Chapter 5

Limited feeding on bacteria by two intertidal benthic copepod species as revealed by trophic biomarkers

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ABSTRACT

Harpacticoids can discriminate between biofilms of different bacterial strains. We investigated whether assimilation of bacteria is selective and whether harpacticoids select the most nutritional bacteria. We specifically focused on the role of bacterial characteristics in copepod food selection. Trophic biomarkers (stable isotopes, fatty acids) were used to test selective assimilation of three bacteria by the harpacticoids *Platychelipus littoralis* and *Delavalia palustris*, all isolated from a salt marsh. The bacteria *Gramella* sp., *Jannaschia* sp. and *Photobacterium* sp. with contrasting ribosomal protein and fatty acid contents, were ^{13}C-labelled and offered in a food-patch choice experiment with monospecific and combination (respectively single and two strains per microcosm) treatments.

Low assimilation of bacterial carbon and lack of significant FA transfer proved that bacteria were a poor food source for the harpacticoids. Assimilation was copepod species-specific and bacteria strain-specific (preference for *Photobacterium*). However, only a low degree of selective feeding occurred; it can partly be explained by extracellular metabolites rather than by biochemical content and densities. Finally, the energetic cost of differential bacterivory resulted in a negative fatty acid balance for *Platychelipus*, while *Delavalia* showed an improved fatty acid profile and thus a positive response to the low-quality bacterial food.

Key words: bacterial grazing; selective feeding; harpacticoid copepods; stable isotopes; fatty acids; nutritional quality, microbe:higher organism interactions

INTRODUCTION

Although microphytobenthos is considered a major food source for harpacticoid copepods (Montagna et al. 1995), copepods can utilize a variety of resources (Hicks & Coull 1983) and their dietary requirements are very species-specific (De Troch et al. 2005). Some harpacticoid copepods are capable of grazing on bacteria (Cnudde et al. 2012) and may even prefer bacteria over diatoms (Vandenberghe & Bergmans 1981, Decho & Castenholz 1986). Good knowledge on their feeding ecology is pivotal as harpacticoids are a favored food source for several juvenile fish (Tsubaki & Kato 2009) and thus transfer autotrophic and heterotrophic production to higher trophic levels.

In addition to sediment characteristics (grain size), other environmental parameters (salinity, pH, temperature, oxygen, nutrients) and species interactions (Hicks & Coull 1983, Dahms 1991, Soetaert et al. 1995, Gonçalves et al. 2010), patchiness of food sources is one of the factors underlying the spatio-temporal distribution of harpacticoids (Carman & Thistle 1985, Azovsky et al. 2005). Moreover, preferences for substrata such as different sediment types or macroalgae can be driven by the microbial coatings covering them (Ravenel & Thistle 1981, Hicks & Coull 1983). The attractiveness of a substratum or a microbial food may be at least as dependent on the identity of the microbiota present as on their mere abundance (Rieper 1982, Dahms et al. 2007), as was also demonstrated for bacterial-feeding nematodes (Moens 1999).

So far, little evidence exists on harpacticoids' ability to feed selectively on specific bacteria and on the biological role of targeting bacteria. For larvae of some benthic invertebrates, bacteria have an informative function as their presence guides the larvae to a suitable substratum to settle (Qian et al. 2003). For benthic copepods, targeting bacteria may serve more than a mere habitat recognition/colonisation function, but may also be related to the selection of food sources that are typically coated with particular bacteria. Moreover, it is possible that selective bacterial feeding could be a strategy independent of feeding on another (larger) food source. In the latter case, the food value of the bacteria *per se* is the main driver of copepod feeding behaviour. The nutritional value of bacteria for copepods remains poorly documented and may differ among copepod life stages (Dahms et al. 2007). Even though most bacteria lack fatty acids such as PUFA and sterols (Cho & Mo 1999) which are vital to copepods, harpacticoids may bioconvert bacteria-derived fatty acids (Desvilettes et al. 1997, De Troch et al. 2012a). Feeding exclusively on a bacterial diet does not necessarily limit copepod development and reproduction (Weiss et al. 1996, Dahms et al. 2007). Application of a bacterial food in mariculture of crustaceans (shrimp) may even promote their growth (see use of biofloc mats) (Crab et al. 2010).

A high degree of feeding selectivity has been demonstrated for calanoid copepods predating on rotifer sibling species (Lapesa et al. 2004). Some calanoid copepods were deterred by toxic cyanobacteria and microalgae species and selectively fed on non-toxic strains/clones (Teegarden et al. 2008, Ger et al. 2011). Harpacticoid copepods respond selectively to the presence of specific bacterial strains (Rieper 1982, Dahms et al. 2007). Selective feeding may underlay resource partitioning between harpacticoid species whose assemblages are often species-rich and attain high abundances in marine sediments (Chertoprud et al. 2010). Therefore, a better understanding of selective feeding modes at the basis of marine food webs is crucial to interpret the energy flow to higher trophic levels.

The use of trophic biomarkers like stable isotopes and fatty acids allows good progress in food selectivity research especially in the case of microbial food sources. These tools have revealed selective feeding by harpacticoids on diatoms, even involving selection for specific growth phases of the diatoms (De Troch et al. 2012b). In addition, the bacterial characteristics at the basis of selective grazing are still under discussion. Characteristics of the cell wall, the produced extracellular substances (Decho & Moriarty 1990) and cell morphology in general (cell shape, pigmentation, motility) may all play a role. More insights into the biochemical characteristics of bacteria should be obtained to unravel the triggers for food selection.

