Chapter 5

The trophic significance of bacterial carbon in a marine intertidal sediment: Results of an in situ stable isotope labeling study

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5.1 Introduction

Deposit feeding organisms face the formidable task of gathering digestible resources that are diluted with minerals and refractory organic matter (Lopez and Levinton, 1987). Bacteria are ubiquitous in marine sediments and because of their high abundance, production and nutritional value they are considered an important resource for sediment dwelling fauna (ZoBell and Feltham, 1937; Gerlach, 1978; Tsuchiya and Kurihara, 1979).

Transfer of bacterial carbon to benthos is often discussed from the bacterial side: is bacterial carbon production a link or sink in the benthic food web (Kemp, 1990)? This bias in favor of the fate of bacterial carbon is also reflected in the methods in use: incubations with ^{14}C organic substrates (Montagna, 1995) or fluorescently labeled bacteria (Starink et al., 1994) measure the amount of bacterial carbon that is grazed per unit of time. These studies show that grazing losses are generally restricted to $<$ 20 % of total bacterial production (Epstein and Shiaris, 1992; Hondeveld et al., 1995; Sundback et al., 1996; Hamels et al., 2001b).

Although bacterial grazing represents a minor fate of bacterial production, one may pose the complementary question whether bacterially derived carbon fulfills a significant part of the total carbon requirements of benthic fauna. However, estimates for the relative importance of bacterial carbon for meiobenthos are scant or are based on qualitative gut content analysis (e.g. for nematodes, Moens and Vincx (1997)). Quantitative data are available for some macrobenthic deposit feeders, based on laboratory measurements of

sediment ingestion rate, bacterial abundance and bacterial digestion efficiency on the one and physiological data on carbon requirements on the other hand (Cammen, 1980; Kemp, 1987; Andresen and Kristensen, 2002). Most of these studies show that bacterial carbon contributes $<$ 10 % of the total requirements. However, deposit feeders are known for their selective uptake of high quality and organic rich particles (Lopez and Levinton, 1987; Neira and Höpner, 1993), which may contain elevated levels of bacteria and therefore the bacterial contribution in their diets may have been underestimated. For example, Hall and Meyer (1998) showed with an in situ ^{13}C-acetate tracer experiment that many stream invertebrates derived more than 50 % of their carbon from bacteria, which is much more than previously anticipated (Baker and Bradnam, 1976). Moreover, the contribution of bacterially derived carbon may differ among the different taxa, for example subsurface deposit feeders that ingest relatively refractory organic matter may be more dependant on bacteria for labile carbon and essential nutrients. Therefore, quantitative data on the bacteria - benthos links are essential for understanding the extent to which this trophic link may structure sediment communities.

In situ stable isotope labeling experiments have proven to be an excellent tool to study element transfer (Blair et al., 1996; Hall and Meyer, 1998; Middelburg et al., 2000). Here we employ stable isotope labeling in a marine intertidal sediment to estimate the amount of carbon that meiobenthic taxa and macrobenthic species derive from bacteria. ^{13}C-glucose was injected into the sediment to tag the bacterial community, incorporation of ^{13}C-glucose by bacteria was traced through ^{13}Cenrichment of bacterial specific phospholipids derived fatty acids (PLFAs). Subsequent transfer of bacterial-derived ^{13}C to benthic fauna was followed through ^{13}C enrichment of hand-picked specimens. A simple isotope model was used to recover the dependency on bacterial carbon from the observed tracer dynamics. Specifically, we will focus on the following questions: (1) How much does bacterial carbon contribute to the carbon requirements of meiobenthic and macrobenthic species? (2) Is the contribution of bacteria derived carbon related to feeding/living depth in the sediment? (3) Are there indications for selective feeding by the benthic fauna?

5.2 Materials and methods

5.2.1 Study site and experimental approach

The Molenplaat intertidal flat was chosen as study site and is located in the saline part (salinity 20-25) of the turbid, heterotrophic and nutrient-rich Scheldt estuary. The sampling site is located in the silty center of the flat (51°26.25' N, 3°57.11' E), which has a median grain size of 77 μm, organic carbon content of $\sim$ 0.5 % wt/wt and exposure time of about 7 hours per tidal cycle (see Herman et al. (2001) for detailed information).

The data presented here have been collected in the frame of an experiment on the fate of bacterial carbon production and two companion papers deal with the fate of bacterial carbon production (Chapter 4) and the fate of bacterial phospholipids, peptidoglycan and proteins (Chapter 6). Methodological details are provided in chapter 4. In short, two 0.25 m^2 square metal frames were inserted in the sediment. On 21 May 2003, the upper 10 cm was labeled with ^{13}C by injecting a glucose solution with a syringe (one injection per 6.25 cm^2), resulting in a flux of 15.3 mmol ^{13}C m^{-2} per labeling day. Labeling was performed daily for 5 consecutive days to ensure sufficient label incorporation by bacteria, but labeling on day 2 had to be canceled due to bad weather. Ten samples (day 0.3, 2, 3, 4, 5, 6, 8, 12, 18 and 36 after the first injection) were collected from each plot in the first weeks from a priori randomly assigned positions. A sampling core (Ø 5 cm) was

inserted 10 cm deep, filled with filtered sea water and closed with a stopper. A metal core (Ø 9 cm) was inserted around the sampling core, which successfully prevented disturbance of the rest of the plot and remained in place during the experiment. The sampling core was carefully withdrawn and transported in a dark cool container to the laboratory. In the laboratory, sediment cores were sliced (0-2, 2-5 and 5-10 cm), homogenized and sampled for δ^{13}C-PLFA, meiobenthic biomass and label incorporation and macrobenthos label incorporation. Samples for d ^{13}C-PLFA were frozen, freeze-dried and stored frozen. Meiobenthic and macrobenthic samples were fixed with formalin (final concentration 4 %). Background δ^{13}C for PLFA, meiobenthos and macrobenthos were taken from the t = 0 sampling core. Some macrobenthos species were not present in the t = 0 samples, in which case background δ^{13}C was taken from Herman et al. (2000). Macrobenthic biomass could not be accurately determined from the small cores taken from the experimental plots and was therefore based on 12 separate cores (Ø 10 cm) taken in close proximity of the experimental plot.

