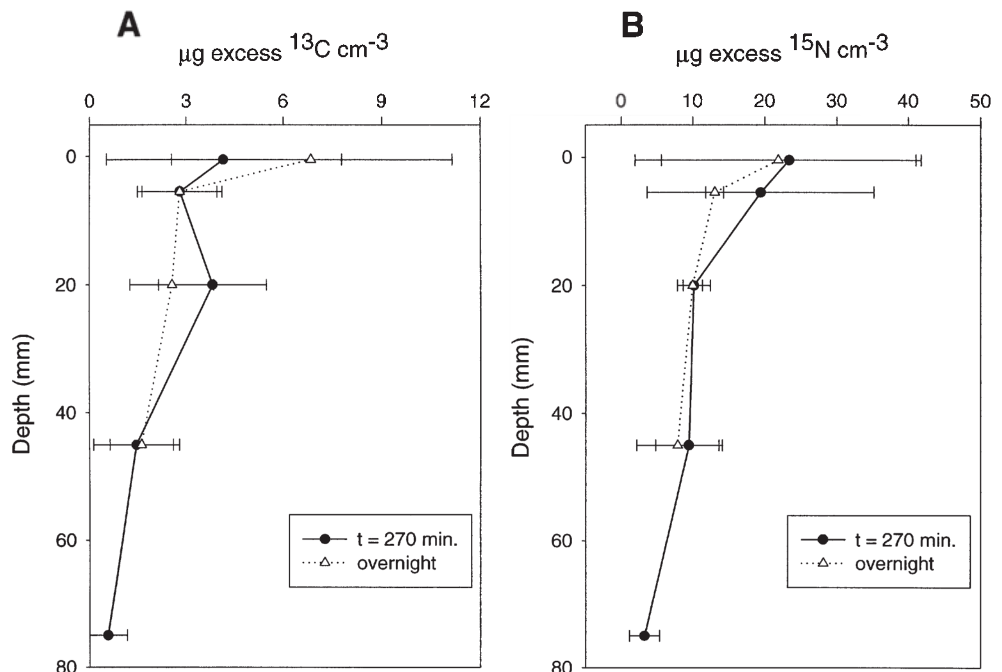

Fig. 4. Dual labelling experiment. Depth distribution within flume sediment of experimentally added label after simulation of a tidal cycle in the flume. ^{13}C was added via incorporation by benthic algae, ^{15}N had been incorporated before the experiment by labelled algae in water added to the flume. Error bars are standard deviations of 3 replicate determinations