This study aimed to assess harpacticoid assimilation of individual bacterial strains in relation to their biochemical characteristics and thus to their potential nutritional value, and to test their ability to select between these bacteria of different nutritional value. This was tested by means of a 9-day food-patch choice experiment with bacterial strains characterized by contrasting ribosomal protein and fatty acid profiles. Bacterial uptake by two harpacticoids, *Platychelipus littoralis* (family Laophontidae) and *Delavalia palustris* (formerly known as *Stenhelia palustris*, family Miraciidae) was assessed by measuring copepod ^{13}C-enrichment after feeding on ^{13}C-prelabelled bacterial biofilms, and the impact of bacterivory (selective or not) on copepod nutritional status was measured in terms of their fatty acid composition. The experiment consisted of monobacterial food treatments (included only one bacterial species), combination food treatments (included two bacterial species) and a starvation treatment (no bacteria) (Fig. S1). Each food treatment contained two food patches, being two monobacterial biofilms grown on PET membranes: monobacterial treatments were composed of two ^{13}C-enriched biofilms from the same species, combination treatments were composed of two biofilms from different bacterial species. In the latter, only one biofilm was ^{13}C-enriched to be able to trace selective uptake.

This up-to-date combination of biochemical screening and the use of biomarkers can provide more insight in 1) the way copepods select food sources with a particular biochemical signature, and 2) whether and how they modify specific biochemical components (fatty acids) for transfer to the next trophic level. More specifically, this study aimed to test (1) whether harpacticoids selectively ingest and assimilate bacterial strains of different biochemical content in monospecific and combination treatments, (2) whether this behaviour is copepod species-specific, which could indicate resource partitioning, and finally (3) to gain further insight into the assimilation of bacterial fatty acids by bacterivores.

EXPERIMENTAL PROCEDURES

Harpacticoid species

Two harpacticoid copepod species were collected from silty sediments of an intertidal creek in the Paulina salt marsh in the polyhaline reach of the Westerschelde estuary (SW of The Netherlands, 51°20'55.4''N, 3°43'20.4''E): *Platychelipus littoralis* (family Laophontidae) and *Delavalia palustris* (formerly known as *Stenhelia palustris*, family Miraciidae). Both species are further referred to by their genus name. These species were chosen based on their co-existence and relatively high abundances within the copepod salt marsh community. They likely occupy different niches/habitats in the salt marsh sediment (Cnudde et al. 2012): *Delavalia* is a well-known tube-dweller (Nehring 1993) but is also capable of active swimming, while *Platychelipus* is a non-swimming, less mobile species (Cnudde C., pers. obs.). The latter species appears tightly linked to the sediment, where it is restricted to the top millimeters in the field (Cnudde C., pers. obs.); in microcosms, it is usually found closely attached to the bottom of the Petri dish. Both species were previously shown to assimilate bacteria, although at low rates (Cnudde et al. 2012). Both 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 over a 250 µm sieve 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 µm Millipore filters and autoclaved; henceforth referred to as 'sterile ASW') and starved for 24 h to allow gut clearance. Finally, copepods were washed once more in sterile ASW before introducing them into the experimental microcosms. Only adult, non-gravid specimens were selected randomly and both sexes were represented in the same proportion as in field samples.

Bacterial species and biofilm culturing

Three marine bacterial strains were selected from an extensive collection, comprising 149 isolates, originally obtained by the first author from the Paulina salt marsh sediment. Isolates were grown on marine agar (37.4 g l^{-1} Marine Broth 2216 Difco™, 20 g l^{-1} Bacto Agar Difco™) and stored at -20°C in a glycerol solution (37.4 g l^{-1} Marine Broth 2216, 15 % glycerol). The complete collection was characterized by ribosomal protein profiling (addendum V, Fig. S1). Ribosomal proteomic analysis was performed using matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). For comparative purposes, standardized culturing conditions were required. All strains were therefore grown on marine agar under aerobic conditions at 20°C for 6 days and subcultured twice prior to harvesting to ensure all isolates were in the same physiological state. Following the protocol of Ghyselinck et al. (2011), bacterial cell extracts were prepared and spotted on a 384 Opti-TOF MALDI-TOF MS target plate. Sample spots were overlaid with a matrix of α-cyano-4-hydroxicinnamic acid (α-CHCA) and analysed using the 4800 *Plus* MALDI TOF/TOF Analyser (AB Sciex). Mass spectra were converted to fingerprints with BioNumerics 5.1 software (Applied Maths, Belgium). Using a Pearson's product moment correlation coefficient and the UPGMA clustering algoritm, similarities between the spectra were calculated and the spectra were clustered (addendum V, Fig. S1). 44 isolates with distinct MALDI-TOF profiles were identified by partial 16S rRNA gene sequencing. The 16S rRNA gene was amplified and partially sequenced as described by Vancanneyt et al. (2004), and PCR products were purified using a Nucleofast 96 PCR clean-up membrane system (Macherey-Nagel, Germany) and Tecan Workstation 200. The V_1-V_3 region of the 16S rRNA gene (± 500 bp) was sequenced using primer BKL1 (Coenye et al. 1999). Obtained fragments were cleaned with the BigDye® XTerminator™ Purification Kit according to the manufacturer's protocol. Sequence analysis was performed using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA). Genus identifications of the sequences were obtained using the classifier of the Ribosomal Database Project (RDP) (Wang et al. 2007). In case the confidence estimate of the genus identification was lower than 80%, higher taxon identification is presented. The sequence data have been deposited in the DDBJ/EMBL/Genbank databases under accession numbers HE999713 – HE999756 (addendum V, Table S1). Subsequently, three bacterial isolates were selected for the current experimental setup based on their contrasting ribosomal protein (addendum V, Fig. S1) and fatty acid (FA) compositions and ecological relevance (i.e. belonging to bacterial taxonomic classes that are abundant in the marine environment): *Gramella* sp. (Flavobacteriaceae, Bacteroidetes), *Jannaschia* sp. (Rhodobacteriaceae, Alphaproteobacteria) and *Photobacterium* sp. (Vibrionaceae, Gammaproteobacteria), in the experimental treatments referred to as G, J and P. These 3 marine genera show distinct differences in fatty acid composition (Yoon et al. 2010, Cho et al. 2011, Kim et al. 2011), as also confirmed later by our FA profiles (see Results and discussion section and Table S2). In order to limit the potential effects of bacterial cell morphology, we chose three strains which were all rod-shaped and Gram-negative. Pigmentation was different with *Gramella, Jannaschia* and *Photobacterium* forming, respectively, yellow, white and brown-white colonies on marine agar. From these three strains the complete 16S rRNA gene sequences were determined (Coenye et al. 1999). The accession numbers of the 16S rRNA gene sequences of *Gramella*, *Jannaschia* and *Photobacterium* strains are HE999723, HE999722 and HE999728.