5.2.2 Analytical procedures

Lipids were extracted from 3 g dry sediment using a Bligh and Dyer extraction, from which the PLFA fraction was isolated. The PLFA extract was derivatized to volatile fatty acid methyl esters and measured by gas chromatography-isotope ratio mass spectroscopy (GC-IRMS) for PLFA isotope values (details in Boschker et al. (1999)). The bacterial isotope signature was determined from the weighted average of the specific bacterial PLFA biomarkers i14:0, i15:0, a15:0, i16:0 and 18:1ω7c (Middelburg et al., 2000). PLFAs are present in the membrane and comprise roughly 6 % of the total carbon in a bacterial cell, the bacterial-specific PLFAs together account for 28 % of the carbon in all bacterial PLFAs (Middelburg et al., 2000). These conversion factors are used to convert PLFA concentration to bacterial biomass and label incorporation in PLFAs to total bacterial label incorporation (Middelburg et al., 2000).

Meiobenthic samples were sieved (38 μm) and sub-sampled. Specimens for stable isotope measurements, typically 15-30, were handpicked, cleaned of adhering detritus, rinsed (0.2 μm filtered water), transferred to silver boats and stored frozen. Processing meiobenthic samples proved to be very time consuming and only one of the two plots was therefore processed for stable isotope and biomass data, as described in Moodley et al. (2000).

Macrobenthic specimens were handpicked, stored in filtered water and preserved with formalin. The sorted sample was transferred to a petri-dish, a species sample was taken, cleaned from debris, rinsed, transferred to a silver boat and stored frozen. Bivalves and Gastropods were placed in an acidified bath (1 mmol HCl) to dissolve their carbonate shell and either whole specimens (*Macoma balthica* ($<$ 7 mm) and *Hydrobia ulvae*) or flesh samples (M. balthica ($\geq$ 7 mm)) were taken. Meiobenthic and macrobenthic samples were thawed, acidified for carbonate removal with 20 μl 2.5 % HCl and oven-dried (50 °C) prior to isotope analysis. Stable isotope ratios were measured by elemental analyzer - isotope ratio mass spectrometry (EA-IRMS) (Middelburg et al., 2000).

Delta values are expressed relative to the carbon isotope ratio (R: $\frac{^{13}C}{^{12}C}$) of Vienna Pee Dee Belemnite (VPDB): $\delta^{13}C = (\frac{R_{sample}}{R_{VPDB}} - 1) \cdot 1000$, with $R_{VPDB} = 0.0112372$. Label uptake by organisms is reflected as enrichment in ^{13}C and is presented as $\Delta\delta^{13}$C, which indicates the increase in δ^{13}C of the sample as compared to its natural background value and is calculated as: $\Delta\delta^{13}C = \delta^{13}C_{sample} - \delta^{13}C_{background}$. Hence, positive $\Delta\delta^{13}$C values indicate that the organism has acquired some of the introduced ^{13}C label.

5.2.3 Tracer model and calibration

The relative contribution of bacteria derived carbon was estimated from comparing the $\Delta\delta^{13}$C of a consumer ($\Delta\delta^{13}C_{con}$) with that of bacteria ($\Delta\delta^{13}C_{bac}$). If the $\Delta\delta^{13}C_{bac}$ and $\Delta\delta^{13}C_{con}$ have reached steady-state, the ratio indicates the fraction of total carbon in the consumer that is derived from bacteria. However, this steady-state assumption is not valid for all organisms within an experimental time frame of several weeks (Hall and Meyer, 1998) and an isotope model that simulates tracer dynamics in a consumer is then a better option (Hamilton et al., 2004). The isotope model can however, only be applied for frequently sampled species due to its higher data requirements. Therefore, dependence on bacterial carbon for species that were encountered repeatedly in the time series samples was estimated by means of the isotope model. For species that were only occasionally encountered, contribution of bacteria derived carbon was estimated from the ratio $\frac{\Delta\delta^{13}C_{con}}{\Delta\delta^{13}C_{bac}}$, because they were not sampled frequent enough to justify fitting the data with the isotope model. The isotope model reads (Hamilton et al., 2004)

$$\frac{d\,\Delta\delta^{13}C_{con}}{d\,t} = k_b \cdot \Delta\delta^{13}C_{bac} - k_c \cdot \Delta\delta^{13}C_{con} \tag{5.1}$$

The first term in the right hand side of the equation denotes label uptake by grazing on bacteria and the second label loss through turnover of the consumer. The dynamics of the PLFA bacterial biomarkers are used as a proxy for $\Delta\delta^{13}C_{bac}$ data and are imposed as a forcing function. The turnover rate constant (k_c) determines the total carbon requirements of the consumer and the ratio $\frac{k_b}{k_c}$ denotes the relative contribution of bacterial carbon to total carbon requirements. If bacteria fulfill all carbon requirements, i.e. $\frac{k_b}{k_c} = 1$, the $\Delta\delta^{13}C_{con}$ approaches the $\Delta\delta^{13}C_{bac}$ with time and when bacteria do not contribute (i.e. $\frac{k_b}{k_c} = 0$), there is no label uptake by the consumer (see Hamilton et al. (2004) for detailed model demonstrations).

Plausible parameter ranges were large to assure a complete coverage of potential growth rates and were 0.05 - 0.50 d^{-1} (k_c) and 0.0 - 0.50 $_{\cdot}$(k_b) for meiobenthos and 0.025 - 0.25 $_{\cdot}$(k_c) and 0.0 - 0.25 d^{-1} (k_b) for macrobenthos. The parameters were calibrated by minimizing the sum of squared differences between the data points and the model prediction.

It proved impossible to calibrate the model parameters k_b and k_c individually, as different combinations gave similar optimal fits, indicating that the parameters are correlated. To resolve this issue we employed Bayesian analysis. Bayesian analysis is a statistical technique that updates a prior probability distribution of a parameter with observations to arrive at a posterior probability distribution (Gelman et al., 2003). The update makes the posterior better constrained than the prior. The prior probability distributions are the initial parameter ranges for which we assume equal probability for each value within this range. The Bayesian analysis starts with a model run with a certain parameter combination, a Markov Chain Monte Carlo technique (Gilks et al., 1998) then takes random steps in parameter space and the model is solved with each parameters set. If a parameter combination gives a better fit to the data than the previous parameter combination, the run is accepted and used as new starting point for a following random step. If the new parameter combination fits worse, it can be accepted with a probability equal to the ratio of probabilities of the tested versus the existing parameter combination. The distribution of parameter values in the set of accepted runs is the posterior probability distribution of each parameter. We ran the model for each species 10000 times, which typically gave $\sim$ 1000 accepted runs. The mean and standard deviation of the ratio

$\frac{k_b}{k_c}$ for a species was then calculated from the accepted model runs. The model was implemented in the freely available simulation environment FEMME (Soetaert et al. (2002), http://www.nioo.knaw.nl/ceme/femme).