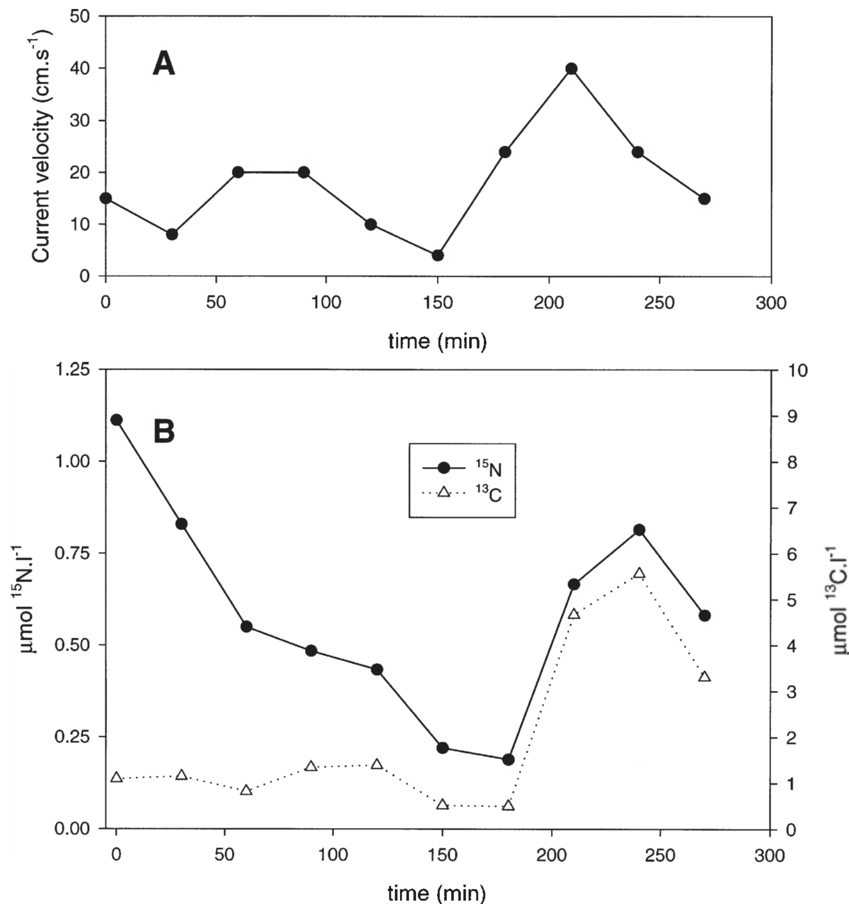

Fig. 5. Dual labelling experiment. (A) Imposed current velocity in the flume. (B) Concentrations of ^{13}C and ^{15}N in suspended matter filtered from flume water. ^{13}C labelled material was resuspended from the sediment. ^{15}N labelled algae were added with flume water at the beginning of the experiment. Scaling of the axes for carbon and nitrogen reflects a C:N ratio of 8

In the course of the simulated tidal cycle in the annular flume (Fig. 5A), there was extensive mixing of the ^{15}N-labelled pelagic algae and the ^{13}C-labelled benthic algae, as evidenced by the labelling of the suspended matter in the flume water (Fig. 5B) and the incorporation of excess ^{15}N into the sediment (Fig. 4B). During the first few hours of the experiment, the ^{15}N-labelled, pelagic suspended matter was gradually removed from the water column and deposited on the bottom by the processes of sedimentation and biodeposition of faecal material by suspension feeders (which were observed to be active during the first 180 min; Fig. 5). Most of this ^{15}N-labelled material was resuspended again at the high current velocities, but some was incorporated into the sediment (Fig. 4B). Substantial amounts of benthic ^{13}C-labelled material was suspended in the water column at high current velocities (>25 cm s^{-1}), but resedimented rapidly. A large fraction of the benthic labelled material was probably associated with rapidly deposited sediment particles. Despite resuspension and sedimentation of both the pelagic and benthic labelled material, the label signatures of the algae in the sediment and in the water column remained generally different. After overnight incubation at low current speed, the $\delta^{13}C$ and $\delta^{15}N$ values of the suspended material were 162 and 3624‰, respectively.

Cell counts of the diatoms confirmed the resuspension of benthic algae at high current velocity (Fig. 6B). The time course of pelagic diatoms shows deposition and resuspension, but the resuspension was less pronounced than for the ^{15}N content. Apparently some sorting of the cells according to size may have taken place. Restriction of the cell counts to diatoms only does not seem to have played a major role. At the start of the experiment, the presence of dinoflagellates in the water column was qualitatively confirmed. However, the resuspended material had a higher fucoxanthin-to-chlorophyll ratio than the material present in the water column at the start of the experiment, and therefore it seems unlikely that resuspension of non-diatoms was more important than resuspension of diatoms.

The signatures of suspended and sediment material have been indicated as vectors in Fig. 7, for comparison with the animal signatures. Theoretically, one expects that an animal feeding on a mixture of suspended and benthic material will form a vector between the vectors of the food source, with the smallest angle between the animal vector and the vector of the dominant food source.

The macrofauna species retrieved from the dual labelling experiment had very different ^{13}C and ^{15}N signatures. The suspension feeder *Cerastoderma edule* had a signature corresponding to the suspended material around the end of the tidal cycle experiment. Epibenthic grazers (*Hydrobia ulvae* and *Bathyporeia* sp.) corresponded to the labelling of organic matter (i.e. the mixture of mainly benthic algae and some phytoplankton algae) in the sediment. This was also the case for *Scoloplos armiger*, but labelling in this species, as in *Anaitides* sp., was weak (see inset in Fig. 7). *Macoma balthica* and *Pygospio elegans* occupied an intermediate position between the benthic and suspended matter.

