

## Chapter 4

# Substrate-dependent bacterivory by intertidal benthic copepods

*Published as:*

*Clio Cnudde, Tom Moens, Anne Willems and Marleen De Troch (2013) Substrate-dependent bacterivory by intertidal benthic copepods. Marine Biology 160(2): 327-341*

### ABSTRACT

The trophic importance of bacteria to harpacticoid copepods in intertidal areas remains poorly understood, and so do the mechanisms of bacterial feeding. It is, for instance, unclear whether harpacticoids directly target bacterial cells, or merely co-ingest them with substrates to which bacterial cells may be attached. Here, we investigate bacterial uptake and substrate requirement for four mud intertidal species (*Microarthridion littorale*, *Platychelipus littoralis*, *Delavalia palustris*, *Nannopus palustris*) by means of <sup>13</sup>C-labeled bacteria and biomarker fatty acids (FA). Bacterial uptake strongly depended on grazing on a primary food source but bacterial ingestion rates were low and no clear indication of copepods directly targeting bacteria was found. *Delavalia* was the only species that accumulated bacteria-derived FA and gained in polyunsaturated FA (PUFA) probably through bioconversion of bacteria-derived FA. In general, however, our results suggest that bacteria represent a relatively minor and low-quality food for intertidal harpacticoid copepods.

### INTRODUCTION

Interest in bacterivory in benthic food webs is closely linked to the need to understand the fate of benthic bacterial biomass. Benthic bacterial densities by far outnumber pelagic densities (Schmidt et al. 1998). The bacterial component of aquatic ecosystems is classically viewed as the main driver of organic matter degradation and nutrient remineralisation processes (Azam et al. 1983). In addition, in pelagic systems a 'feedback loop' from heterotrophic bacteria to the grazer food web, with flagellates as the principal bacterivores, has been demonstrated (microbial loop concept, Azam et al. 1983). In marine sediments, bacterivorous protists can potentially structure the bacterial community (Kemp 1988, Epstein & Shiaris 1992, Hondeveld et al. 1992, Epstein 1997). In addition to microbenthos, organisms in other size classes, i.e. the meio- and macrobenthos, may graze on bacterial biomass. Quantitative information on meiobenthic bacterivory is scant and has been estimated to remove 0.03-6.5% of bacterial standing stock per day (Epstein & Shiaris 1992, Epstein 1997). Furthermore, these 'bulk' rates of bacterivory may conceal more specific meiobenthos-bacteria interactions. Species-specific responses towards, and feeding selectivity among, different bacterial strains refute indiscriminate feeding behavior of nematodes (Montagna et al. 1995, Moens et al. 1999b) and harpacticoid copepods (Rieper 1978, Vandenberghe & Bergmans 1981, Rieper 1982, Carman & Thistle 1985, Montagna et al. 1995, Moens 1999, Moens et al. 1999c, Dahms et al. 2007) on bacteria. Moreover, De Troch et al. (2009) illustrated the close relationship between harpacticoid copepods and bacteria on their fecal pellets. Mechanisms as bacterial gardening and trophic upgrading underline the potential role of bacteria at the basis of marine food web.

Nevertheless, several basic questions on harpacticoid bacterivory remain poorly studied: (1) are bacteria an essential component of the harpacticoid diet, and (2) are bacteria ingested directly or merely co-ingested during the uptake of substrates with attached bacteria, such as sediment grains or diatoms? Harpacticoid copepods are an important link to higher trophic levels (Fujiwara & Highsmith 1997), but the contribution of bacteria to the nutritional status of copepods remains largely unknown. The work of Rieper (1978) formed a baseline study for the role of bacterivory by copepods. Later on, Souza-Santos et al. (1996, 1999) underlined the role of bacteria associated to diatoms to rear copepods and the use of bacteria as a potential food source. Harpacticoid copepods are rich in polyunsaturated fatty acids such as EPA (eicopentosaenoic acid) and DHA (docosahexaenoic acid) which are essential to their consumers (e.g. juvenile fish) and which they probably largely obtain from consumption of diatoms or other 'high-quality' food sources such as PUFA-rich dinoflagellates (Veloza et al. 2006, Chen et al. 2012). Bacteria generally lack these fatty acids and are therefore considered a low-quality food source (Chen et al. 2012), even though Rieper (1978) observed 'normal' copepod growth and reproduction on an exclusive diet of bacteria. On the other hand, planktonic and benthic copepods are known to produce these vital fatty acids through bioconversion of short-chain FA to long-chain FA (Desvillettes et al. 1997, De Troch et al. 2012a).

In addition, it remains unclear whether copepods directly target bacterial cells or merely co-ingest them while feeding on other sources. Marine sediments contain various types of bacteria-rich sources, from macroalgae (Hicks 1977) and microalgae (Sapp et al. 2008) to phytodetritus (Perlmutter & Meyer 1991), animal carcasses (Tang et al. 2006), fecal pellets (De Troch et al. 2010) and sediment grains (Griebler et al. 2001). Hicks (1977) and Perlmutter and Meyer (1991) indicated that not the substrate but the substrate-bound bacteria attract copepods. Therefore, the present study aims to unravel the assimilation of bacterial carbon in relation to the presence of a substrate. Assimilation of bacterial carbon will be quantified by means of trophic markers.

Direct measurement of grazing through the use of biomarkers has become widely applied in feeding ecology studies (Boecklen et al. 2011, Kelly & Scheibling 2012). Combining trophic markers like stable isotopes and fatty acid profiles offers new opportunities to unravel trophic interactions at the basal level of marine food webs such as the bacteria-meiofauna link. Carbon isotopes may offer tools for a direct measurement of the assimilation of bacterial carbon, while fatty acid contents of grazers are indicative of the grazer's nutritional condition after feeding on a bacterial diet.

The specific objectives of the present study were (1) to evaluate the nutritional value of bacteria for harpacticoid copepods; (2) to assess whether bacterivory is copepod species-specific; (3) to assess whether bacterivory by harpacticoid copepods is an independent feeding strategy or merely a passive consequence of the ingestion of substrata; and (4) to test if the requirement for any substrate is purely physical (only bacteria attached to larger particles can be ingested) or food-quality dependent (only bacteria attached to high-quality food particles can be ingested). Bacterivory was estimated by means of  $^{13}\text{C}$  labeled bacteria and by fatty acid analysis. The third and fourth objective were achieved by conducting a laboratory experiment in which copepods were incubated with bacteria in the absence and presence of sediment or diatoms as colonisable substrate. Species-specific differences in bacterivory were assessed by comparing the responses of four naturally co-occurring copepod species from a temperate salt marsh intertidal community.

## **MATERIAL AND METHODS**

### ***Harpacticoid copepod species***

Copepods were collected from silty sediments of a small intertidal creek in the Paulina salt marsh in the Westerschelde estuary (SW Netherlands, 51°20'55.4"N, 3°43'20.4"E). In total, four harpacticoid copepod species with distinct differences in body morphology (body shape and size) and motility/emergence behavior, and belonging to different families, were selected. Two epibenthic harpacticoid species were

included: *Microarthridion littorale* (family Tachidiidae) and *Platychelipus littoralis* (family Laophontidae). The former is small (~0.5 mm length, Lang 1948) and an active swimmer in near-bottom water (McCall 1992). The latter is a larger (~0.9 mm length, Lang 1948), sluggish, non-swimming copepod (C. Cnudde pers observ). The two other species, *Nannopus palustris* (family Huntemaniidae) and *Delavalia palustris* (formerly known as *Stenhelia palustris*, family Miraciidae), are large epi-endobenthic copepods (~0.6 to 0.8 mm length, Lang 1948). *Nannopus* is a burrower (Santos et al. 2003), while *Delavalia* is a tube-dweller and tube-builder (Nehring 1993); both species have good swimming abilities and are quite mobile (C. Cnudde pers observ). Copepod species will further be referred to by their genus names. All copepod species were field-caught two days before the start of the experiment and extracted alive from the sediment using a mixed technique of sediment decantation and extraction based on their movement towards white light. Copepods were washed multiple times in sterile artificial seawater (Instant Ocean synthetic salt, salinity: 28, filtered over 0.2  $\mu\text{m}$  Millipore filters and autoclaved) (henceforth referred to as 'sterile ASW') and starved for 24 h. Finally, copepods were washed once more in sterile ASW before introducing them into the experimental microcosms. Only adult, non-gravid specimens were selected and both sexes were represented in the same proportions as in the field samples.