To test whether dependence on bacterial carbon for meiobenthos increases with depth in the sediment, the data of a meiobenthic group of a respective depth interval was fitted with the bacterial $\Delta\delta^{13}$C from the respective interval. For macrobenthos it is difficult to directly link their presence in a certain depth interval with their feeding depth because the size of these species ($\sim$ cm) is comparable to that of the depth intervals. Therefore we used the feeding classification for macrobenthos species (Fauchald and Jumars, 1979; Pearson, 2001) and compared surface deposit and subsurface deposit feeders. When appropriate, data from all depth layers were pooled and used to calculate dependence on bacterial carbon.

5.3 Results

5.3.1 Label incorporation by bacteria

The $\Delta\delta^{13}$C of different bacterial PLFAs were weighted with their respective concentration to obtain a proxy for bacterial $\Delta\delta^{13}$C. Dynamics of bacterial $\Delta\delta^{13}$C were very consistent between plots and intervals for the upper two depth intervals (0-2, 2-5 cm) but the deepest interval (5-10 cm)showed differences between the plots and was somewhat higher labeled than the upper two intervals (Fig. 5.1A). Due to the lower concentration of PLFAs in the deepest interval (374, 181, 227 mmol C m^{-2} in the depth intervals 0-2, 2-5 and 5-10 cm, respectively), its influence on the average bacterial $\Delta\delta^{13}$C is limited (Fig. 5.1A). The average forcing function, weighted with concentration and thickness of the depth interval, for bacterial $\Delta\delta^{13}$C was used in the isotope model or ratio calculations, because the results did not depend critically on whether distinctions were made among intervals or plots (see 5.4 Discussion). The bacterial $\Delta\delta^{13}$C increased during and shortly after the ^{13}C-glucose injection period, peaked at 519 ‰ on day 5 and decreased to 173 ‰ at day 36.

5.3.2 Meiobenthic and macrobenthic biomass

Meiobenthic biomass was 188 mmol C m^{-2} and was dominated by nematodes (35 %), hard-shelled foraminifera (33 %) and juvenile *Heteromastus filiformis* (18 %) (Table 5.1). Copepods, soft-bodied foraminifera, small polychaetes and turbellaria each comprised $\leq$ 5 % of the meiobenthic biomass. The meiobenthic biomass was highest in the top interval (0 - 2 cm, 72 % of total meiobenthic biomass) and all meiobenthic groups were present here (Table 5.1). The number of meiobenthic groups decreased with depth, with only nematodes, hard-shelled and soft-bodied foraminifera present in the deepest layer. Nematodes dominated biomass in the top two intervals (37 % and 58 %, respectively), whereas hard-shelled foraminifera clearly dominated in the deepest interval (91 %) (Table 5.1). *H. filiformis* juveniles represented a significant amount of the meiobenthic-sized biomass in the top layer (24 %), but they vanished in the middle depth interval.

Macrobenthic biomass was 1684 mmol C m^{-2}, label uptake was measured in species representing 96 % of the biomass, albeit with different frequencies. The remaining 4 % of the biomass was made up of species that were not sampled with the experimental cores. Large specimens of the bivalve *Macoma balthica* ($\geq$ 7 mm, 41 %) and the polychaetes

Heteromastus filiformis (37 %) and *Pygospio elegans* (13 %) dominated the macrobenthic biomass (Table 5.1). Macrobenthic biomass did not show a pronounced trend with depth in the sediment, because the biomass important species *M. balthica* ($\geq$ 7 mm) and *H. filiformis* have their biomass maxima in the middle and deepest depth interval, respectively. This compensates the strong decrease in biomass with depth for other species, such as *P. elegans*, *Polydora cornuta*, *M. balthica* ($<$ 7 mm), *Hydrobia ulvae* and *Corophium* spp., which all had more than 80 % of their biomass in the top layer. The species *Arenicola marina*, *Eteone* spp. and *Cyatura carinata* and *M. balthica* ($\geq$ 7 mm) had their highest biomass in the middle depth interval.

5.3.3 Meiobenthic and macrobenthic label incorporation

All sampled species acquired ^{13}C label, but $\Delta\delta^{13}$C dynamics differed among groups and species (Fig. 5.1). Among the meiobenthos, polychaetes (Fig. 5.1N) and copepods (Fig. 5.1I) only marginally increased in $\Delta\delta^{13}$C and remained below 20 ‰ during the experiment. Juvenile *Heteromastus filiformis*, soft-bodied and hard-shelled foraminifera (Fig. 5.1L, K, J) showed similar label dynamics with a steady increase to 60 ‰ followed by an exponential-like decrease to almost background levels at day 36. Among the macrobenthos, *Macoma balthica* ($<$ 7 mm, Fig. 5.1E) attained highest $\Delta\delta^{13}$C values (217 ‰ at day 7), but returned to almost background values at day 18 (22 ‰). In contrast, *H. filiformis* incorporated label slowly and its $\Delta\delta^{13}$C remained constant at $\sim$ 50 ‰ over a month (Fig. 5.1D). Labeling of large *M. balthica* ($\geq$ 7 mm, Fig. 5.1F) specimens was highly variable, but overall lower than labeling of small specimens (Fig. 5.1E). Label incorporation by *Corophium* spp. was very rapid and peaked at 82 ‰, but its $\Delta\delta^{13}$C signal rapidly decreased in an exponential fashion when the ^{13}C-glucose injection had ended (Fig. 5.1B). The $\Delta\delta^{13}$C dynamics of *Polydora cornuta* and *Pygospio elegans* resemble each other with a steady increase during the first 10 days, followed by a slow decrease (Fig. 5.1G, H).

5.3.4 Dependence on bacterial carbon

As pointed out earlier, fitting the individual parameters k_b and k_c of the isotope model proved impossible due to the strong correlation between both parameters. For example, many combinations give an acceptable fit of the observed $\Delta\delta^{13}$C values for *Heteromastus filiformis* (Fig. 5.1D, 5.2A). However, the relative contribution of bacterial carbon to the consumers total carbon requirements, i.e. $\frac{k_b}{k_c}$, is our prime interest and this ratio is much better constrained than the individual parameters (Fig. 5.2). The distribution of the ratios in the accepted set of Bayesian runs approximates a normal distribution, from which the average and standard deviation can be derived (Fig. 5.2B). The histograms for the other species/groups are not shown, but all give a similar picture. The $\Delta\delta^{13}$C dynamics of most consumers could be readily fitted with the simple isotope turnover model (Fig. 5.1). Good visual fits were however not obtained for *Macoma balthica*, in particular the small ($<$ 7 mm) specimens (Fig. 5.1E), and *Corophium* spp. (Fig. 5.1B), for which peak labeling and label loss rate were underestimated by the model.