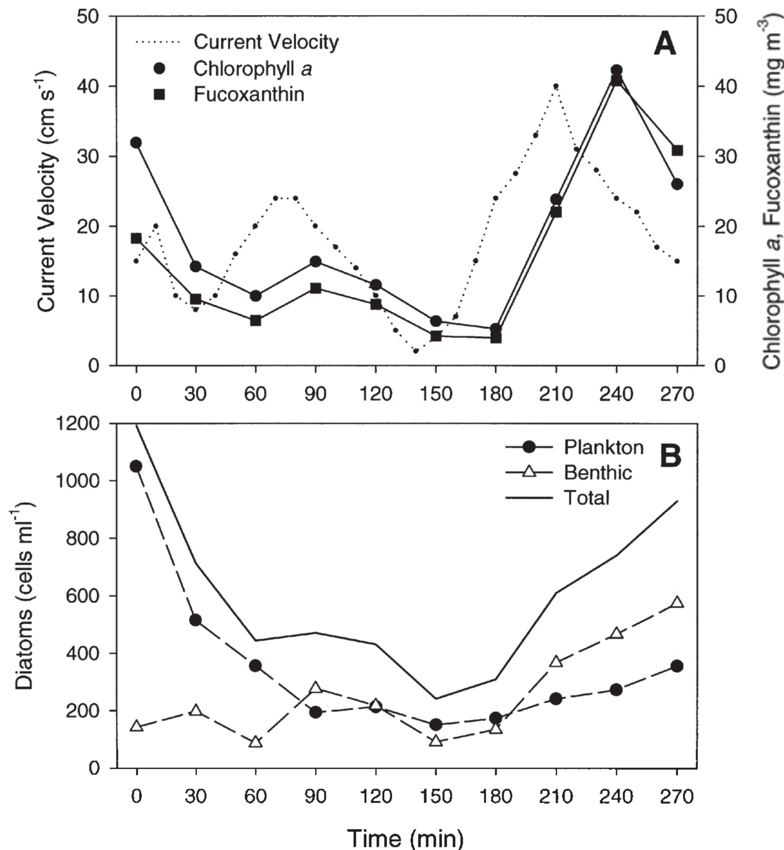

Fig. 6. Dual labelling experiment. (A) Chlorophyll *a* and fucoxanthin in suspended matter of the flume; (B) numbers of benthic and pelagic diatoms in suspended matter

On the basis of field biomass data of June 1997, the macrobenthic species sampled during the experiment incorporated 8.2 mg excess ^{13}C m^{-2} during the experiment, i.e. 5.5% of the label incorporated by the benthic algae, consistent with the *in situ* labelling study.

DISCUSSION

The 3 approaches to revealing the relative importance of microphytobenthos production as a food source for macrobenthos each have a number of limitations and advantages. The natural stable-isotope signatures of the species integrate over relatively long periods of time, and thus represent a weighted signal. However, their interpretation depends on the assumptions of (1) constant or at least consistently different stable-isotope ratios in the food sources (benthic and planktonic algae, and POM in sediment and water), (2) constancy over time and space in the use of these food sources by the species (within the time scales set by tissue turnover time of the animal and the spatial scale set by the movement range of the animal), and (3) consistent fractionation from one trophic level to the next. For a complicated open system such as an intertidal flat, it is very difficult to test the validity of these assumptions.