Bacterial isolates were cultured as monostrain biofilms on a membrane surface. Membrane-associated biofilms were produced by means of ThinCert™ cell culture inserts (Greiner Bio-One) consisting of a PET membrane (diameter 24.85 mm, 0.4 µm-sized pores, pore density: $1x10^8$ cm^{-2}) to improve cell adhesion. First, bacterial isolates were grown on marine agar for 5 days at 15°C in the dark to check purity of each isolate. Bacterial biomass was then harvested from the agar plates and brought into suspension in sterile ASW. A total of 117 ThinCert™ inserts (39 inserts for each strain) and their wells were filled with a liquid growth medium, consisting of autoclaved ASW (salinity: 28), beef extract (DIFCO, 3 g L^{-1}), bacto peptone (DIFCO, 5 g L^{-1}) and one third of the membranes each was inoculated with a different bacterial isolate by adding 10 µl of the respective strain suspension. The 6-well plates with membranes were incubated for 3 days. Subsequently, bacterial biofilms were ^{13}C-labelled by replacing the liquid growth medium by a 20-fold diluted medium containing ^{13}C glucose at a concentration of 0.5 g L^{-1} (D-glucose, U-$^{13}C6$, 99%,

Cambridge Isotope Laboratories, Inc.). This was done for 24 biofilms of each bacterial isolate. The remaining 15 biofilms were further grown on an equivalent medium with ^{12}C glucose. ^{13}C labelling and continued growth of the bacterial biofilms lasted for 2 days and the ^{13}C-labelled medium was refreshed once in between. Specific uptake of bacterial strains measured 7276 ± 49 ‰ for G, 1002 ± 124 ‰ for J and 8341 ± 1311 ‰ for P (mean ± 1 SD, n=3) corresponding to an atomic % ^{13}C of 8.5 ± 1.0 %, 2.2 ± 0.2 % and 9.5 ± 1.2 % (mean ± 1 SD, n=3), respectively.

Selection experiment

A short-term food-choice experiment was carried out to quantify selective uptake of one bacterial species over the others and this by means of direct measurement of bacterial carbon assimilation by the copepods (^{13}C-tracing). For this purpose, field-caught harpacticoid copepods (not cultured, not preconditioned) were offered 3 salt marsh bacterial strains in the form of living biofilms, set up as food patches in two different Petri dish (5.2 cm diameter) microcosm setups (see below) (Fig. 1). Each monostrain biofilm represented one 'food patch' in the experimental microcosms. Selective feeding was evaluated by offering copepods two food patches representing two different bacterial strains (combination treatment). Strain-specific feeding was also observed in absence of a second strain (a monobacterial treatment), thus offering two food patches of the same strain, to keep bacterial density constant over treatments. Consequently, the experimental design consisted of the following 10 treatments:

(1) 3 monobacterial treatments: with a single bacterial strain as food (G*, J*, P*). * refers to the ^{13}C prelabelling of the bacterial cells;
(2) 6 pairwise combination treatments: three types of 1:1 combinations were prepared containing one ^{13}C prelabelled bacterial strain (G*J, G*P, J*P; and their complement GJ*, GP*, JP*)
(3) 1 starvation treatment: empty membranes were inserted, copepods had no access to bacteria or any other food.