Both the results from the isotope model (Eqs. 5.1) and ratio estimates ($\frac{\Delta\delta^{13}C_{con}}{\Delta\delta^{13}C_{bac}}$) show that dependence on bacterial carbon was limited (Table 5.1). The dependencies for meiobenthos were $\leq$ 0.14 and averaged at 0.08. The estimates for macrobenthos were more variable, ranging from 0.00 to 0.23, but averaged at 0.11 (Table 5.1). The dependence for small *M. balthica* ($<$ 7 mm) was higher (0.36), but we consider this estimate unreliable

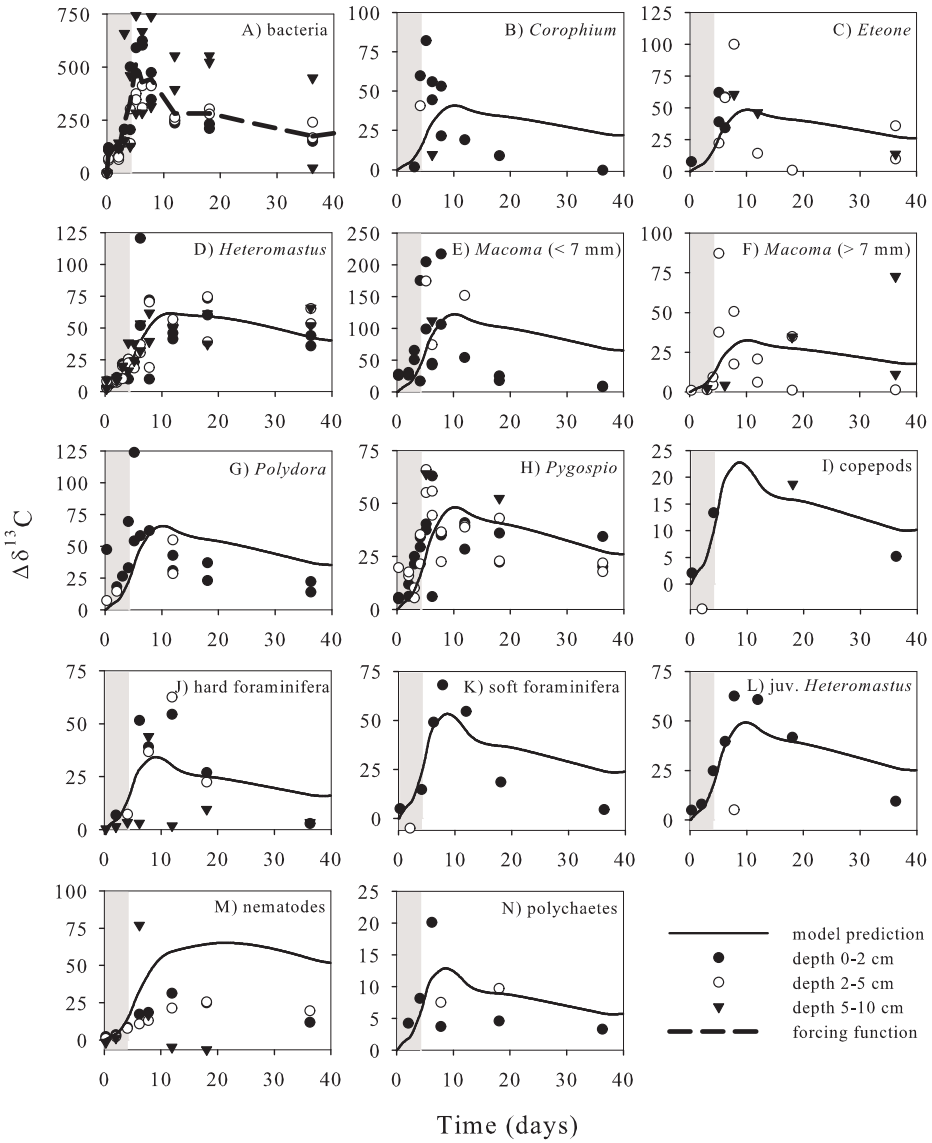

Figure 5.1: A) Observed data and forcing function of $\Delta\delta^{13}$C of bacteria. B-N) Observations and best-fit for the macrobenthos species (B) *Corophium* spp. (n = 12), (C) *Eteone* spp. (n = 15), (D) *Heteromastus filiformis* (n = 58), (E) *Macoma balthica* ($<$ 7 mm) (n = 23), (F) *M. balthica* ($\geq$ 7 mm) (n = 18), (G) *Polydora cornuta* (n = 19), (H) *Pygospio elegans* (n = 41) and meiobenthic groups (I) copepods (n = 5), (J) hard-shelled foraminifera (n = 20), (K) soft-bodied foraminifera (n= 8), (L) juvenile *H. filiformis* (n = 9), (M) nematodes (n = 22) and (N) small polychaetes (n = 8). Shown are data pooled from both plots in the three depth intervals. Shaded area indicates period of ^{13}C-glucose injection.

because of the poor fit to the data (see 5.4 Discussion). Despite the variability in the $\Delta\delta^{13}$C data, bacterial carbon contributions to diets could be readily estimated for most species, as most standard deviations are between 25 and 30 % of the mean.