There is strong evidence that benthic algae have, in general, a different $\delta^{13}C$ signal than phytoplankton. Polar lipid-derived fatty acid (PLFA) biomarkers specific for diatoms showed a $\delta^{13}C$ of −15 to −16‰ for benthic microalgae at Stn 2 (Middelburg et al. 2000). Values for phytoplankton in this section of the Westerschelde are around −21 to −23‰, assuming that phytoplankton is slightly heavier than the bulk suspended organic matter in this region of the estuary (Middelburg & Nieuwenhuize 1998) and taking into account suspended POM $\delta^{13}C$ values of −23.9‰ (June 1998), −22.6‰ (April 1997) and −23.8‰ (June 1996) (Middelburg unpubl. data). It is difficult to partition phytoplankton from other suspended POM in this region of the Westerschelde because size fractionation is complicated by high turbidity levels, and differences in $\delta^{13}C$ with particle-specific gravity are only small (Middelburg & Nieuwenhuize 1998). Other potential food sources were not considered in this study. Macroalgae have negligible biomass in the Westerschelde. The flat is isolated by deep gullies from salt marshes, preventing salt marsh plants (e.g. *Spartina* spp.) to directly reach the experimental plots. Moreover, the net export from salt marshes in the Westerschelde is very limited (Hemminga et al. 1993). Any detritus derived from these sources or from terrestrial organic matter should moreover have shown up in POM. Our study of natural stable-isotope ratios showed that most macrobenthic species have a significantly heavier carbon signature than the phytoplankton and, *a fortiori*, the suspended POM in the water column. Therefore, microphytobenthos production contributes to their diet. Remarkable is also that even deep deposit feeders such as *Heteromastus filiformis* have a much heavier signature than the bulk organic matter in the sediment, which at Stn 2 was −23.0‰, very close to the signature of the suspended POM in the water column. At Stn 4, the bulk organic matter in the sediment was slightly heavier (average −21.8‰), and especially the superficial 1 mm was more influenced by the microphytobenthos signature (−19.3‰). At this sandy station, organic carbon concentration was 1 order of magnitude lower than at the muddy station (Stn 2); therefore, the products of microphytobenthos primary production may constitute a larger fraction of the POM.

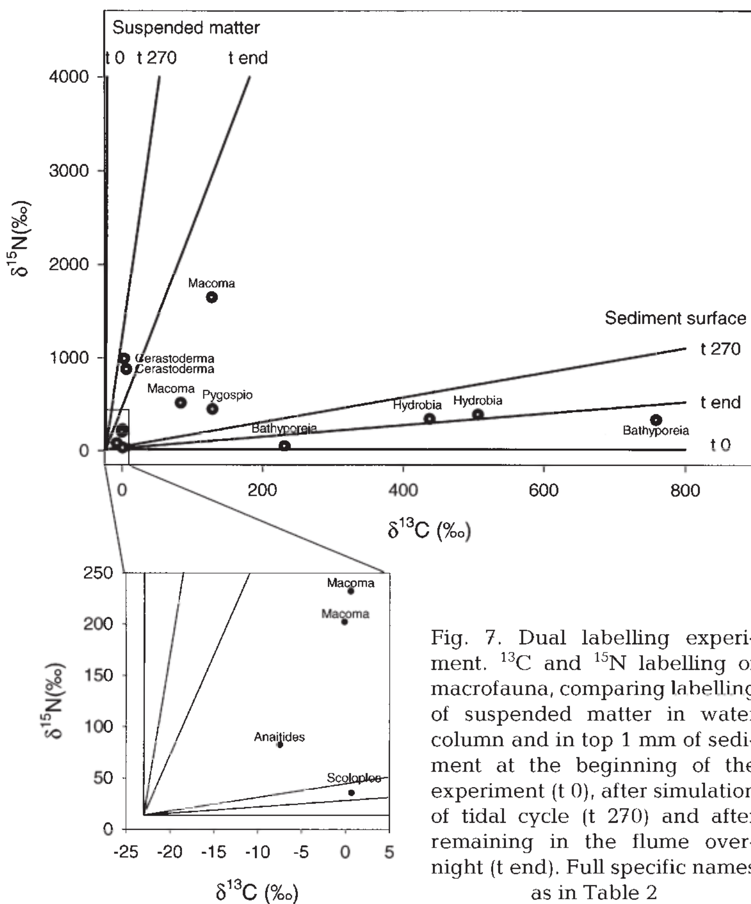

Fig. 7. Dual labelling experiment. ^{13}C and ^{15}N labelling of macrofauna, comparing labelling of suspended matter in water column and in top 1 mm of sediment at the beginning of the experiment (t 0), after simulation of tidal cycle (t 270) and after remaining in the flume overnight (t end). Full specific names as in Table 2