### **Bacterial cultures**

Within 3 h after sediment collection, a bacterial suspension was prepared by adding 10 mL sterile artificial seawater (ASW, Instant Ocean synthetic salt, salinity: 28) to a small sediment aliquot (5 mL wet sediment) and thoroughly vortexing and shaking by hand; this mixture was finally filtered over a 3  $\mu\text{m}$  Millipore filter to remove flagellates and eukaryotes. From 100  $\mu\text{l}$  of the obtained bacterial suspension a  $10^{-1}$  to  $10^{-5}$  dilution series in ASW was prepared and inoculated on marine agar (Marine Broth 2216, Difco™) and incubated for 7 days at 20°C in the dark. Dilutions  $10^{-2}$  and  $10^{-4}$  showed well isolated colonies, different arrays of colony morphologies and substantially higher colony diversities than other dilutions. These dilutions resulted in colonies of different color (e.g. white, pink, yellow), shape (circular, irregular), elevation (raised, convex, flat) and colony margin (entire, undulated, curled) as inspected under binocular. The  $10^{-2}$  and  $10^{-4}$  mixed cultures were harvested from the agar plates by means of a cell scraper and both cultures were separately inoculated in a liquid growth medium consisting of autoclaved ASW (salinity: 28), beef extract (DIFCO, 3 g L<sup>-1</sup>) and bacto peptone (DIFCO, 5 g L<sup>-1</sup>). They were incubated for 3 days at 20°C on a mechanical shaker. The cultures resulting from the  $10^{-2}$  and  $10^{-4}$  dilutions were harvested through centrifugation at 2500 rpm for 10 min and resuspended in new growth medium, which was 20-fold diluted compared to the above-described medium, and to which 0.5 g L<sup>-1</sup> <sup>13</sup>C glucose (D-glucose, U-<sup>13</sup>C6, 99%, Cambridge Isotope Laboratories, Inc.) was added. As a control for the effectiveness of <sup>13</sup>C-labeling, parallel bacterial cultures were incubated in an identical liquid medium with <sup>12</sup>C glucose. After 24 h of growth, the growth medium was replaced by sterile seawater to remove any remaining <sup>13</sup>C. This labeling technique yielded a specific uptake (see further under 'Stable isotopes analysis') in the bacteria between 8530 ‰ and 8700 ‰ corresponding to ca 9.7 atomic %. Prior to the experiment, bacteria were repeatedly rinsed with sterile ASW to remove the bacterial growth medium, and absence of flagellate and ciliate was controlled using a phase-contrast microscope. Bacterial densities were estimated by epifluorescence microscopy after staining with 10  $\mu\text{g mL}^{-1}$  DAPI (final concentration) for 10 min, modified protocol after Porter and Feig (1980). It is clear that our bacterial inoculum differed from the natural bacterial community since the culturing and labeling steps on artificial media inevitably reduced bacterial diversity and also cell size of cultured bacteria could differ from the natural bacterial size range. We nevertheless advocate the use of cultured and prelabeled bacteria in laboratory experiments since this procedure yields much higher specific uptake of bacteria ( $\delta^{13}\text{C} > 8000\text{‰}$ ) due to high activity of bacterial cells in culture compared to direct labeling of uncultured sediment bacteria where only a minor proportion of bacteria are able to incorporate the label, allowing a more sensitive detection of low rates of bacterivory. Secondly, prelabeling the bacteria avoided potential biases such as unspecific labeling of copepods (due to direct ingestion of the suspended label or protist grazing) or ectosymbiotic bacteria of copepods (Carman 1990), making interpretation of grazer enrichment easier.

### ***Diatom culture***

The benthic diatom *Seminavis robusta* has repeatedly been shown to be a suitable food source for a variety of harpacticoid copepods (De Troch et al. 2008, Cnudde et al. 2011). *Seminavis robusta* strain 84A was obtained from the diatom culture collection of the Laboratory for Protistology and Aquatic Ecology (Ghent University). The original strain was isolated from a sample collected in November 2000 from the 'Veerse Meer', a brackish water lake in Zeeland, The Netherlands (Chepurnov et al. 2002). At the time of our experiment, the cells measured  $35.7 \pm 3.1$   $\mu\text{m}$  in length. The diatom cultures were grown non-axenically in tissue culture flasks (175  $\text{cm}^2$  surface) with  $20\text{mL L}^{-1}$  f/2 medium (Guillard 1975) based on sterile ASW (salinity: 28) during 10 days at 16-18 °C with a 12:12-h light:dark period and 25-50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

At the start of the experiment, the diatom growth medium was replaced by sterile ASW to prevent any additional growth. Two additional washes were performed to remove loosely attached bacteria by centrifugation at 2500 rpm for 5 min. Diatom cell densities were estimated under an inverted light microscope (Zeiss Axiovert 40C).

### ***Substrate experiment***

The aim of this experiment was to assess the dependence of direct bacterial feeding by harpacticoid copepods on the presence of a physical substrate, either another food source (diatoms) or a sediment matrix. Experimental microcosms contained only copepods and living bacteria and, depending on the treatment, sediment grains and/or diatoms. Indirect uptake of bacterial carbon, for instance through predation on bacterivorous ciliates and flagellates, was excluded. The two harpacticoid copepod species with the highest abundances in the field samples were used here, i.e. *Microarthridion* and *Platychelipus*. In view of their different mobility and behaviour (see above), we expected differences in their dependence on substrate presence. *Microarthridion* may feed more on suspended particles (Decho 1986), whereas *Platychelipus* is more constrained to feeding in the sediment matrix. As the latter only moves its appendages to feed and hardly changes its endobenthic position (C. Cnudde, pers observ), we expect it to be mainly dependent on food sources in the sediment. Bacterial uptake by the copepods was analysed by providing a  $^{13}\text{C}$ -labeled bacterial mix into 4 treatments, corresponding to each of four different substrate conditions:

- (1) without any substrate, so with the bacterial suspension and copepods directly added to Petri dishes (treatment B);
- (2) in the presence of muffled sediment, a substrate without any nutritional value (treatment BS);
- (3) in the presence of diatoms, a substrate with nutritional value to copepods (treatment BD);
- (4) in the presence of both muffled sediment and diatoms (treatment BDS).

Each treatment was replicated four times. Petri dishes (diameter: 5.2 cm) were filled with 15 mL of sterile ASW (salinity: 28). In the sediment treatments BS and BDS, a sediment layer of 2 mm thick (ca 3 g dry weight) was added to the Petri dishes. This sediment was pretreated at 550°C for 4h to remove all organic carbon, and homogenized. Treatments BD and BDS were supplied with diatoms at a density of  $2.5 \times 10^6$  diatom cells/Petri dish. All experimental units received  $1 \times 10^{11}$   $^{13}\text{C}$ -labeled bacterial cells. After allowing diatoms and bacteria to settle on the Petri dish or sediment surface, copepods were added. We used 45 and 60 specimens per Petri dish for *Platychelipus* and *Microarthridion*, respectively. 20 specimens from each dish were used for stable isotope analysis and the remaining copepods of the 4 replicate dishes were pooled into one sample for fatty acid analysis. The experimental units were incubated for 4 days in a climate room at 16-18°C with a 12:12-h light:dark regime.

### ***Time-series experiment***

The aim of this experiment was to assess the nutritional value of bacteria for three harpacticoid copepod species by evaluating a change in total fatty acid content as an index of copepod nutritional status, and by investigating the incorporation of bacteria-specific FA biomarkers in addition to measuring uptake of  $^{13}\text{C}$ -enriched bacterial biomass. Based on the outcome of the first experiment (see results section), we chose two incubation times of 4 ( $T_4$ ) and 9 days ( $T_9$ ), respectively, the latter mainly because shifts in copepod FA patterns tended to become pronounced only after several days (De Troch pers. obs.).

Based on the low assimilation rates obtained in sediment treatments in the substrate experiment, we restricted the time-series experiment to the B and BD treatments. There were four replicates per treatment and time. All copepods of the fourth replicate were used for fatty acid analysis. The treatments were set up with three copepod species: *Nannopus*, *Delavalia* and *Platychelipus*, with 60 specimens per microcosm. *Microarthridion* was omitted from this experiment in view of its poor performance in the substrate experiment (see results section). Diatom and bacterial cultures were obtained as described before, starting from a new diatom stock culture and a freshly collected sediment sample for preparation of a bacterial suspension, respectively. The same bacterial and diatom cell densities and incubation conditions were applied as described for the substrate experiment.

### ***Sample processing***

After assessing the mortality of copepods in each microcosm, samples of 15 (*Platychelipus*) or 20 (*Microarthridion*, *Nannopus*, *Delavalia*) copepod specimens from each replicate microcosm were prepared for stable isotope analysis. Copepods were washed in sterile ASW, starved overnight to empty their guts and temporarily stored at  $-20^\circ\text{C}$  till further processing. Remaining copepods of these three replicates, together with all copepods of the fourth replicate, were pooled, cleaned while alive, and stored on a Whatman filter at  $-80^\circ\text{C}$  for fatty acid extraction. This yielded 60 to 120 specimens per sample for fatty acid analysis. For each treatment, we thus had three independent samples for stable carbon isotope analysis and one for fatty acid analysis. Control fatty acid samples of diatoms, bacteria and copepods were prepared in triplicate at the beginning of the experiment ( $T_0$ ).