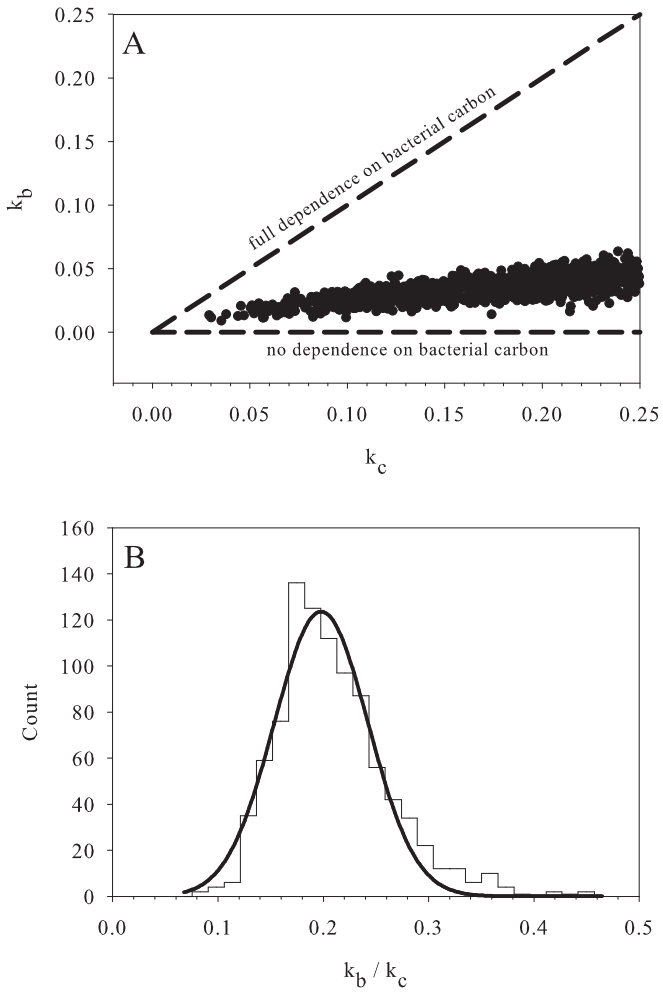

Figure 5.2: A) Scatter plot of the accepted Bayesian runs of the model parameters k_b and k_c for *Heteromastus filiformis*, the linear relationship among both parameters shows that the ratio of the parameters is better constrained than the individual parameter values. B) histogram of the ratio $\frac{k_b}{k_c}$ from the data in Fig. 5.2A and the fitted normal distribution. Histograms for the other organisms fit the normal distribution equally or better.

Nematodes and hard-shelled foraminifera were encountered frequently enough in all depth interval samples to use the isotope model to examine whether dependence on bacterial carbon changed with depth in the sediment. Nematodes did not show differences with regard to living depth (0.08 ± 0.02, 0.08 ± 0.02 and 0.06 ± 0.04 for 0-2, 2-5 and 5-10 cm, respectively). Hard-shelled foraminifera dependence on bacteria was similar for the upper two sediment layers (0.13 ± 0.03 and 0.15 ± 0.05), but was much lower in the deepest sediment layer (0.03 ± 0.02), where they dominated meiobenthic biomass. Other meiobenthic groups had ≤ 3 observations in the middle or deepest depth layer, which does

not allow reliable fitting with the isotope model. However, the ratio $\frac{\Delta\delta^{13}C_{con}}{\Delta\delta^{13}C_{bac}}$ of all samples was smaller or similar to estimated dependence on bacterial carbon estimated for the top sediment layer for each group (data not shown), indicating no increase in dependence with depth for these meiobenthic groups.

There were also no clear differences in dependence on bacterial carbon among macrobenthic feeding modes (Table 5.1), although the subsurface feeders *Heteromastus filiformis* (0.21) and *Arenicola marina* (0.23) seem to have a slightly higher dependence on bacterial carbon as compared to surface feeders (typically between 0.10 and 0.15, table 5.1). As an additional check, the ratio $\frac{\Delta\delta^{13}C_{con}}{\Delta\delta^{13}C_{bac}}$ of all macrobenthic species was evaluated in each depth layer and this confirmed that there was no trend in dependence on bacterial carbon with respect to depth (data not shown).

Table 5.1: Dependence on bacterial carbon by the benthic community. Biomass (mmol C m^{-2}) is shown as depth integrated (B) and partitioned over the three depth intervals (d1: 0-2 cm, d2: 2-5 cm, d3: 5 - 10 cm). Feeding modes (FM) for macrobenthos are surface deposit feeder (SDF), predator (P), subsurface deposit feeder (SSDF), suspension feeder (SF) or omnivore (O). The ratio $\frac{k_b}{k_c}$ is the contribution of bacterial carbon to total carbon demands as average $\pm$ standard deviation derived from Bayesian analysis (see 5.2.3 Tracer model and calibration). Numbers in italic indicate a poor model fit (see section 5.4). The ratio $\frac{\Delta\delta^{13}C_{con}}{\Delta\delta^{13}C_{bac}}$, with bracketed day number, is shown for all sampled meiobenthos and macrobenthos.

Species/group	**B**	**d1**	**d2**	**d3**	**FM**	$\frac{k_b}{k_c}$	$\frac{\Delta\delta^{13}C_{con}}{\Delta\delta^{13}C_{bac}}$
copepods	3	2	0.1	-		0.06±0.02	0.03 (36)
foraminifera (hard)	62	31	6	25		0.09±0.03	0.02 (36)
foraminifera (soft)	5	4	0.5	0.6		0.14±0.04	0.03 (36)
juv *H. filiformis*	34	32	2	-		0.14±0.03	0.05 (36)
nematodes	67	50	15	2		0.06±0.02	0.09 (36)
small polychaetes	4	4	0.2	-		0.03±0.01	0.02 (36)
turbellaria	4	4	-	-			0.14 (18)
unknown species	9	7	2	-			0.02 (36)
Corophium spp.	4	3	1	0.2	SDF	*0.12±0.05*	0.00 (36)
Eteone spp.	15	2	13	0.3	P	0.15±0.04	0.11 (36)
Heteromastus filiformis	597	10	194	393	SSDF	0.21±0.05	0.30 (36)
M. balthica ($<$ 7 mm)	28	28	0.2	-	SDF	*0.36±0.09*	0.05 (36)
M. balthica ($\geq$ 7 mm)	671	-	612	59	SDF	0.11±0.04	0.16 (36)
Polydora cornuta	34	33	0.5	-	SF/SDF	0.20±0.04	0.10 (36)
Pygospio elegans	215	185	30	0.1	SDF	0.15±0.03	0.14 (36)
Arenicola marina	0.2	0.09	0.1	-	SSDF		0.23 (8)
Cyatura carinata	7	3	4	0.2	P		0.00 (6)
Hydrobia ulvae	24	24	-	0.2	SDF		0.01 (18)
Nereis (Hediste) spp.	21	0.3	-	21	O		0.05 (36)
Streblospio benedicti	1	0.2	0.8	-	SDF		0.01 (18)
Tharyx marioni	3	0.3	2	-	SDF		0.06 (36)

5.4 Discussion

Sediment organic matter is a complex mixture of pools that differ in lability, nutritional value and origin and it is very difficult to link carbon sources to the sediment dwelling community in situ. We have successfully employed a long-term stable isotope labeling approach to quantify the importance of bacteria as a carbon source for the benthic community.