The nitrogen-isotope signatures of the benthic animals differed between Stns 2 and 4. At Stn 2 $\delta^{15}N$ seems to increase with trophic level only (as is generally expected), whereas at Stn 4 $\delta^{15}N$ seems to increase with $\delta^{13}C$. It is very unlikely that a grazer like *Hydrobia ulvae* would occupy a higher trophic level at one station than at another. Published $\delta^{15}N$ values of microphytobenthos are more variable than those of $\delta^{13}C$. Currin et al. (1995) reported a range of –1 to 6‰; Créach et al. (1997) found +6‰ but with a standard deviation of almost 2‰; Riera (1998) found values between 4.1 and 6‰, and Riera et al. (1999) 4.5‰. Most probably, the $\delta^{15}N$ of both phytoplankton and microphytobenthos in the Westerschelde are considerably higher. Estuarine POM at our study zone has a $\delta^{15}N$ varying between +7‰ during a bloom (April 1997) to +14‰ (June 1998) and 13 to 15‰ (June 1996) (Middelburg unpubl. data). It is therefore likely that the highly enriched inorganic nitrogen sources in the Westerschelde give rise to high $\delta^{15}N$ values in the microphytobenthos. The difference between the 2 stations is probably related to differences in the nitrogen cycling between the 2 different sediment types.

The *in situ* labelling experiment offered the opportunity to demonstrate the direct transfer of carbon from the benthic microalgae to the macrobenthic organisms. For all species investigated, some increase in $\delta^{13}C$ was demonstrated. Quantitatively important transfers were only evident for *Macoma balthica* (where it was most pronounced in small specimens), *Nereis succinea* and *Pygospio elegans*. Labelling was generally poorest in the suspension feeders and in the deep deposit-feeding species *Heteromastus filiformis*. In its natural stable-isotope signatures, especially at Stn 2, *Nereis succinea* bore a strong microphytobenthos $\delta^{13}C$ signature. The slightly elevated $\delta^{15}N$ in the natural stable-isotope signature of the species, as compared with *Hydrobia ulvae*, *P. elegans* or *M. balthica*, suggests that part of the transfer from the microalgae to *N. succinea* is through predation on grazing organisms. The species is generally classified as an omnivore (Goerke 1971) with evidence of surface deposit-feeding (Fauchald & Jumars 1979). Middelburg et al. (2000) showed very rapid label transfers through the microheterotrophic food web. After only a few hours, a predatory nematode was already significantly labeled. Similarly, Moodley et al. (2000) showed rapid transfer of label from algal material to foraminiferans. The strong labelling of the (at least partly) predatory polychaete *N. succinea* confirms that trophic transfers in this benthic community are operating fast.

In contrast to the measurements of natural stable-isotope ratios, the labelling experiments may have been biased because no separation was made between gut contents and animal tissue. Consequently, the label uptake rates reported probably overestimate the amount of label actually incorporated into animal tissue. In studies of uptake of xenobiotics, this has been reported to result in considerable overestimation of body burdens (e.g. Cain et al. 1995) or estimates of uptake rate (e.g. Odin et al. 1997). However, as these studies show, the problem becomes smaller as the bioaccumulation of the compound becomes more important and (for bioaccumulating materials) as the experimental uptake time increases: at a constant gut content, more bioaccumulating materials have higher tissue concentrations over time. For labelled algal carbon and experiments lasting at least a number of hours, the bias due to gut contents is likely to be minor. The following simple model can be used to derive an estimate. Assume label is ingested into the gut at a constant rate, U, and that during the passage of food

through the gut the concentration of label decreases linearly from C to $C \times (1 - \alpha)$, where α = absorption efficiency. The amount of label in the gut contents is then given by $C \times U \times \text{GPT} \times (1 - \alpha/2)$, where GPT = gut passage time. The amount of label absorbed per unit time is given by $C \times U \times t \times \alpha$. The fraction of gut content label in the whole body label (including gut contents) is then given by $\text{GPT} \times [1 - \alpha/2] / [\alpha t + \text{GPT}\ (1 - \alpha/2)]$.

With a GPT of 1 h (e.g. *Arenicola marina* [Plante & Mayer 1994], the nematode *Monhystera disjuncta* [Herman & Vranken 1988]) to a few hours (e.g. Bock & Miller 1999) for deposit feeders and up to 4 h for bivalve suspension feeders (Hawkins & Bayne 1984), and an absorption efficiency for algal carbon of 50 to 80%, the fraction of gut content in the whole body will be between 3 and 20% after 24 h and between 0.8 and 6% after 96 h. The model used here may have to be extended or modified to represent faithfully the ingestion and absorption process in particular species, but different models yield similarly low relative importance of the gut content in the whole body label when the label uptake period is several days, as was the case for our macrobenthic data in the *in situ* labelling experiment.