These treatments were set up for both harpacticoid species and each treatment was replicated three times. Membranes covered with biofilms were cut out of the Thincert™ inserts using a scalpel and were gently dipped in 3 separate washing baths (preventing cross contamination) filled with sterile ASW in order to remove the labelled medium. The combination treatments consisted of a labelled biofilm of one bacterial strain and an unlabelled biofilm of another bacterium. Monobacterial treatments contained two labelled biofilms of the same bacterium (Fig. 1a). The two membranes together covered half of the microcosm surface, were placed opposite of each other and against the border of the dish wall, leaving a free space of about 3 mm between the borders of the membranes and a distance of 2.8 cm between the food patch centers. The membranes were held in place by a precut plastic foil (Fig. 1b). Before adding sterile ASW and copepods to the Petri dishes, a PVC ring was used to cover the outer circular edge of the foil (Fig. 1b). This setup, including culture membranes, plastic foil and PVC ring, was necessary (1) to obtain evenly distributed biofilms and (2) to anchor biofilms firmly to the dish surface. Petri dishes were gently filled with 15 ml of sterile ASW (salinity: 28) and 40-45 copepod specimens were added to each dish. The experimental units were randomly placed on a shelf in a climate room and incubated for 9 days at 15°C with a 12:12-h light:dark regime (Fig. 1c). A 9-day incubation was chosen since this is the minimum time needed to detect any changes in copepod fatty acids under similar feeding conditions (De Troch et al. 2012a). A few additional biofilm membranes of each strain were stored in 2 % gluteraldehyde-NaCl solution at 4°C and bacterial densities of each strain biofilm were determined after experimental setup. These are expected to be dissimilar since division rate and biofilm formation (membrane attachment) of the strains are likely to be strain-specific. For counting purposes, membrane-attached bacteria were suspended by sonicating them twice for 30 s in a sonication bath (37 Hz, 70W) and then manually scraping the membranes with a glass coverslide. Bacterial densities were estimated by epifluorescence microscopy after staining with 4 µg mL^{-1} DAPI (final concentration) for 10 min (modified after Porter & Feig 1980). Per filter, a horizontal and vertical transect with a minimum of 200 cells were

counted. These counts are conservative estimates since (1) bacterial detachment from the membranes may have been incomplete and (2) a proportion of the cells might have been degraded due to preservation in the fixative (Kamiya et al. 2007).

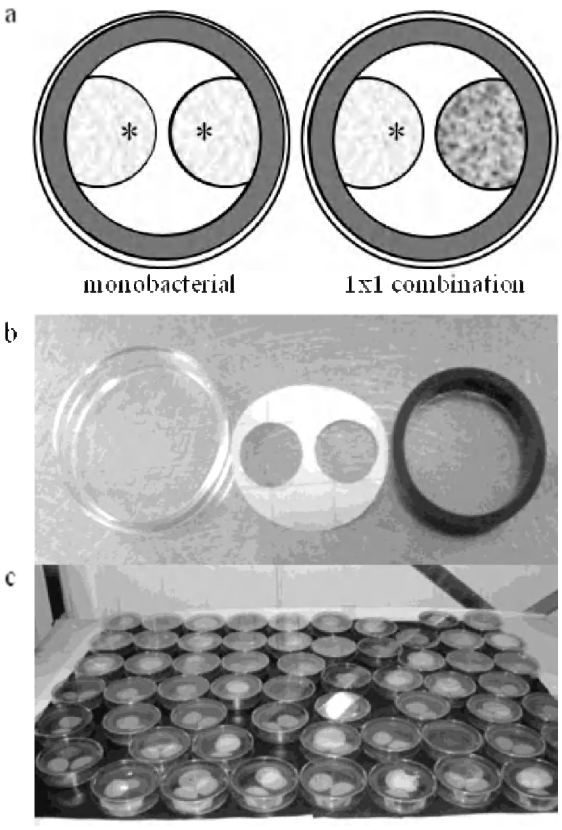

Fig. 1. Experimental setup: (a) two types of food microcosms, a monobacterial treatment (left) and a combination treatment (right), containing two biofilms of one bacterial species or of two different bacterial species, respectively [asterisk (*) indicating the ^{13}C-labelled biofilms]; (b) microcosms consisted of two membranes covered with biofilm (not shown) placed into a Petridish (5.2 cm diameter) and held in place by a plastic foil and PVC ring, (c) running experiment, incubated in climate room.

Sample processing

Mortality of copepods in each microcosm was assessed at the end of the experiment. Surviving copepods were collected from the Petri dishes and used for stable isotope analysis and fatty acid analysis. From each replicate microcosm, 15 copepod specimens were taken at random, washed in sterile ASW, starved overnight to empty their guts and temporarily stored at -20°C till further processing for isotope analysis. All remaining copepods of the three replicates were pooled for the preparation of a fatty acid sample. For combination treatments, the three replicates and the complementary treatments were pooled (e.g. G*J and GJ*) to increase sample content, since due to mortality the number of copepods available for fatty acid analysis was otherwise not always sufficient. Copepods were cleaned while alive by multiple transfers through sterile ASW, collected on a Whatman filter and frozen at -80°C prior to fatty acid extraction. Depending on copepod numbers lost due to mortality, this yielded one sample with 35 to 105 specimens for fatty acid analysis per treatment. Hence, for each treatment, three independent samples for stable carbon isotope analysis and one for fatty acid analysis were obtained. At the start of the experiment, control samples of bacteria (labelled and unlabelled) and copepods were prepared in triplicate for each type of analysis. Bacteria control samples were subsamples of the laboratory cultures of G, J, P en G*, J*, P*.

Copepods for isotope analysis were thawed, rinsed several times in MilliQ water and transferred to muffled aluminum capsules (6x2.5 mm). The overall procedure was executed within 2 hours after thawing to minimize leakage of ^{13}C from the copepod body (Moens et al. 1999d). Copepods in capsules were oven-dried overnight at 60°C, pinched closed and stored under dry atmosphere until analysis. Control samples were prepared in the same manner.