One of the assumptions of the isotope turnover model is that bacteria are the only ^{13}C source for the benthic fauna. However, benthic organisms might have acquired label by direct utilization of the added ^{13}C-glucose, which would complicate the interpretation of the label dynamics. In this respect it is relevant to compare the time scale of the experiment ($\sim$ weeks) with turnover times of ^{13}C-glucose ($\sim$ minutes, Sawyer and King (1993)) and bacteria ($\sim$ weeks, Schallenberg and Kalff (1993)). These time scale differences imply that direct ^{13}C-glucose uptake would be characterized by immediate labeling that stops shortly after the glucose injection period, whereas ^{13}C-bacteria uptake would be characterized by a somewhat delayed but longer lasting label uptake.

Small *Macoma balthica* ($<$ 7 mm)and *Corophium* spp. showed rapid labeling with peak labeling shortly after the end of the ^{13}C-glucose injection and a very rapid loss of label which cannot be explained by bacterial grazing. These are indications of direct ^{13}C-glucose uptake. Indeed, the fact that the data could not be fitted with the bacterial $\Delta\delta^{13}$C dynamics as forcing function (Fig. 5.1B and E), suggest alternative routes of label uptake. For these reasons we regard the estimates for *M. balthica* ($<$ 7 mm) and *Corophium* spp. as unreliable. However, these results do also suggest that only these two species may utilize dissolved organic matter (DOC), whereas this was not evident for other species. Data on $\Delta\delta^{13}$C of DOC are not available, so that the relative importance of this carbon source for *M. balthica* ($<$ 7 mm) and *Corophium* spp. cannot be evaluated.

Most species continued to take up label after completion of label injection and some $\Delta\delta^{13}$C trajectories approached a rather constant level (Fig. 5.1). These are both indications of transfer of bacterial ^{13}C rather than direct ^{13}C-glucose uptake and suggest that our estimates of dependence on bacterial carbon are reliable. The ratio $\frac{\Delta\delta^{13}C_{con}}{\Delta\delta^{13}C_{bac}}$ was used to quantify dependence on bacterial carbon for infrequently sampled groups/species, which is valid approach only at or close to steady-state. Data availability limited quantitative evaluation of the $\Delta\delta^{13}C_{con}$ dynamics to assess possible inference of ^{13}C-glucose uptake or whether or not steady-state has been reached and these estimates should therefore be viewed with more caution as compared to estimates derived from the isotope model.

Another potential problem is uptake of ^{13}C-glucose by epicuticular bacteria (i.e. bacteria attached to the body surface), a potential artifact that was reported for ^{14}C-acetate in copepods bacterivory experiments (Carman, 1990). However, to explain a $\Delta\delta^{13}$C of 50 ‰ (a typical value for benthos, Fig. 5.1) and assuming that epicuticular bacteria have a $\Delta\delta^{13}$C similar to sedimentary bacteria (280 - 519 ‰), thoroughly cleaned meiobenthic and macrobenthic specimens would have consisted of 11 to 22 % of epicuticular bacteria. Therefore, although epicuticular bacteria might elevate the $\Delta\delta^{13}$C signal of consumers, it is unlikely that this would explain the dominant part of the signal. Moreover, arguments with regard to the short turnover time of ^{13}C-glucose as given above, also apply here.

Although not considered a major artifact here, direct uptake of ^{13}C-glucose and via epicuticular bacteria uptake would result in an overestimation of the dependence on bacterial carbon. As a final remark, model simulations taking possible DOC uptake into account, indicate that the total meiobenthic and total macrobenthic derive 10 % and 20 %, respectively, of their total carbon demands from bacteria (Chapter 4), consistent with the

results presented here for the individual groups/species.

$\Delta\delta^{13}$C labeling patterns of bacteria were very consistent between the two plots for the upper two depths, but were more variable for the deepest depth layer (Fig. 5.1A). The average bacterial $\Delta\delta^{13}$C captures the overall dynamics well, particularly for the upper two depths where most of the grazing has taken place. Nevertheless, labeling of some grazers was rather variable (Fig. 5.1), though at a level which is typical for in situ labeling experiments (Hall and Meyer, 1998; Middelburg et al., 2000). To examine the effect of this variability on our results, the observations of the frequently sampled *Heteromastus filiformis* were fitted separately for each plot with the bacterial $\Delta\delta^{13}$C for the respective plot as forcing function. The derived dependencies were similar for both plots (0.21 ± 0.04 versus 0.21 ± 0.06). Because other species have a similar level of variability, we assert that our results are robust, despite the high variability inherent in this type of experiments.

5.4.1 Bacteria as a carbon source

Due to methodological difficulties in measuring bacterivory and total carbon demand requirements simultaneously, there are few studies that have quantified the importance of bacterial carbon for the benthic community. Sundback et al. (1996) measured grazing rates on microphytobenthos and bacteria by the meiobenthic groups nematodes, harpacticoids and 'others' in a microtidal sandy sediment. Grazing on microphytobenthos exceeded that on bacteria to the extent that the contribution of bacterial carbon was generally restricted to $<$ 10 %. Our results agree very well with these estimates, especially for nematodes and other meiobenthic groups. The nematode community at our study site was dominated by *Tripyloides gracilis*, *Viscosia viscosa*, *Ptycholaimellus ponticus* and *Daptonema tenuispiculum* (Steyaert et al., 2003). Moens and Vincx (1997) used gut contents analysis to identify particulate food sources of nematodes and report no or only a limited importance of bacteria for these species. We find a contribution of bacterial carbon of 0.06 ± 0.02 and thus corroborate the results from gut content analysis. It will be interesting to see whether this type of labeling approaches confirm the importance of bacterial carbon for nematodes that have been classified as bacterivores. Moens and Vincx (1997) suggested that DOC uptake may potentially be important for some nematodes, this was however not evident in our study (Fig. 5.1M).

Among meiobenthic groups, highest dependence on bacterial carbon was found for hard-shelled and soft-bodied foraminifera (9 and 14 %, respectively, Table 5.1). These protozoans gather food particles through a network of pseudopodia and actively select particles before they are ingested (Moodley et al. (2000) and references therein). The selected nutritious particles might be highly populated by bacteria, which might explain their relatively high dependence on bacterial carbon.

Most estimates of importance of bacteria as carbon source exist for macrobenthic deposit feeders and are based on measured sediment ingestion rate and bacterial abundance in relation to carbon requirements assessed from physiological measurements or literature data (Cammen, 1980; Kemp, 1987; Plante et al., 1989; Cheng and Lopez, 1991; Andresen and Kristensen, 2002). Cammen (1980) found that bacteria supply between 7 and 10 % of the carbon requirements of the deposit feeder *Nereis succinea*. Due to fragmentation of the specimens we were not able to distinguish between *N. diversicolor* and *N. succinea*, but the ratio $\frac{\Delta\delta^{13}C_{con}}{\Delta\delta^{13}C_{bac}}$ of 0.05 (day 36) confirms the limited dependence of *Nereis (Hediste)* spp. on bacterial carbon (Table 5.1).