The major limitation of the *in situ* experiment is its limited scope in space and time. The quadrants sprayed and labelled were only ¼ m^2 in surface. Surface-dwelling organisms (e.g. *Hydrobia ulvae*, *Bathyporeia* sp.) could freely move in and out of the quadrant and had not necessarily been feeding on the labelled algae for the whole 3 to 4 d period. The same may have been true for deep-burrowing species such as *Arenicola marina*, since the quadrants were lined by a barrier of only 8 cm deep. Body-size dependence of the labelling, as demonstrated for *Macoma balthica*, may also have influenced the results. On the one hand there may be an ontogenetic shift in dependence on benthic algae as a food source (see e.g. Hentschel 1998). On the other hand weight-specific activity is known to decrease with increasing weight and the poor labelling of large specimens may partly depend on this. Similar to our finding for *M. balthica*, Kang et al. (1999) described a shift in cockles *Cerastoderma edule* from a $\delta^{13}C$ close to that of microphytobenthos for juveniles to a value close to suspended POM for adults. In our study, we only sampled relatively large specimens (around 10 mm) of the cockles.

Limitation by the time scope of the experiment may be particularly important for the deep deposit-feeding species *Heteromastus filiformis*, and probably also for *Arenicola marina*. The natural abundance signatures of these species suggest a moderate (*H. filiformis*) to strong (*A. marina*) dependence on microphytobenthos. It is likely that *H. filiformis* is less dependent on the fresh algae than on detritus derived from these algae. The difference between its $\delta^{13}C$ and that of the bulk POM in the sediment suggests a strong selectivity in uptake for algal detritus. *A. marina* is able to subduct surface sediment very rapidly in its burrow (Rijken 1979), and may therefore ingest benthic algae much more directly.

Because of time and space constraints, the 4 to 7% uptake of the microphytobenthic primary production over a 4 d period, as found in the *in situ* experiment, is probably an underestimate of the rate at which algae and algal detritus are taken up by macrobenthos.

The dual labelling experiment provides information on a limited set of species, due to the relatively small surface of sediment in the flume and the shallow depth (10 cm) to which the sediment was sampled. It offers the advantage that the animals were constrained to the experimental surface, which may have been responsible for the much stronger labelling of *Hydrobia ulvae* and *Bathyporeia* sp. in this experiment than in the *in situ* experiments. For the *in situ* experiment at Stn 4, we estimate that mobile *H. ulvae* incorporated 0.08% of the fixed label over a 4 d period, whereas in the dual labelling experiment, they incorporated 5% in <1 d. Such differences were not observed, however, for the more sedentary species *Cerastoderma edule*, *Macoma balthica*, *Pygospio elegans* and *Scoloplos armiger*. The dual labelling experiment allowed a direct appraisal of selectivity of the species for benthic or pelagic algae, when both were present in a realistic simulation of sedimentation and resuspension. Despite the heavy resuspension of benthic material during the brief period when currents increased to 40 cm s^{-1}, this material appears to settle out of the water column rapidly. Most algae in this sandy sediment may be tightly associated with sediment grains and have a fast sinking velocity (Herman et al. 1999). This association may also have prevented uptake of the benthic algae by the suspension feeder *C. edule* for 2 reasons. Their fast disappearance from the water column limited the time period of their availability to the suspension feeder, and their association with coarse grains may have hindered uptake by the cockles, which exhibit a preference for smaller-sized 'pure' algal particles (Navarro & Iglesias 1993). As a consequence, the cockles were labelled almost exclusively by the pelagic algae, in marked contrast to the benthic grazers. The experiment also confirmed the intermediate position of *M. balthica* and *P. elegans*, with uptake of both types of algal material, as was also suggested by their natural abundance, stable-isotope signatures. This may reflect their direct grazing on benthic algae as well as their grazing on pelagic algae within faecal biodeposits of suspension feeders.