Stable isotope analysis

$\delta^{13}C$ values of bacteria (natural and enriched) and harpacticoid copepods (expressed in ‰) and copepod carbon content (expressed as µg C sample^{-1} of x copepods) were obtained from the Davis Stable Isotope Facility (University of California, USA) using a Europa Integra isotope ratio mass spectrometer. Uptake of bacterial ^{13}C by the copepods is expressed as total ^{13}C uptake per individual. For this purpose $\delta^{13}C$ values were converted to carbon uptake according to Middelburg et al. (2000). Total uptake of ^{13}C per copepod (in µg ^{13}C copepod^{-1}) is calculated as the product of excess ^{13}C (above background, E) and mean individual copepod biomass (organic carbon) per sample. Excess ^{13}C is the difference between the fraction ^{13}C (F) of the control (here copepods at the beginning of the experiment) and of the sample (copepods after experimental treatment), where $F = {}^{13}C/({}^{13}C+{}^{12}C) = R/(R+1)$. The carbon isotope ratio (R) was derived from the measured $\delta^{13}C$ values as $R = (\delta^{13}C/1000 + 1) \times R_{VPDB}$, with $R_{VPDB} = 0.0112372$, being the carbon isotope ratio of the reference material (Vienna PDB). Because ^{13}C labellling of the 3 strains resulted in pronounced differences in atomic % ^{13}C of bacterial biomass (see before), ^{13}C uptake values were multiplied by 11.8, 45.5 and 10.6, respectively, for treatments containing labelled bacteria G*, J* or P*, thus converting ^{13}C assimilation to total carbon assimilation (in µg C copepod^{-1}). Finally, total carbon uptake per unit copepod biomass (in µg C) was calculated by dividing uptake by copepod biomass. Individual carbon contents of the copepod species were 1.57±0.15 and 1.09±0.1 µg C copepod^{-1} (mean ± 1 SD, n=33) for *Platychelipus* and *Delavalia*, respectively. Natural carbon isotopic signals of both copepod species were -14.6 ± 0.5 ‰ and -13.8 ± 0.9 ‰ (n = 3) for *Platychelipus* and *Delavalia*, respectively.

FA analysis

Hydrolysis of total lipids of bacteria 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. 2012a). The boron trifluoride-methanol reagent was replaced by a 2.5 % H_2SO_4-methanol solution since BF_3-methanol can cause artefacts or loss of PUFA (Eder 1995). The fatty acid Methylnonadecanoate C19:0 (Fluka 74208) was added as an internal standard to allow later quantification. The obtained FAME were analysed using a gas chromatograph (HP 6890N) coupled to a mass spectrometer (HP 5973). The samples were run in splitless mode (5 µL injected per run), at an injector temperature of 250°C using a HP88 column (Agilent J&W; Agilent Co., USA). The oven temperature was programmed at 50°C for 2 min, followed by a ramp at 25 °C min^{-1} to 175 °C and then a final ramp at 2°C min^{-1} to 230°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 250 µg mL^{-1}) of external standards (Supelco # 47885, Sigma-Aldrich Inc., USA).

Shorthand FA notations of the form A:BωX 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

Bacterial FA are expressed as relative FA compositions. These relative multivariate FA data of the three bacterial strains were analysed using a one-factor Permutational ANOVA (PERMANOVA) with factor strain; 9999 permutations were run with the raw data. The assumption of homogeneity of multivariate dispersion was tested with PERMDISP. The subsequent pair-wise PERMANOVA test consisted of only 10 unique permutations and p-values were drawn from Monte Carlo permutations (P_{MC}). FA highly responsible for the differences in bacterial FA profiles were provided by a SIMPER analysis for which a 70% contribution cut-off level was used.

For the analysis of copepod mortality and carbon assimilation data two-way Analysis of Variance (ANOVA) was applied with fixed factors copepod species and treatment, to asses differences between the copepod species and between all treatments (starvation, mono and combination treatments). The assumptions of homogeneity of variances and normality were respected, as tested, respectively, with the Levene's test and Shapiro-Wilks test. Mortality data were arcsin-root transformed, carbon assimilation data were $\log_{(X+1)}$ transformed. The Tukey's HSD *post hoc* test was applied to detect pairwise differences in mortality and bacterial uptake, using 95% confidence limits.

Differences in FA composition between copepods were visualized by Principal Coordinates Analysis (PCO) based on Bray-Curtis similarities. PCO visualised those copepod FA that correlated >70% with one of the first two PCO axes.

All ANOVA analyses were performed using the software package R, version 2.14.1 (R Development Core Team 2012). PERMANOVA, SIMPER, MDS and PCO analyses were performed within PRIMER v6 with PERMANOVA add-on software (Clarke & Gorley 2006, Anderson et al. 2008).

RESULTS AND DISCUSSION

Bacterial strains and their nutritional content

Three bacterial strains were chosen from an isolate collection originating from salt marsh sediment including members of the Alfaproteobacteria, Gammaproteobacteria, Actinobacteria and Flavobacteria (Bacteroidetes) (Table S1): *Jannaschia* sp. (family Rhodobacteriaceae, Alfaproteobacteria), *Photobacterium* sp. (family Vibrionaceae, Gammaproteobacteria) and *Gramella* sp. (family Flavobacteriaceae, Flavobacteria). No representative of the Gram-positive Actinobacteria was included since the difference in cell wall could interfere with the aim to interpret copepod selectivity for bacterial biochemical properties. Cultured bacterial biofilms obtained relative densities of 53:1:3 for *Gramella*:*Jannaschia*:*Photobacterium*, so *Gramella* attained much higher abundances than the other two strains. *Gramella* has a high surface-adhering ability (Bauer et al. 2006), which explains its comparatively high cell densities. No difference in cell size was observed and bacteria contained an equally low C:N ratio of 4:1. For algal food sources, a low C:N content was found to be indicative of high food quality for copepods (Jones & Flynn 2005) but C:N is not consistently reliable as food quality indicator (Hatcher 1994).