After thawing, samples for isotope analysis were processed by rinsing copepods several times in MilliQ water to remove adhering particles. The copepods were transferred to aluminum capsules (6 x 2.5 mm). The overall procedure was executed within 2 hours after thawing to minimize leakage of  $^{13}\text{C}$  from the copepod body (Moens et al. 1999d). Subsequently, the aluminum capsules were oven-dried overnight at  $60^\circ\text{C}$ , pinched-closed and stored under dry atmospheric conditions until analysis. Additionally, 3 capsules for stable isotope analysis were prepared with subsamples of the bacterial cultures.

### ***Stable isotopes analysis***

$\delta^{13}\text{C}$  values and carbon content of samples were measured using an isotope ratio mass spectrometer (type Europa Integra) at the Davis Stable Isotope Facility (University of California, USA). Uptake of  $^{13}\text{C}$  label is expressed as specific uptake ( $\Delta\delta^{13}\text{C} = \delta^{13}\text{C}_{\text{sample}} - \delta^{13}\text{C}_{\text{control}}$ ) and as  $^{13}\text{C}$  uptake per unit copepod biomass. The control signal refers to the  $\delta^{13}\text{C}$  value of the copepods or bacteria at time  $T_0$ . These data were converted to carbon uptake according to Middelburg et al. (2000), expressed as total uptake of  $^{13}\text{C}$  (I, in  $\mu\text{g }^{13}\text{C}$ ), calculated as the product of excess  $^{13}\text{C}$  (above background, E) and mean individual copepod biomass (organic carbon) per sample. Excess  $^{13}\text{C}$  is the difference between the fraction  $^{13}\text{C}$  of the control ( $F_{\text{control}}$ ) and the sample ( $F_{\text{sample}}$ ), where  $F = \frac{^{13}\text{C}}{^{13}\text{C} + ^{12}\text{C}} = \frac{R}{R+1}$ . The carbon isotope ratio (R) was derived from the measured  $\delta^{13}\text{C}$  values as  $R = (\delta^{13}\text{C}/1000 + 1) \times R_{\text{VPDB}}$ , with  $R_{\text{VPDB}} = 0.0112372$ . Subsequently, total  $^{13}\text{C}$

uptake was converted to total bacterial carbon assimilation per unit copepod carbon (in  $\mu\text{g C}$ ), calculated by dividing uptake I by sample biomass (organic carbon content) and taking into account the atomic  $\% \text{ }^{13}\text{C}$  in bacterial biomass (9.7 %). Individual carbon contents of the copepod species were, in decreasing order,  $1.51 \pm 0.10 \mu\text{g C}$ ,  $1.05 \pm 0.06 \mu\text{g C}$ ,  $0.85 \pm 0.04 \mu\text{g C}$  and  $0.55 \pm 0.02 \mu\text{g C}$  (mean  $\pm$  1 SD, N=4) for *Platychelipus*, *Nannopus*, *Delavalia* and *Microarthridion*, respectively.

### **FA analysis**

Hydrolysis of total lipids of bacteria, diatoms and copepods and methylation to fatty acid methyl esters (FAME) was achieved by a modified one-step derivatisation method after Abdulkadir and Tsuchiya (2008) (De Troch et al, 2012). The boron trifluoride-methanol reagent was replaced by a 2.5 %  $\text{H}_2\text{SO}_4$ -methanol solution since  $\text{BF}_3$ -methanol can cause artefacts or loss of PUFA (Eder 1995). The obtained FAME were analysed using a gas chromatograph (HP 6890N) with a mass spectrometer (HP 5973). The samples were run in splitless mode (for copepods, 5  $\mu\text{L}$  injected per run) and split mode (for bacteria, 0.1  $\mu\text{L}$  injected per run), at an injector temperature of 250  $^\circ\text{C}$  using a HP88 column (Agilent J&W, Agilent Co., USA). The oven temperature was programmed at 50  $^\circ\text{C}$  for 2 min, followed by a ramp at 25  $^\circ\text{C min}^{-1}$  to 175 $^\circ\text{C}$  and then a final ramp at 2  $^\circ\text{C min}^{-1}$  to 230 $^\circ\text{C}$  with a 4-min hold. The FAME were identified by comparison with the retention times and mass spectra of authentic standards and mass spectral libraries (WILEY, NITS05), and analysed with the software MSD ChemStation (Agilent Technologies). Quantification of individual FAME was accomplished by linear regression of the chromatographic peak areas and corresponding known concentrations (ranging from 5 to 150  $\mu\text{g mL}^{-1}$ ) of external standards (Supelco # 47885, Sigma-Aldrich Inc., USA).

Shorthand FA notations of the form A:B $\omega$ X were used, where A represents the number of carbon atoms, B gives the number of double bonds and X gives the position of the double bond closest to the terminal methyl group (Guckert et al. 1985).

### **Statistical data analysis**

Differences in bacterial carbon uptake by copepods and in copepod mortality among treatments were analysed by means of two-way analysis of variance (ANOVA) for the substrate experiment with fixed factors copepod species and substrate and three-way ANOVA for the time-series experiment with fixed factors copepod species, substrate and time. The Tukey's HSD *post-hoc* test was applied to detect pairwise differences, using 95% confidence limits. Prior to ANOVA, Levene's test was used to check the assumption of homoscedasticity; if the data did not fulfill this requirement, data of carbon uptake and mortality were  $\log_{(x+1)}$  transformed and arcsine square root transformed, respectively. All data analyses were performed using the software package R, version 2.14.1 (R Development Core Team 2009).

Differences in FA composition between food sources (bacteria and diatoms) and between natural copepods (copepods at  $T_0$ ), were analysed using a PERMANOVA with, respectively, factor food and factor copepod species, based on a Bray-Curtis resemblance matrix constructed from untransformed relative FA values. Depending on the number of unique permutations of each dataset, P-values (P) or Monte Carlo P-values ( $P_{MC}$ ) were interpreted. The assumption of homogeneity of the multivariate dispersions was checked using PERMDISP. FA responsible for group differentiation were identified using Similarity Percentage analysis (SIMPER). Principal Coordinates Analysis (PCO) using Bray-Curtis similarity ( $n = 21$ ) was performed to visualize FA composition of copepods at  $T_0$ ,  $T_4$  and  $T_9$ . Individual FA contributing highly to the variation explained by PCO were represented as vectors.

## RESULTS

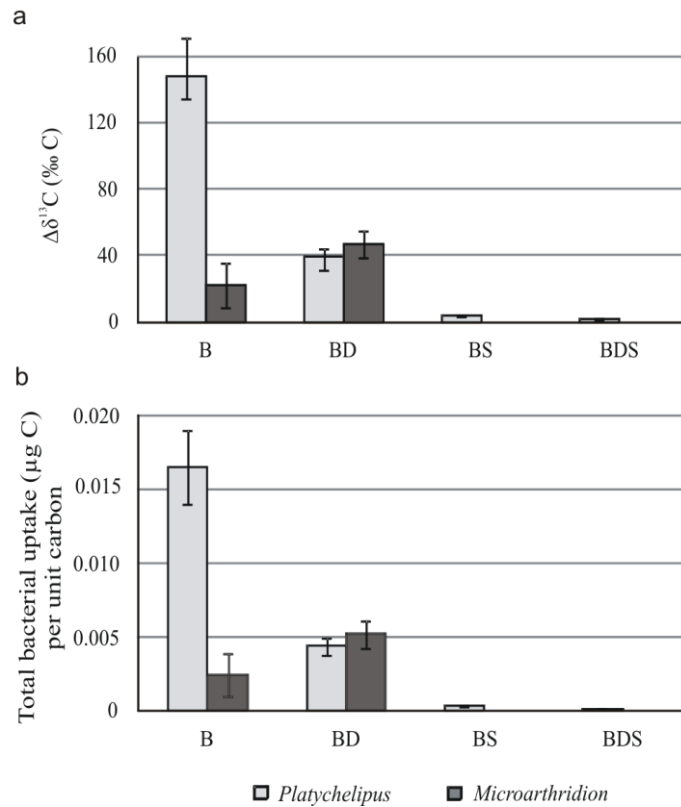
### *Substrate experiment*

Mortality after 4 days incubation differed between harpacticoid species and treatments ( $p < 0.001$  for substrate, copepod species and copepod x substrate). The mortality of *Platychelipus* was relatively limited (Table 1) and independent of the substrate ( $p > 0.05$ ). *Microarthridion* had a similar mortality as *Platychelipus* in the absence of sediment, but a very high mortality (i.e. 96-100 %) in presence of sediment. Mortality in both species was not influenced by the presence of diatoms ( $p > 0.05$ ).