Our experimental approaches confirmed the interpretation of natural stable-isotope ratios in terms of

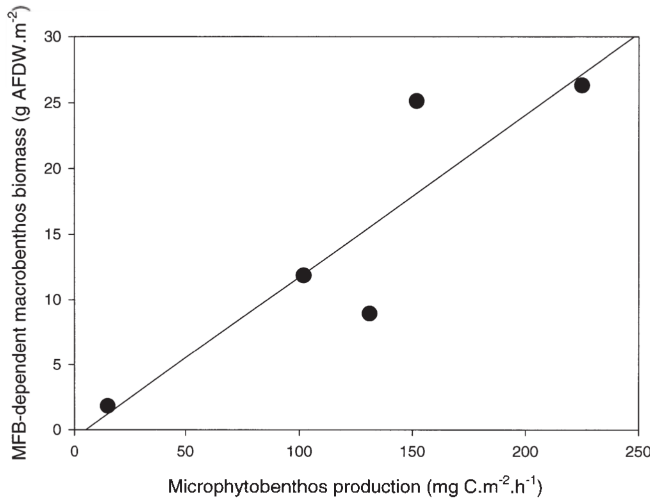

Fig. 8. Relation between microphytobenthic primary production (Hamels et al. 1998, C. Barranguet unpubl. data) and macrobenthic biomass that is calculated to be directly dependent on microphytobenthos (MFB) (see Table 2 for parameters and 'Discussion' for calculation). Linear correlation coefficient is 0.91 (n = 5, p = 0.034). AFDW: ash-free dry wt

the relative importance of microphytobenthos- versus phytoplankton-derived food. The general picture from these measurements was that the $\delta^{13}C$ of the suspension feeders (*Cerastoderma edule*, *Mya arenaria*) was very close to that of pelagic algae, whereas the benthic grazers (e.g. *Hydrobia ulvae*, *Bathyporeia* spp.) had a $\delta^{13}C$ close to that of the benthic algae. A number of deposit-feeder species had intermediate values. Similar to our findings, Riera et al. (1999) found the suspension feeder *Mytilus edulis* closest to the pelagic algae, and the interface feeders *Scrobicularia plana* and *Macoma balthica* intermediate between pelagic and benthic algae.

We used the natural stable-isotope ratios to calculate the relative dependence of the different species on microphytobenthos as food. Assuming a $\delta^{13}C$ of −15‰ for microphytobenthic algae and of −21‰ for phytoplankton algae, the relative dependence of the species on microphytobenthos was calculated as $(X + 21)/6$, where X was the average $\delta^{13}C$ of the species at Stns 2 and 4. This expression yielded a coefficient between 0 (for species entirely dependent on phytoplankton) and 1 (entirely dependent on microphytobenthos).

Multiplying the coefficients in Table 2 with the average biomass of the different species at the 5 stations, we calculated the macrobenthic biomass that is dependent on microphytobenthic primary production. Our measured coefficients covered >95% of the total biomass. For the rest of the biomass we used the weighted average of the coefficients. Over the 5 stations, the microphytobenthos-dependent biomass relates very well to the relative primary productivity of the microphytobenthos, as measured in June 1996 by oxygen microelectrodes (Hamels et al. 1998, Barranguet unpubl. data) (Fig. 8; $r = 0.91$; $n = 5$; $p = 0.034$). The remainder of the biomass is dependent on pelagic primary production or sedimenting allochthonous POM, either directly or indirectly via biodeposition by suspension feeders. It has no significant correlation with microphytobenthos productivity or standing stock; other governing factors are thought to influence this variable.

Herman et al. (1999) made a comparison of macrofauna biomass in different ecosystems and observed that the suspension feeders seem to constitute the most variable part. Deposit feeders are much more evenly distributed over space within an estuary, and their biomass is much less variable from one system to another than the biomass of suspension feeders. This pattern may be due to the different dependence of these broad groups on microphytobenthos production. In this study, we showed that epibenthic grazers and, to a large extent also deposit feeders, depend directly on microphytobenthic production. As this production is relatively constant over a broad range of environments and over the seasonal cycle (Colijn & de Jonge 1984, Steward et al. 1992, Barranguet et al. 1998, Underwood & Kromkamp 1999), a relative constancy of the macrofauna groups dependent on this source may be expected. Suspension feeders, on the other hand, were shown to depend mainly on pelagic food sources in our field observations and experiments. As a consequence, they will depend on different primary production and transport processes operating on much larger spatial scales and implying very different competitive interactions.

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