Bacterial strains differed in nutritional content by their ribosomal protein profiles (addendum V, Fig S1) and by their fatty acid (short notation: 'FA') content (main test, pseudo-F = 52.53, p_{perm} = 0.0034; PERMDISP F= 4.08, p_{perm} = 0.13), *Gramella* deviating strongly from both *Jannaschia* and *Photobacterium* (pair-wise test, G-J: t = 6.86, p_{MC} = 0.0011; G-P: t = 12.49, P_{MC} = 0.0001; J-P: t = 2.73, P_{MC} = 0.0242). *Gramella* contained only saturated FA with large amounts of C16:0 and C18:0 and also C14:0 and C15:0 (Table S2). *Jannaschia* and *Photobacterium* also contained saturated FA such as C16:0 and C18:0 but were characterized by the unsaturated C16:1ω7 and C18:1ω9 (SIMPER). Differences between both bacteria

were rather subtle with *Jannaschia* containing lots of C18:0 and *Photobacterium* having considerably more C16:0 and small amounts of C17:0 and C17:1ω7.

FA content is often used to assess food quality as PUFA (polyunsaturated fatty acids) are much more absorbed than nitrogen or carbon (Mayor et al. 2011); they are also vital FA for harpacticoids. Only a minority of marine bacteria contain PUFA (Cho & Mo 1999) and our three strains also lacked PUFA. Recent research indicates that harpacticoids are capable of PUFA synthesis through bioconversion (De Troch et al. 2012a) and do not necessarily obtain all long-chained PUFA directly from their food.

Quantitative and qualitative importance of bacterivory for harpacticoid copepods

The two copepod species differed in their bacterial carbon assimilation rate. Assimilation further depended on bacterial identity (copepod species: $p < 0.01$, treatments: $p < 0.001$, copepod species x treatment: $p < 0.05$) (Fig 1). After an incubation of 9 days, *Platychelipus* assimilated carbon in all but one food treatment (J*P), while *Delavalia* did not exhibit significant assimilation in any treatment (all $p > 0.05$) (Fig. 2). Bacterial assimilation was consistently very low (see further) and bacteria can therefore be considered a poor food source for these harpacticoids. Nevertheless, *Platychelipus* exhibited a strain-specific carbon uptake in the monotreatments, with mean carbon uptake levels decreasing from *Photobacterium* (6.35 ng C) over *Gramella* (2.88 ng C) to *Jannaschia* (1.14 ng C). In fact, only the uptake of *Photobacterium* was significantly above control values (Tukey HSD test, $p < 0.001$) (Fig. 2). Hence, bacterial uptake in our study was not proportional to bacterial abundance. For example, *Gramella* was the strain reaching by far the highest densities in our biofilms, but it was not assimilated the most. Similarly, Dahms et al. (2007) offered bacterial biofilms of unequal densities to the harpacticoid copepod *Schizopera* sp. and did not find the strongest response to the biofilm with the highest cell density. *Delavalia* also tended to utilize more *Photobacterium* than other bacteria ($0.05 < p < 0.1$) in the monotreatments, but none of the strains yielded assimilation which was significantly above background. In combination treatments with two bacterial strains, total assimilation of *Platychelipus,* calculated as the sum of mean uptake values of complementary labeled treatments (e.g. J*G and JG*), was in most cases higher than in the monobacterial treatments (Fig. 3).

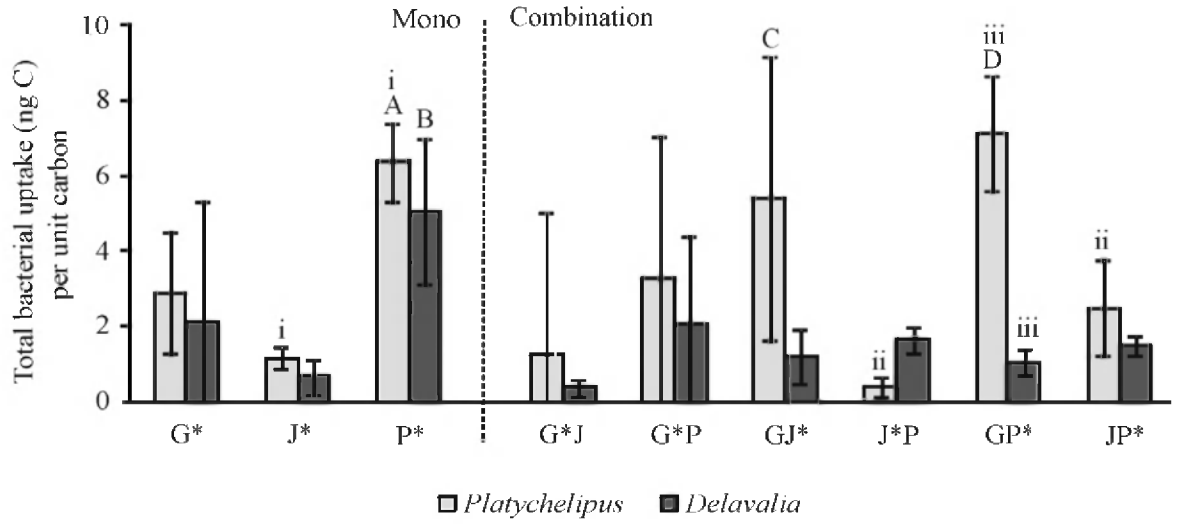

Fig. 2. Carbon uptake by *Platychelipus* and *Delavalia* in each bacterial treatment expressed as total carbon uptake per unit copepod carbon. Monotreatments included 2 monobacterial biofilms of one strain (G* = *Gramella*, J* = *Jannaschia*, P* = *Photobacterium*, with * indicating the ^{13}C labelling). Combination treatments were composed of 2 monobacterial biofilms of 2 different strains and strains were alternately labelled (GJ = *Gramella-Jannaschia,* GP = *Gramella-Photobacterium*, JP = *Jannaschia-Photobacterium*). Significant

uptake in bacterial treatments compared to the starvation treatment (A to D) and between bacterial treatments (i to iii) are indicated.