**Table 1.** Copepod mortality percentage ( $\pm 1$  SD,  $n = 4$ ) in the substrate experiment and in the time-series experiment

Mortality (%)		B	BD	BS	BDS
<b>Substrate exp.</b>					
<i>Platychelipus</i>	T <sub>4</sub>	8 ( $\pm 7$ )	25 ( $\pm 16$ )	19 ( $\pm 6$ )	24 ( $\pm 3$ )
<i>Microarthridion</i>	T <sub>4</sub>	13 ( $\pm 9$ )	12 ( $\pm 3$ )	100 ( $\pm 0$ )	96 ( $\pm 3$ )
<b>Time-series exp.</b>					
<i>Platychelipus</i>	T <sub>4</sub>	3 ( $\pm 3$ )	3 ( $\pm 2$ )		
	T <sub>9</sub>	18 ( $\pm 7$ )	2 ( $\pm 2$ )		
<i>Nannopus</i>	T <sub>4</sub>	1 ( $\pm 1$ )	3 ( $\pm 2$ )		
	T <sub>9</sub>	6 ( $\pm 3$ )	2 ( $\pm 2$ )		
<i>Delavalia</i>	T <sub>4</sub>	8 ( $\pm 9$ )	5 ( $\pm 5$ )		
	T <sub>9</sub>	34 ( $\pm 12$ )	9 ( $\pm 5$ )		

The specific  $^{13}\text{C}$  uptake levels ( $\Delta\delta^{13}\text{C}$ ) of *Platychelipus* and *Microarthridion* (Fig. 1a) proved species-specific bacterial carbon uptake and its substrate-dependence ( $p < 0.005$  for factors copepod, substrate and copepod x substrate). Both copepod species were able to assimilate bacteria in absence of a substrate (Fig. 1a, treatment B), though *Platychelipus* reached a significantly higher specific uptake than *Microarthridion* ( $p < 0.001$ ). The sediment substrate (Fig. 1a, treatments BS and BDS) almost completely inhibited bacterial feeding: specific  $^{13}\text{C}$  uptake was low for *Platychelipus* whilst most *Microarthridion* died in treatments with sediment (up to 100 % mortality; Table 1). In the presence of diatoms equal bacterial assimilation was observed for both species ( $p > 0.05$ ). However, presence of diatoms enhanced bacterial assimilation by *Microarthridion* (*post hoc* test,  $p < 0.01$ ) while the opposite was observed for *Platychelipus* (*post hoc* test,  $p < 0.001$ ). The same trends were observed for the total bacterial carbon uptake per unit copepod carbon (Fig. 1b). Overall, highest bacterial uptake per unit carbon was found for *Platychelipus* in absence of a substrate, while *Microarthridion* had a much lower assimilation than *Platychelipus* in the B treatment, but an equal uptake in the BD treatment (Fig. 1b).



**Fig. 1.** Substrate-dependent assimilation of bacterial carbon (mean  $\pm$  1 SD,  $n = 4$ ) by *Platychelipus* and *Microarthridion* after 4 days of grazing on a bacterial mixture without a primary substrate (treatment B) and in the presence of diatoms (treatment BD), sediment (treatment BS) and the combination diatoms+sediment (treatment BDS). Assimilation of bacterial carbon is expressed as (a) specific uptake  $\Delta\delta^{13}\text{C}$  and (b) total uptake of bacterial carbon per unit copepod carbon

Total FA content (fatty acid content) of both copepods decreased in both treatments with bacteria as the only food source (Table 2; B and BS). *Platychelipus* lost 7 and 11 %, respectively, of its initial fatty acids in the B and BS treatment, and *Microarthridion* lost 25 % in the B treatment. In bacterial-diatom treatments (BD and BDS), total FA content of *Platychelipus* doubled (+122 %) and quadruplicated (+287 %), respectively, without and with sediment, whilst *Microarthridion* gained 79 % in the BD treatment compared to the control.

### Time-series experiment

After 4 days of feeding, mortality did not differ between copepod species ( $p > 0.05$ ), or food treatments ( $p > 0.05$ ) (Table 1). Mean mortality was  $4 \pm 4$  % (mean  $\pm$  1 SD,  $n = 21$ ). Mortality remained unaltered over time in the bacteria-diatom treatment (*post hoc* test,  $p > 0.05$ ). In treatments with only bacteria, mortality of the copepod species *Platychelipus* and *Delavalia* had increased after 9 days, with mean mortalities of  $18 \pm 7$  % and  $34 \pm 12$  % ( $n = 4$ ), respectively (*post hoc* test,  $0.05 < p < 0.1$  and  $p < 0.01$ , respectively). Mortality of *Nannopus* remained low ( $6 \pm 3$  %,  $p > 0.05$ ).



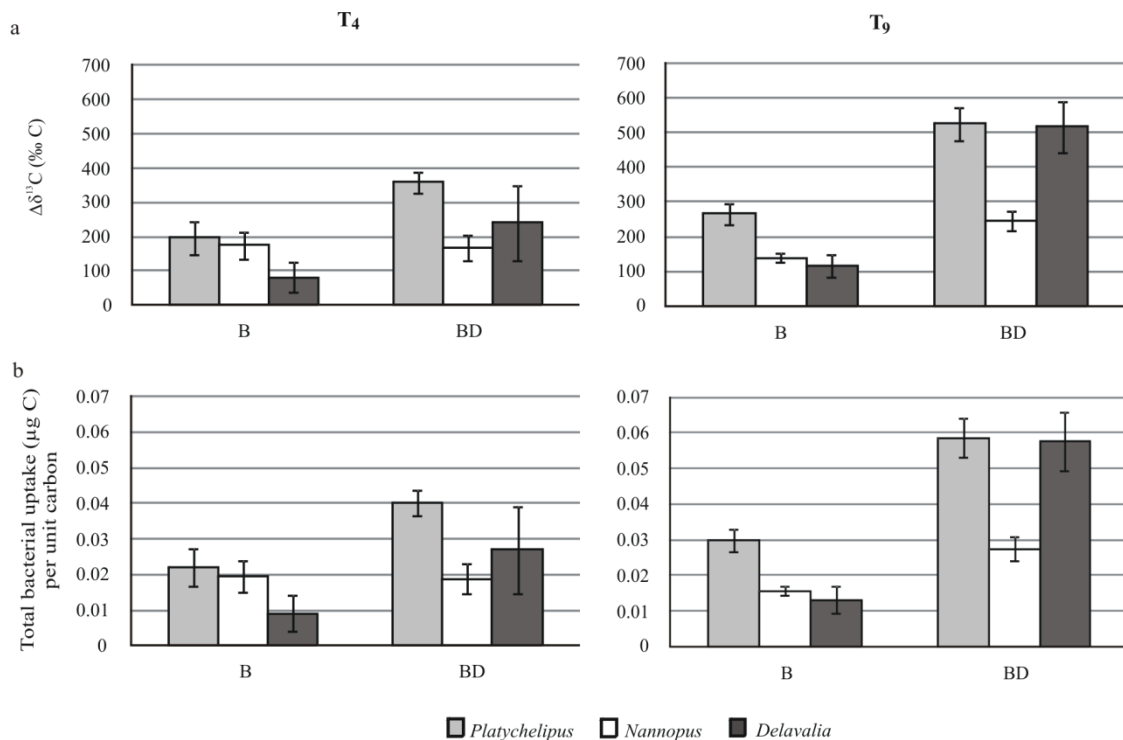
**Table 2.** Total fatty acid content (FA) of copepods from the substrate experiment and time-series experiment, before (control) and after grazing (treatments B, BD, BS, BDS) in comparison to their initial fatty acid content (control) before grazing (%)

		Total FA (%)
Substrate exp.	<b><i>Platychelipus</i></b>	
	control	0
	B	-7
	BD	+122
	BS	-11
	BDS	+257
	<b><i>Microarthridion</i></b>	
control	0	
B	-25	
BD	+79	
Time series exp.	<b><i>Platychelipus</i></b>	
	control	0
	B-T4	-41
	B-T9	-56
	BD-T4	+71
	BD-T9	+117
	<b><i>Nannopus</i></b>	
	control	0
	B-T4	-59
	B-T9	-61
	BD-T4	-44
	BD-T9	-31
	<b><i>Delavalia</i></b>	
	control	0
	B-T4	-27
B-T9	+21	
BD-T4	+67	
BD-T9	+240	