Arenicola marina exhibits strong bacteriolytic activity in its midgut section (Plante and Mayer, 1994), reducing the ambient bacterial density up to 70 % during transition of

the digestive tract (Aller and Yingst, 1985; Grossmann and Reichardt, 1991). However, gut contents analysis and subsequent carbon budget calculations show that bacteria fulfill only 3 - 8 % of the total carbon requirements of *A. marina* (Andresen and Kristensen, 2002). Due to the low density of *A. marina* at our study site we obtained only one $\Delta\delta^{13}$C observation (day 8), from which we estimate a contribution on bacterial carbon of 0.23 (Table 5.1). This figure should be taken with extreme caution due to limited sampling, but suggests that bacteria might sometimes be a more important carbon source. It will be interesting to apply the labeling approach in areas densely populated with *A. marina* to examine in situ the importance of bacterial carbon.

Clough and Lopez (1993) investigated the importance of potential carbon sources for *Heteromastus filiformis*. Bacterial carbon was not considered important, because only 26 % of the ingested bacteria were assimilated during gut passage. This figure alone is not sufficient to quantify dependence on bacterial carbon, because the ingestion rate of bacterial carbon is also required. Following their budget calculations for organic matter and assuming that bacterial carbon is 1 % of sedimentary organic carbon one arrives at a contribution of $\sim$ 3 % in the budget of *H. filiformis*. This is much lower than our estimate of 21 % (Table 5.1). Fecal casts of *H. filiformis* are several times enriched in organic carbon, nitrogen and protein content relative to sediments at feeding depth, which clearly shows selective feeding capabilities of *H. filiformis* (Neira and Höpner, 1994; Wild et al., 2005). If these worms select preferentially reactive organic matter with associated bacteria, this could explain why we find higher dependence on bacterial carbon as compared to the dependencies based on indiscriminate feeding in the budget calculations. Moreover, Aller and Yingst (1985) reported that bacterial densities are greatly reduced in fecal pellets as compared to the surrounding sediment, implying efficient use of bacterial carbon. Because uptake of several potential carbon sources was insufficient to account for the carbon requirements of *H. filiformis*, Clough and Lopez (1993) and Neira and Höpner (1994) suggested that dissolved organic carbon (DOC) might be an important additional carbon source. There were however no signs of direct ^{13}C-glucose uptake in our experiment (Fig. 5.1D). In fact, *H. filiformis* was sampled very frequently (n = 58) and these observations could be accurately fitted with bacterial $\Delta\delta^{13}$C as forcing function (Fig. 5.1D). Therefore we surmise that it is unlikely that DOC is an important carbon source of *H. filiformis*.

Another way to follow the carbon sources utilized by organisms is to examine their fatty acid composition in which specific biomarker fatty acid of different sources such as algal, bacterial or vascular plants can be traced (e.g. Meziane et al., 1997)). Bacterial specific fatty acids have consistently been found in fatty acids of macrobenthos from mangroves (Meziane and Tsuchiya, 2000; Bachok et al., 2003) and intertidal sediments (Meziane et al., 1997), comprising roughly 5 to 15 % of the total macrobenthic fatty acids. Similarly, bacterial specific fatty acids were encountered in all foraminifera at our study site (Moodley et al. in prep). However, converting specific fatty acids to a contribution of bacterial carbon in diets is not straightforward. Conversion factors are needed to upscale specific fatty acids to total carbon contribution, assimilation efficiencies may differ among fatty acids and assimilated fatty acids can be metabolized or stored in adipose tissue by the consumer (Iverson et al., 2004). Therefore a direct comparison with our data is cumbersome, but the presence of bacterial specific fatty acids in benthic fauna evidently confirms transfer of organic compounds from bacteria to benthos.

The amount of labile carbon usually decreases with sediment depth and therefore we hypothesized that subsurface feeders would have a higher dependence on labile bacterial carbon as compared to surface feeders. Nematodes did not show important differences

with regard to living depth (0.08 $\pm$ 0.02, 0.08 $\pm$ 0.02 and 0.06 $\pm$ 0.04 for 0 - 2, 2 - 5 and 5 - 10 cm, respectively). Hard-shelled foraminifera dependence on bacteria was similar for the upper two sediment layers (0.13 $\pm$ 0.03 and 0.15 $\pm$ 0.05), but contrary to our expectations was lower for the deepest sediment layer (0.03 $\pm$ 0.02). There were also no clear differences in dependence on bacterial carbon among macrobenthic feeding modes (Table 5.1). Although the subsurface feeders *Heteromastus filiformis* (0.21) and *Arenicola marina* (0.23) seem to have a somewhat higher dependence on bacterial carbon as compared to surface feeders (0.10 to 0.15 on average), it remains to be seen whether these small differences are ecological relevant. We conclude that there are no clear differences in the dependence on bacterial carbon with respect to depth for metazoan meiobenthos nor macrobenthos.

Although our results show that the contribution of bacterial carbon to total carbon requirements of intertidal meiobenthos and macrobenthos is limited to $<$ 10 - 15 %, bacteria might be a source of essential compounds (Lopez and Levinton, 1987). For example, some foraminifera only reproduce when bacteria are present as food source (Muller and Lee, 1969). Advances in the analysis of stable isotopes in lipids (Boschker and Middelburg, 2002) and amino acids (Veuger et al., 2005) may be used to address this issue. For example, when applying an isotope tracer approach, compounds that are predominantly derived from bacteria are expected to have an elevated $\Delta\delta^{13}$C as compared to the $\Delta\delta^{13}$C of compounds that are assimilated from other resources. Furthermore, it remains to be established whether our results also apply in sediments that receive more refractory organic matter (e.g. deep-sea sediments). From the information available, we found on the one hand, no effect of living/feeding depth, which suggests that more refractory organic carbon does not increase dependence on bacterial carbon. On the other hand, many invertebrates of a small leaf-litter dominated stream food web relied for $>$ 50 % on bacterial carbon, as shown by a stream water ^{13}C-acetate enrichment (Hall and Meyer, 1998). Their estimates are consistently higher than ours, which does suggest that dependence on bacteria is more important in systems that are dominated by refractory organic matter with a high C:N ratio. This comparison is admittedly crude and more data sets are required to single out the factors that control dependence on bacterial carbon in other benthic systems.