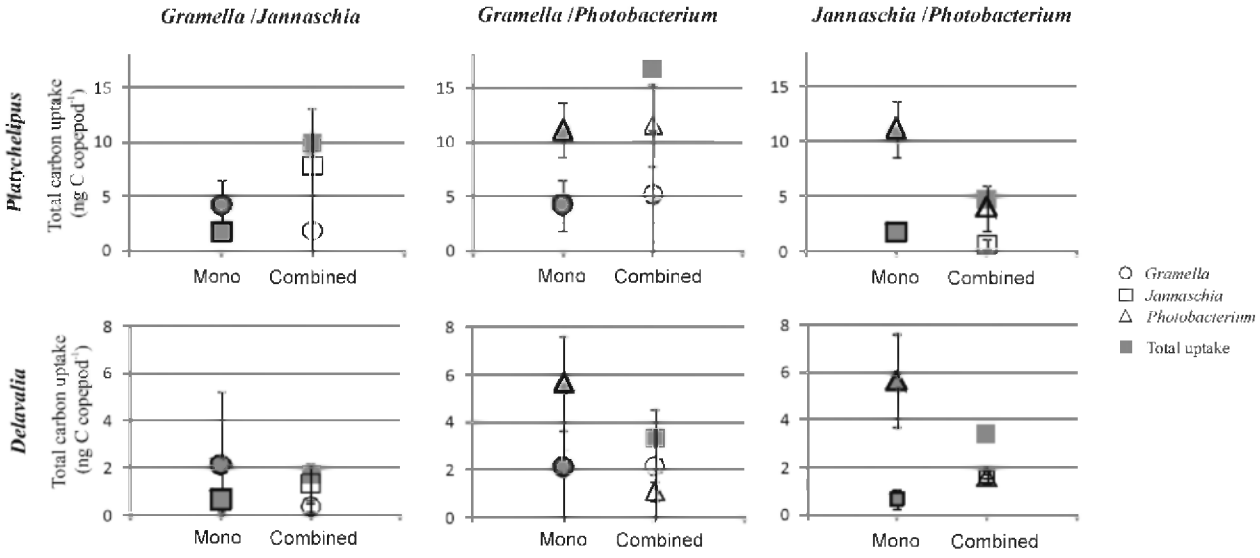

Fig. 3. Total carbon uptake values (per copepod) of each bacterial strain (symbols ○, □, Δ) by *Platychelipus* and *Delavalia* in mono versus combined treatments. Total bacterial uptake in each treatment is indicated by grey squares (■). For combination treatments, total bacterial uptake is calculated as the sum of the averaged uptake of each strain, deduced from 2 complementary treatments (X*Y and XY*).

Overall, the bacterial assimilation rates by both copepods in this study were very low, with a maximum of 16.8 ng C copepod^{-1} for *Platychelipus* and 5.7 ng C copepod^{-1} for *Delavalia*. When assuming a minimal daily carbon requirement of only 1 % of copepod body carbon (values up to 30 % have been reported by e.g. Debs 1984, Froneman 2004), the daily assimilation estimated here could only cover 0.1% of their carbon requirements. However, quantifying carbon assimilation rates in meiobenthic organisms from incubation experiments lasting several days may severely underestimate real carbon assimilation rates, because such experiments only measure incorporation resulting from anabolic processes while a major part of assimilated carbon could have served the energy requirements for catabolic processes, its fate thus being CO_2 lost through respiration (Moens et al. 1999d). Considering that *Production=Assimilation–Respiration* and given that copepod biomass remained unaltered after 9 days and no reproduction was observed (data not shown), carbon respiration more or less equalled carbon assimilation. In contrast, Drillet et al. (2011) also found low assimilation of marine bacteria by copepods, but did observe an increase in copepod adult body size, egg production and hatching success, leading the authors to suggest that bacteria play a probiotic role rather than directly contributing to copepod nutrition.

Furthermore, maximum assimilation varies depending on experimental conditions. Assimilation by *Platychelipus* and *Delavalia* in this experiment was two to three times lower than values recorded for the same harpacticoid species in Cnudde et al. (2012). The latter study differed from the present one in that (a) copepods were fed a bacterial mixture, and (b) bacteria were offered as a suspension rather than as a biofilm attached to a filter substratum. Bacterial mixtures have been shown to be more attractive to harpacticoid copepods than single bacterial strains (Dahms et al. 2007). Our previous work has also demonstrated that these copepods consume considerably higher amounts of bacteria – 5.5 and 9 times higher for *Platychelipus* and *Delavalia*, respectively – in presence of another food source such as diatoms, indicating that much of the uptake of bacteria is due to co-ingestion (Cnudde et al., 2012).