### *<sup>13</sup>C uptake and total fatty acid content as tracers of bacterivory*

At the start of the experiment, copepods exhibited species-specific differences in their natural  $\delta^{13}\text{C}$  ( $p < 0.001$ , *post hoc* test  $p < 0.05$ ):  $-14.9 \pm 0.2\text{‰}$ ,  $-16.1 \pm 0.4\text{‰}$  and  $-17.1 \pm 0.3\text{‰}$  ( $n = 3$ ) for *Platychelipus*, *Nannopus* and *Delavalia*, respectively. At the end of the experiment (at T<sub>9</sub>), all three copepod species showed  $^{13}\text{C}$ -enrichment resulting from uptake of labeled bacteria (Fig. 2). Overall, a three-way ANOVA showed a copepod species-specific uptake of bacteria, significant differences depending on substrate type and over time and significant pair-wise interactions between the factors (Table 3; factors copepod species, substrate and time,  $p < 0.001$ ). More specifically, copepod specific uptake is found in the B treatment (*post hoc* test copepod x substrate,  $p < 0.01$ ). After 4 days feeding (Fig. 2a, T<sub>4</sub>), *Delavalia* showed the lowest specific uptake ( $81 \pm 44 \text{‰}$ ), which was nevertheless still higher than the specific uptake by *Microarthridion* ( $22 \pm 13 \text{‰}$ ) in the corresponding treatment of the substrate experiment. The specific uptake by *Platychelipus* was the highest ( $196 \pm 48 \text{‰}$ ) among the three species, and closely resembled that in the substrate experiment ( $148 \pm 22 \text{‰}$ ) (Fig. 1a). This copepod specific pattern is also present after 9 days feeding. In the presence of diatoms, bacterial assimilation by *Platychelipus* and *Delavalia* was significantly higher than by *Nannopus* (*post hoc* test, copepod x substrate,  $p < 0.001$ ). Prolongation of the feeding period to 9 days (Fig. 2a) did not result in any significant increase or decrease in  $^{13}\text{C}$  uptake in the B treatment for any of the three species (*post hoc* test substrate x time,  $p > 0.05$ ). In the BD treatment  $^{13}\text{C}$ -enrichment of *Platychelipus* and especially *Delavalia* strongly increased with time (*post hoc* test copepod x

time, resp.  $p < 0.01$  and  $p < 0.001$ ). Due to diatom presence, total bacterial uptake per unit copepod carbon (Fig. 2b) doubled for *Platychelipus* (from 0.03 to almost 0.06  $\mu\text{g C}_{\text{bact}}$  per  $\mu\text{g C}_{\text{cop}}$ ) and quintupled for *Delavalia* (from 0.01 to 0.06  $\mu\text{g C}_{\text{bact}}$  per  $\mu\text{g C}_{\text{cop}}$ ). For *Nannopus* the uptake of bacteria was twice as high (from 0.015 to almost 0.03  $\mu\text{g C}_{\text{bact}}$  per  $\mu\text{g C}_{\text{cop}}$ ) in the presence of diatoms but of no significance, not time-related or substrate-dependent.



**Fig. 2.** Assimilation of bacterial carbon (mean  $\pm$  1 SD, N = 3) by *Platychelipus*, *Nannopus* and *Delavalia* after 4 days (T<sub>4</sub>) and 9 days feeding (T<sub>9</sub>), in the absence of a substrate (treatment B) and in the presence of a diatom substrate (treatment BD). Assimilation is expressed as (a) specific uptake  $\Delta\delta^{13}\text{C}$  and (b) total uptake of bacterial carbon per unit copepod carbon

Copepods showed an important change in total FA content compared to their initial FA content ('control') (Table 2). *Platychelipus* and *Nannopus* in the B treatment showed a reduction in FA content during the first 4 days (up to -59 %) and an additional but minor loss during the following days (up to day 9) (Table 2). *Delavalia* exhibited a comparatively small decrease in FA content after 4 days (-27 %), but after 9 days it had a 21 % higher FA content than the T<sub>0</sub> specimens. Species-specific differences also occurred in the BD treatment: *Delavalia* and *Platychelipus* showed a considerable increase in FA content by 67-71 % and by up to 240 %, respectively, after 4 and 9 days, while *Nannopus* showed loss of FA in both treatments (B, BD), independent of the duration of the incubation.

### Individual fatty acid biomarkers

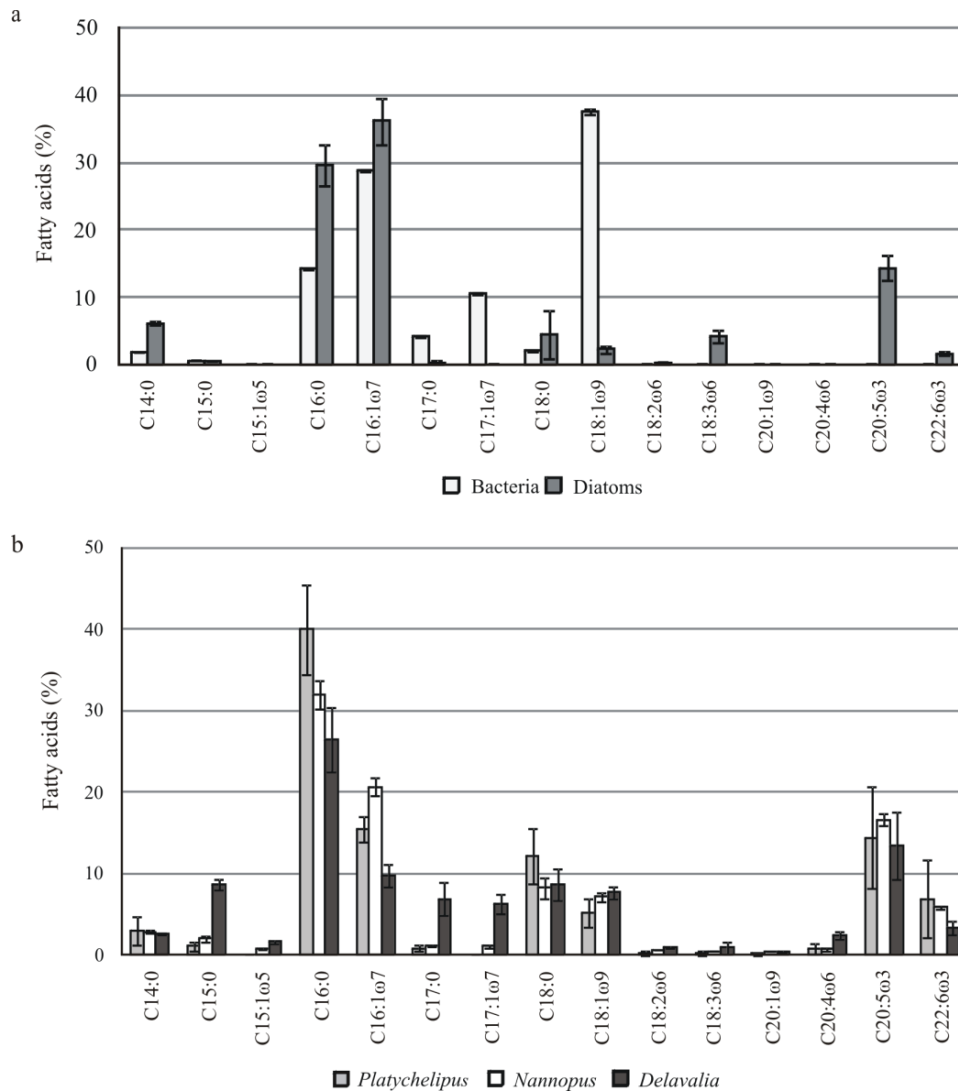
FA composition clearly differed between bacteria and diatoms (Fig. 3, main test, pseudo-F 185.54,  $P_{\text{MC}} = 0.001$ ), with a dissimilarity of 50.5 % (SIMPER, data not shown). C16:1 $\omega$ 7 and C16:0 (Fig. 3; ESM Table 1) were the main FA (with relative abundance >10 %) shared by both food sources. Major bacteria-specific FA, henceforth referred to as bacterial biomarkers, were C18:1 $\omega$ 9 (relative abundance 37.6%, also found in diatoms but in very low amounts) and C17:1 $\omega$ 7 (relative abundance 10.7 %), whereas C20:5 $\omega$ 3 (eicosapentanoic acid, EPA, relative abundance 14.3 %) was a diatom-specific FA ('diatom biomarker').

Other biomarkers, though of lower relative abundance (2-10 %) (Fig. 3), were C17:0 for bacteria and C18:3 $\omega$ 6 and C22:6 $\omega$ 3 (docosahexaenoic acid, DHA) for diatoms.

The three copepod species, whilst originating from the same sampling station, had distinct FA compositions at T<sub>0</sub> (Fig. 3) (main test, pseudo-F= 8.77, P = 0.0036; see also ESM Table 1). The main differences were found between *Delavalia* on the one hand and *Platychelipus* and *Nannopus* on the other (Table 3). *Delavalia* contained higher amounts of C17:0, C17:1 $\omega$ 7 and also C15:0 (Fig. 3). Odd-numbered FA in general, also including 15:1, are FA produced by marine bacteria (Kelly & Scheibling 2012). The elevated amounts of these three bacterial-derived substantially contributed to the dissimilarity in FA composition between *Delavalia* and *Platychelipus* + *Nannopus* (Table 4). The total relative concentration of bacterial FA (sum of C15:0, C15:1 $\omega$ 5, C17:0 and C17:1 $\omega$ 7) for *Delavalia*, *Nannopus* and *Platychelipus* was, respectively, 23.4  $\pm$  2.5 %, 4.8  $\pm$  0.6 % and 1.8  $\pm$  0.7 % (n=3), confirming that *Delavalia* has a stronger bacterial signature compared to the other species (p < 0.001).