5.4.2 Selective feeding

The marginal dependence on bacterial carbon was surprisingly general among the benthic fauna encountered in the intertidal sediment (Table 5.1). This generality hints at a mechanism that prevents a greater exploitation of bacterial carbon. One possible explanation may be the dilution of bacterial carbon due to its attachment to inedible sediment grains. As suggested by Cammen (1980), a greater bacterial carbon exploitation may be limited by the processing rate of sediment particles by the benthos. For our study site, we estimated fauna processing rates as follows: total meiobenthic and macrobenthic biomass in the upper 10 cm of the sediment was 1872 mmol C m^{-2}, which translates to a volume of $\sim$ 300 cm^3 m^{-2} (0.5 g C = 1 g DW, 0.15 g DW = 1 g WW and 1 g WW = 1 cm^3). If we assume a relative gut volume of 0.10 (Kooijman, 2000) and a gut residence time of 2 hour (Bock and Miller, 1999), 0.36 % of the sediment passes a digestive tract on a daily basis. If we further assume a homogeneous bacteria - sediment mixture, indiscriminate feeding by benthos results in a grazing rate of 0.0036 d^{-1} of the bacterial stock. With the average bacteria biomass of 781 mmol C m^{-2} (Chapter 4), the expected grazing rate from indiscriminate feeding is 2.8 mmol C m^{-2} d^{-1}. Assuming maintenance carbon

requirements of 19 mmol C m^{-2} d^{-1} (0.01 of benthic biomass d^{-1}, Nielsen et al. (e.g. 1995)), bacterial grazing of 2.8 mmol C m^{-2} d^{-1} is far below maintenance requirements alone and shows that processing rates by indiscriminate feeding by benthos may indeed limit a greater exploitation of bacterial carbon.

However, benthic fauna do not feed indiscriminately (Lopez and Levinton, 1987) and we therefore examined our data for evidence of selective feeding. From quantitative modeling the experimental data, we derived a grazing rate of 18 mmol C m^{-2} d^{-1} on bacteria by the benthic community (Chapter 4), which is about 6× the expectation of indiscriminate feeding (2.8 mmol C m^{-2} d^{-1}), clearly showing selective ingestion by benthic fauna. However, since bacteria comprise only a small part of the total carbon requirements of benthos, it is likely that selection occurs for food patches that have a higher organic matter content or quality rather than selection for bacteria as such. The contribution of bacterial carbon to organic matter increases with increasing quality (Findlay et al., 2002; Fischer et al., 2002), therefore one may use dependence on bacteria as proxy for the quality of the organic matter that is selected. As a result, feeding on high quality organic matter would result in a concomitant increase in dependence on bacterial carbon.

Foraging theory predicts that feeding niches are defined by body size: small organisms rely on small but high quality patches and larger organisms on larger but lower quality patches (Ritchie, 1998; Ritchie and Olff, 1999). From this prediction a decreasing dependence on bacterial carbon with increasing body size is expected. The expected relationship was not evident in our data (Fig. 5.3). Although this exercise is speculative, it suggests that benthic fauna collectively select for high quality resources.

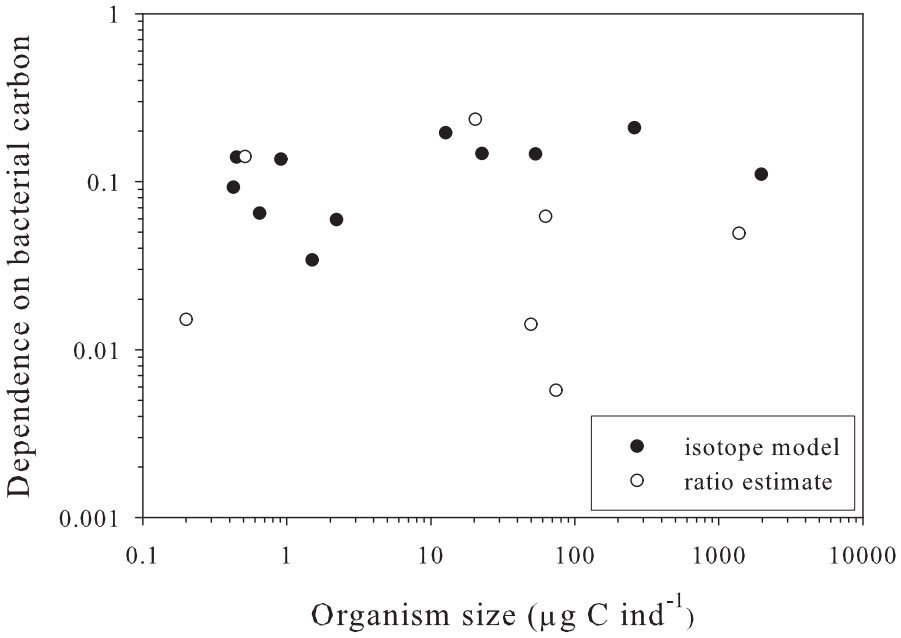

Figure 5.3: Relative dependence on bacterial carbon as a function of body size.

5.4.3 Implications of the weak bacteria - benthos interaction

In accordance with earlier observations, our results show that bacteria are a limited carbon source benthic fauna. The limited transfer of bacterial carbon to the benthos implies that there is no important direct influence of bacterial abundance or production on the dynamics of benthic fauna. Bacteria are found to be dominant processors of labile microphytobenthic carbon (Middelburg et al., 2000) and phytodetritus (Moodley et al., 2002, 2005), but also of refractory lignocellulosic detritus from vascular plants (Benner et al., 1986). On the one hand, this suggests a competitive interaction between bacteria and benthic fauna for labile carbon resources, given that also the latter rapidly assimilate labile phytodetritus (Blair et al., 1996; Herman et al., 2000; Moens et al., 2002; Witte et al., 2003). The limited transfer of bacterial carbon implies that labile carbon assimilated by bacteria, is effectively lost for the metazoan food web. On the other hand, transfer of bacterial carbon might provide a means through which refractory and otherwise indigestible detritus may enter the traditional metazoan food web (Hall and Meyer, 1998). The characteristics of the benthic system, in particular the lability of the detritus pool, will eventually determine whether the bacteria - benthos interaction will be a net gain or loss for the benthic fauna.

In terms of food web relations in intertidal sediments, the observed weak bacteria - benthos interaction should not be viewed as a predatory relation, but is more likely to be of competitive nature with respect to labile carbon sources. As described earlier, benthic fauna fed selectively, but the lack of a relationship between body size and dependence on bacterial carbon suggests that benthic organisms compete for organic matter of similar quality. Therefore, intertidal benthic food webs seem to be regulated predominantly by competitive interactions for labile resources among bacteria, meiobenthos and macrobenthos.