**Table 3.** Results from 1-factor PERMANOVA analysis: pairwise tests of copepod species at T<sub>0</sub> for differences in natural fatty acid composition, based on the Bray-Curtis resemblance matrix. Significant differences among copepod species are deduced from Monte-Carlo p-values (P<sub>MC</sub>). Significance codes: '\*' P<sub>MC</sub> < 0.05, '\*\*' P<sub>MC</sub> < 0.01, '\*\*\*' P<sub>MC</sub> < 0.001

Groups	t	P	Unique perms	P <sub>MC</sub>
DEL-NAN	4.1788	0.1015	10	0.0034 **
DEL-PLA	3.1305	0.0974	10	0.0098 **
NAN-PLA	1.7609	0.1035	10	0.0955



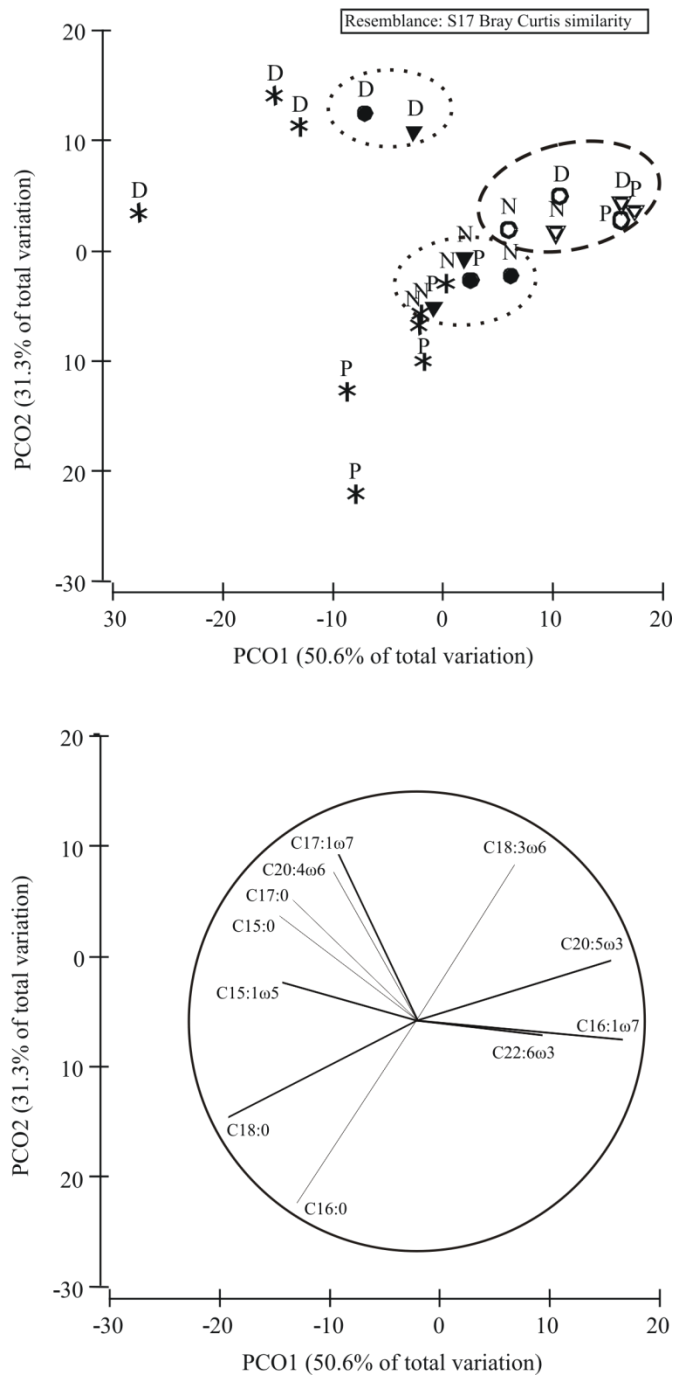
**Fig. 3.** Relative fatty acid composition (%) of (a) the food sources (bacteria and diatom monoculture) and of (b) the copepod species at time  $T_0$

FA composition of experimental copepods deviated from their initial FA composition ( $T_0$ ), as shown by the PCO plot (Fig. 4). The first two axes of the PCO explained more than 80% of the variation in copepod FA patterns and 11 FA, represented as vectors, are recognized as main contributors to changes in FA patterns. Changes in FA composition differed depending on the copepod species and on the food treatment (Fig. 4). Copepod species fed with bacteria showed limited FA changes compared to their original FA pattern. Copepods fed with diatoms grouped together at the upper-right side of the PCO, showing elevated levels of C16:1 $\omega$ 7 (found in both diatoms and bacteria) and three PUFA (C18:3 $\omega$ 6, C20:5 $\omega$ 3, 22:6 $\omega$ 3), especially for *Delavalia* and *Platychelipus*. The detailed changes of these and other FA are visualized in Fig. 5. In the B treatment, only *Delavalia* showed increased levels of FA in contrast to the  $T_0$  levels and this after 9 days: C16:1 $\omega$ 7, the bacterial biomarker C18:1 $\omega$ 9, and PUFA (e.g. EPA, DHA). PUFA were not obtained by feeding since the bacterial food lacked PUFA, so bioconversion must have occurred. Other species showed strong reductions of almost all FA, including the listed biomarkers and PUFA. In BD treatments (Fig. 5), copepod FA composition was more profoundly influenced, in particular for *Platychelipus* and *Delavalia*, showing elevated levels of both C16:0 and C16:1 $\omega$ 7, of diatom biomarker FA (C20:5 $\omega$ 3, C18:3 $\omega$ 6, C22:6 $\omega$ 3, C14:0) and of the bacterial biomarker C18:1 $\omega$ 9. No gain in C17:1 $\omega$ 7, an exclusive bacterial biomarker, was observed. These FA changes were already visible after 4 days of feeding (Fig. 5). For *Delavalia* in the BD

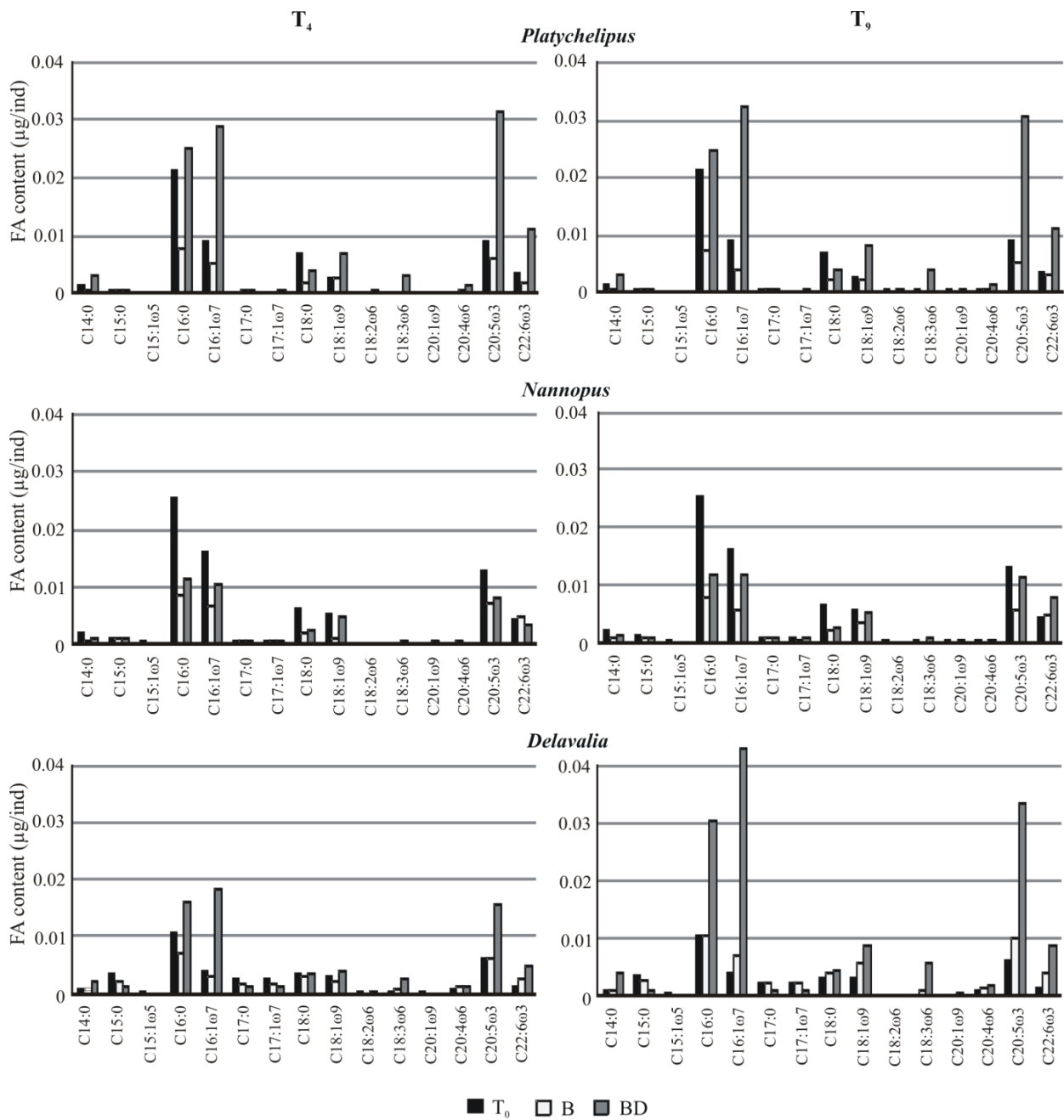
treatment, FA levels increased until the end of the incubation (Fig. 5, left-right panels), whereas they leveled off after 4 days in *Platychelipus* and decreased from the start in *Nannopus*.

**Table 4.** Partial result from SIMPER analysis: dissimilarity percentages between the fatty acid composition of copepod species at T<sub>0</sub>, based on Bray-Curtis similarity. Fatty acids contributing to dissimilarity between copepod FA composition are presented (Contrib%), using a cut-off of 70%

	Fatty acids	Contrib%
DEL-NAN	C16:1 $\omega$ 7	23.26
	C15:0	14.49
	C17:0	12.3
	C16:0	12.08
	C17:1 $\omega$ 7	11.14
DEL-PLA	C16:0	22.13
	C15:0	12.61
	C17:1 $\omega$ 7	10.29
	C17:0	9.89
	C16:1 $\omega$ 7	9.29
	C20:5 $\omega$ 3	8.43
NAN-PLA	C16:0	24.51
	C16:1 $\omega$ 7	15.95
	C20:5 $\omega$ 3	13.02
	C18:0	12.8
	C22:6 $\omega$ 3	9.96



**Fig. 4.** Principal Coordinate (PCO) analysis of *Platychelipus* (P), *Nannopus* (N) and *Delavalia* (D) based on their natural relative fatty acid composition (\*) and composition after experimental grazing during 4 days (○) and 9 days (▽ (■ - grazing on bacteria, □ - grazing on bacteria and diatoms). Changes in copepod FA profiles after feeding on bacteria and diatoms are encircled by dotted and broken lines, respectively. The vectors represent individual fatty acids with a Spearman-Rank correlations of > 50% to one of the first two PCO axes



**Fig. 5.** Fatty acid composition ( $\mu\text{g ind}^{-1}$ ) of the copepods *Platyhelipus*, *Nannopus* and *Delavalia* after grazing during 4 days ( $T_4$ ) and 9 days ( $T_9$ ) on bacteria without an additional substrate present (treatment B) and with a diatom substrate (treatment BD), compared to fatty acid profiles of copepods before grazing ( $T_0$ )

## DISCUSSION

### *Substrate-dependent bacterivory*

Harpacticoids are considered substrate browsers (Marcotte 1977, Hicks & Coull 1983, Huys et al. 1996), except for the two more primitive families Longipediidae and Canuellidae which are filter-feeders. Harpacticoid copepods collect their food from so-called large substrates (such as sediment and detrital particles) by point-feeding, edge-scraping, sweeping food of a planar surface into their mouth, or scraping food from depressions in sand particles. Feeding on smaller substrates (solid-feeding) is done by crushing the food, by sphere-cleaning where food is cleaned from surfaces by rotating spheres or organic floccules in the copepod's mouth, and by rubble-sorting where food is cleaned of organic debris by passing it over the mouth parts and organic debris is passed back through the arch of the swimming legs.

To our knowledge, no studies have specifically addressed the requirement of a physical substrate during bacterial feeding of harpacticoid copepods vs direct bacterial targeting. In calanoid copepods, filter-feeding is a common mechanism for capturing small-sized particles, but even for this group only a few studies have focused on ingestion of particles smaller than 2  $\mu\text{m}$  (Berggreen et al. 1988, Turner & Tester 1992, Roff et al. 1995). In the present study, we focus on bacterial grazing by harpacticoid copepods in treatments with and without a substrate (sediment or diatoms) and further test whether it is merely the physical presence of the substrate that matters or its nutritional value. Our study shows that all tested harpacticoid copepods ingest and assimilate bacteria in absence of a substrate, albeit at species-specific and generally low rates. This low bacterial assimilation could be linked to morphological constraints on feeding, limiting capture efficiency of free-living bacteria. However, only a few studies (Seifried & Dürbaum 2000, Michels & Schnack-Schiel 2005, De Troch et al. 2006) have linked morphology of the harpacticoid feeding apparatus to food utilization and in general, mouth morphology is not applied as a predictor of food utilization by harpacticoids (De Troch et al. 2006). It seems plausible, though, that harpacticoids are incapable of actively capturing micro-particles ( $< 5\mu\text{m}$ ), implying that bacterivory is passive (Ling & Alldredge 2003). For example, for the non-motile *Platychelipus*, bacterial ingestion could be achieved by flapping of the feeding appendages (pers obs). Alternatively, however, bacteria may be actively ingested by grazing on larger-sized bacterial aggregates or on fecal pellets, a substratum produced by the copepods themselves and which rapidly becomes coated with bacteria (De Troch et al. 2010). Based on dual labelling (both N and C), Leroy et al. (2012) found that larvae of the gastropod *Crepidula fornicata* were able to ingest particles of typical bacterial size. As in the present study, their results however suggested that the gastropod larvae preferentially used diatoms and showed that the supply of free bacteria did not alter the uptake of diatoms. They further concluded that bacteria may constitute a complementary resource for the larvae when phytoplankton is abundant and may become a substitute resource at low phytoplankton concentrations.

The presence of sediment as a substratum had a negative impact on bacterial uptake. *Microarthridion* suffered from very high mortality in treatments with sediment, while *Platychelipus* did not. This outcome can likely be ascribed to the ecology (motility) of the copepod species. The swimming species *Microarthridion* can easily avoid less preferred sediments (habitat selection) such as muffled sediment though it probably still depended on the sediment for food uptake (C. Cnudde, pers. obs.). *Platychelipus* does not emerge from the sediment into the water. It is clearly well adapted to live in or on top of the sediment. In spite of its high survival in the muffled sediment, *Platychelipus* showed only a marginal bacterial uptake. Rieper (Rieper 1978) reported slightly increased rates of bacterivory for *Tisbe holothuriae* and *Paramphiascella vararensis* when bacteria were mixed with beach-sand grains. For muddy sediments, as in our study, bacterivory was not stimulated by the presence of grains. Here, the negative effects of sediment could result from sediment pretreatment. Muffling altered grain size as the muffled sediment had a median grain size of 45  $\mu\text{m}$  and a 62 % mud fraction, compared to natural sediment from the copepod habitat with a median grain size 126  $\mu\text{m}$  and a mud fraction of 30 %. The presence of a higher fraction of very fine sediment particles and their looser texture and structure may have interfered with



normal feeding, for instance by clogging the feeding apparatus or by accumulation inside the mouth cavity or intestine. This was also suggested as an explanation for the observation that fine sediment clearly interfered with grazing of copepods (*Paramphiascella fulvofasciata*, *Nitocra spinipes*) on diatoms (De Troch et al. 2006).

On the other hand, the increased FA content of *Platyhelipus* in the BDS versus BD treatment suggests that the presence of sediment stimulated diatom assimilation by *Platyhelipus*. Hence, unlike in *Microarthridion*, the lack of bacterial uptake by *Platyhelipus* in the sediment treatments is unlikely to have resulted from a negative impact of the sediment on copepod feeding activity, but rather indicates that this species is incapable of efficiently accessing bacteria that are mixed with sediment. Cultured bacteria are effective short-term sediment stabilizers through production of exopolymer secretions (EPS) during grain adhesion, implying that bacterial attachment to sediment grains likely occurred during the experiment. The observation that *Platyhelipus* hardly assimilated bacterial carbon in our sediment treatments suggests that this species does not scrape off bacteria from sediment grains nor exhibits unselective ingestion of sediment particles and its associated microorganisms, even though exploitation of particle-bound bacteria through substrate ingestion and stripping in the gut has been reported for *Leptocaris brevicornis* (Decho & Castenholz 1986). Judging from the FA profiles, *Platyhelipus* did, however, efficiently graze on diatoms and probably co-ingested and co-assimilated bacteria with diatoms, resulting in a higher uptake of bacterial carbon in the BD treatment. Co-ingestion of bacteria while feeding on diatoms was found in three out of four copepod species tested here, i.e. *Platyhelipus*, *Delavalia* and *Microarthridion*, while in the fourth species, *Nannopus*, no apparent diatom grazing occurred even after prolonged incubation (9 days). This study indicates that for diatom-feeding harpacticoids, bacterial grazing is strongly dependent on diatom ingestion as a significant part of bacterial grazing is realised through co-ingestion of bacteria with diatoms. Diatoms are thus expected to be the primary food source, even though the reverse, i.e. assimilation of bacteria and egestion of undigested diatom cells, has also been reported for *Leptocaris brevicornis* (Decho & Castenholz 1986).

For *Platyhelipus* the effect of diatom presence on the bacterial uptake rate was incoherent between the two experiments, in spite of copepod total FA content demonstrating active feeding on diatoms in both setups. Specific and biomass-specific uptake were similar for the B treatment in both experiments, but were much lower in the BD treatment of the first experiment compared to the second. We have no clear explanation for this discrepancy.

Microphytobenthos, mainly diatoms, are known as a primary food source for intertidal saltmarsh meiofauna (Pinckney et al. 2003, Galvan et al. 2008). Few field studies have addressed seasonal food availability in relation to population dynamics and feeding ecology of individual harpacticoid species or genera. *Microarthridion littorale* and *Nannopus palustris* are known as diatom feeders (Santos et al. 1995). This is supported by the natural  $\delta^{13}\text{C}$  of both species in the current study (ca. -16 ‰), which is very close to that of microphytobenthos at the Paulina field site (Moens et al. 2002, Moens et al. 2005a), and by the high values of FA C16:1 $\omega$ 7 and C20:5 $\omega$ 3, known as characteristic for Bacillariophyceae (Kelly & Scheibling 2012). The lack of FA accumulation by *Nannopus* in the BD treatment may point at feeding selectivity, with the diatoms used in our experiment perhaps being an unsuitable source for this species. Data on the importance of microphytobenthos for *Platyhelipus littoralis* and *Delavalia palustris* and their general trophic ecology is, however, lacking. The abundances of Bacillariophyceae FA in these species varied considerably. Their natural  $\delta^{13}\text{C}$  signals (*Platyhelipus* ca. -15 ‰ and *Delavalia* ca. -17 ‰) were, however, very close to these of *Microarthridion* and *Nannopus* and within the range of microphytobenthos values known for the study site (Moens et al. 2002, Moens et al. 2005a). The somewhat more depleted  $\delta^{13}\text{C}$  values of *Delavalia* nevertheless suggest some contribution of settled phytoplankton detritus (Moens et al. 2002). Moreover, this is the only species out of the four species tested that showed high abundances of odd-branched bacterial biomarker FA (C15:0, C17:0, C17:1 $\omega$ 7). *Delavalia* is a typical tube-builder and tube-dweller (Nehring 1993) and covers the inner tube wall with a mucoid substance secreted by cuticular glands (Williams-Howze & Fleeger 1987). Mucus secretion and the presence of secretory mucus

glands has been reported for only a few harpacticoid copepods: *Heteropsyllus nunni* (Coull & Grant 1981), *Pseudostenhelix* sp. (Williams-Howze & Fleeger 1987) and *Diarthrodes nobilis* (Hicks & Grahame 1979). The former two have a largely sessile life, respectively involving dormancy and tube-building, and mucus functions in copepod 'housing' (dormancy capsule, tube) but may also be used for bacterial gardening, as also observed for nematodes (Moens et al. 2005b). The latter copepod species uses a mucus capsule as temporary protective shield while feeding on algae. Nevertheless, mucus may also be involved in feeding, since some juvenile *Diarthrodes* were found to survive and develop inside the capsules, obtaining their energy from the bacteria-rich mucus and entrapped organic debris (Hicks & Grahame 1979). *Delavalia* is not permanently residing in its tubes and after some time in experimental settings we could observe that this species 'sticks' to the bottom of Petri dishes. This might suggest the production of adhesive mucus (C. Cnudde pers observ). Since our data demonstrates a copepod-bacteria trophic interaction for *Delavalia* only, we hypothesize that *Delavalia* may apply such a microbial gardening strategy, the importance of which is, however, not as strong as for the above mentioned sessile copepods.

### ***Direct transfer of bacterial FA to harpacticoids and other insights into FA dynamics***

For three out of four harpacticoid species a marked impoverishment in total FA content was observed when offered bacteria as the only food, but not so for *Delavalia*. In contrast, all species except *Nannopus* accumulated FA on a mixed diet of bacteria and diatoms.

FA loss in copepods in the bacteria treatment is mainly noticeable in the highly abundant FA such as the C16 FA and PUFAs. C16 FA, which were among the main FA in the bacterial inoculum, were not incorporated, except by *Delavalia*. *Delavalia* was the only species that acquired dietary FA from an exclusively bacterial diet (final FA content: 121 %). This species mainly incorporated the most abundant FA in the bacterial inoculum: C16:1 $\omega$ 7 and C18:1 $\omega$ 9. Little or no incorporation of less abundant FA (C16:0, C17:1 $\omega$ 7 and C17:0) was measured. Bacterial FA contribute more than 15 % to *Delavalia* FA, while less than 5% for *Nannopus* and *Platychelipus*. Studies on the lipid dynamics in marine copepods are mostly focusing on calanoid copepods, particularly on their even-chain FA, and do not report bacterial FA (Pasternak et al. 2009). One of the few studies dealing with the natural FA content of harpacticoids is Leduc et al. (2009), in which the proportion of bacterial FA in 'bulk' harpacticoids, mainly consisting of *Parastenhelia megarostrum*, was < 5 %. This is in agreement with the bacterial FA found here in *Nannopus* and *Platychelipus* but considerably less than in *Delavalia*. In mucus-producing *Heteropsyllus nunni* the total of C15:0 and C17:0 comprised almost 10 % of FA content (Williams & Biesiot 2004). Here we demonstrate that bacterial FA can be directly transferred to harpacticoid copepods. Furthermore, *Delavalia* increased its PUFA content on this bacterial diet, as levels of EPA and DHA were elevated in the B treatment already after 4 days. PUFA were absent from the bacterial diet and we can provide no other explanation than *de novo* production of these PUFA by *Delavalia* (bioconversion). These are vital fatty acids, required for somatic growth and membrane functioning, making copepods a highly nutritional prey for juvenile fishes (Rajkumar & Vasagam 2006). Since higher trophic levels depend on dietary omega-3 PUFA, tracing these long-chain FA provides valuable information about how carbon is channeled through marine food webs (Brett & Muller-Navarra 1997).

In the treatment with diatoms, *Platychelipus* and *Delavalia* incorporated a broad spectrum of FA. The elevated levels of the diatom-characteristic PUFA and to a lesser extent of C18:3 $\omega$ 6 are indicative of the nutritional value of diatoms for these two copepods. No incorporation of bacteria-specific C17 FA was detected and increases of FA such as C14:0 and C18:1 $\omega$ 9 could originate from diatoms as well as from bacteria.

Overall, *Delavalia* was able to derive FA from both diatoms and bacteria, *Platychelipus* and *Microarthridion* only from diatoms, and *Nannopus* did not accumulate FA from diatoms or bacteria. In all, these results suggest that bacteria represent a low-quality food and at best an additional food source for the

harpacticoid copepods used here. This is in line with the main findings of Souza-Santos et al. (1999) as they concluded that diatoms were the main dietary component and bacteria are a source of vitamin. Only in *Delavalia*, bacterivory was accompanied by clear assimilation of dietary FA. Therefore, proper assessment of the importance of bacterivory for harpacticoid copepods should not merely focus on uptake and assimilation, but also on incorporation of FA by the consumers. It is important to note that consumer FA profiles do not necessarily reflect those of their food source as important modifications may take place. It is therefore essential to consider the possibility of bioconversion (e.g. elongations of short-chain FA) (De Troch et al, 2012) rather than merely focusing on the direct transfer of bacteria-specific FA to grazers.

### **Conclusion**

The four sediment-dwelling intertidal harpacticoid copepods consumed bacteria at low rates, partly due to passive ingestion and partly by co-ingestion with benthic diatoms, but not by co-ingestion with sediment. This study demonstrates that bacterial FA can be directly accumulated by only some harpacticoids (*Delavalia*), whereas diatom FA were assimilated by three species. *Delavalia* also produced polyunsaturated FA from a bacterial diet lacking these FA. Generally, however, our results indicate that bacteria represent a minor and low-quality food for intertidal harpacticoid copepods.

### **ACKNOWLEDGEMENTS**

The first author acknowledges a PhD grant of IWT (Institute for the Promotion of Innovation through Science and Technology in Flanders). MDT is a postdoctoral researcher financed by the Special Research Fund at the Ghent University (GOA project 01GA1911W). Financial support was obtained from the Flemish Science Foundation through project 3G019209W and from the research council of Ghent University through project project BOF09/24J/148. Special thank goes to Ir. Dirk Van Gansbeke (Marine Biology, UGent) for analysing the fatty acids and Annick Vankenhove for her help with sorting the copepods. The authors thank two anonymous reviewers for their constructive remarks that contributed to the improvement of the manuscript