Although *Platychelipus* actually consumed bacteria (carbon incorporation), bacteria did not appear to contribute to its nutrition; on the contrary, FA and PUFA content have proportionally diminished after 9 days of feeding on bacteria. However, under starvation, *Platychelipus* FA content remained balanced (Table 1). In sharp contrast, *Delavalia* obtained elevated absolute FA and PUFA contents in all bacterial treatments as well as under starvation. Since no prominent transfer of bacterial FA to this copepod was detected and the bacterial strains lacked PUFA, this indicates that PUFA were obtained through bioconversion of assimilated short-chained bacterial FA. De Troch et al. (2012a) demonstrated by means of compound-specific FA analysis that harpacticoid copepods are able to bioconvert FA and that this process is mainly governed by the FA content of the food sources. Alternatively, protein reserves might be exploited, fatty acids being synthesized through conversion of amino acids, a pathway suggested for the nematode *Caenorhabditis elegans* (Brooks et al. 2009).

Bacterial feeding thus resulted in an opposite response in FA content for both copepod species, irrespective of the identity and nutritional content (i.e. ribosomal protein and lipid content) of the bacterial food. A Principal Co-ordinates analysis (PCO; Fig. 4a) of copepod FA composition indeed revealed a strong shift in FA that can mainly be explained by PUFA (e.g. 20:5ω3 and 22:6ω3). For *Delavalia*, the increase in PUFA is clearly shown by the separate grouping of FA samples before the start (T_0) and at the end of the 9-day experiment. *Platychelipus* samples are scattered in the PCO plot. The substantial scatter of the T_0 samples of both copepod species shows a high heterogeneity in field-collected copepod FA, which likely reflects a diet composed of an array of food sources rather than a very specialized one dominated by a single food source. Copepods may feed on PUFA-rich diatoms and dinoflagellates or on PUFA-poor food sources such as detrital organic matter, cyanobacteria and heterotrophic bacteria. Although an effect of feeding on bacteria occurred in *Platychelipus* in the form of a loss of FA, this effect does not show up in the PCO since their relative FA composition remained constant. The PCO after exclusion of the PUFA (Fig. 4b), indicated that some minor FA changes occurred, like e.g. an increase in C16:1ω7 in both copepods and decreased levels of C15:0, C17:0 and C17:1ω7 in *Delavalia*. In particular for *Platychelipus*, the comparatively high bacterial consumption in treatments P, GP and GJ (see before; Fig 3) did not result in a pronounced increase of bacteria-derived FA (fig 4a). Only for treatment P, a substantial increase of C16:0 (present in *Photobacterium*) was found but on the other hand, no elevated levels of other *Photobacterium* FA such as C16:1ω7 and C18:1ω9c were found.

The limited transfer of trophic biomarkers (^{13}C carbon and fatty acids) underlines the limited nutritional value of bacteria *sensu stricto*, as source of compounds exploited in copepod anabolism. *Sensu lato*, bacteria could be a source of vitamins or other metabolites (Yu et al. 1988, Gorospe et al. 1996, Gapasin et al. 1998); moreover, their exopolymer secretions (EPS) may be of trophic importance to copepods. Evidence for the role of the bacterial cell wall and its extracellular substances in the attractiveness of bacteria to copepods has been reported (Decho & Moriarty 1990). However, since bacterial carbon assimilation by copepods in the present experiment, where bacteria were offered as biofilms and hence cells were embedded in an EPS matrix, was low compared to our previous experiment which used a bacterial suspension (Cnudde et al., 2012), our data do not support the idea that extracellular substances are a strong driver of bacterivory. Nevertheless, EPS could have played a role in bacterial differentiation/selection since the EPS-rich *Photobacterium* (see further) was more assimilated than the other strains. Likewise, De Troch et al. (2012b) reported EPS as one of the factors governing harpacticoid selection among different diatoms, those species producing more copious EPS being favoured more.

Table 1. Copepod nutritional status: mortality and fatty acid content (total FA and PUFA) of copepods at the beginning of the experiment (T_0) and in each experimental treatment after 9 days incubation. FA and PUFA content are represented as absolute values (ng per individual) and as proportional changes compared to FA characteristics at T_0.

		MORTALITY (%)	FATTY ACID CONTENT				
			ABSOLUTE VALUES (ng ind^{-1})			Changes (%)	
			∑ FA	∑ PUFA	% PUFA	in FA	in PUFA
Platychelipus	T_0	0	73.57 (30.61)	40.15 (22.47)	55 (12)	0	0
	starved	1.5 (1.3)	73.10	45.01	62	-1	+12
	G	10.6 (5.1)	60.40	35.75	59	-18	-11
	J	11.0 (3.8)	39.87	17.03	43	-46	-58
	P	23.5 (17.3)	66.09	34.58	52	-10	-14
	GJ	2.8 (4.9) to 3.7 (3.4)	34.88	19.99	57	-53	-50
	GP	12.6 (14.0) to 12.8 (18.5)	50.22	28.13	56	-32	-30
	JP	12.5 (5.1) to 21.8 (17.9)	30.08	15.12	50	-59	-62
Delavalia	T_0	0	54.00 (13.42)	23.44 (8.22)	43 (5)	0	0
	starved	5.0 (4.3)	100.32	53.62	53	+86	+119
	G	0.8 (1.3)	94.01	59.42	63	+74	+154
	J	2.5 (0.2)	90.58	48.05	53	+78	+105
	P	25.7 (20.6)	98.93	55.66	56	+83	+138
	GJ	3.5 (3.1) to 6.8 (3.7)	104.01	61.95	60	+93	+164
	GP	4.3 (5.3) to 10.7 (2.5)	69.63	38.79	56	+29	+66
	JP	11.4 (15.2) to 14.9 (19.6)	97.96	56.59	58	+81	+141

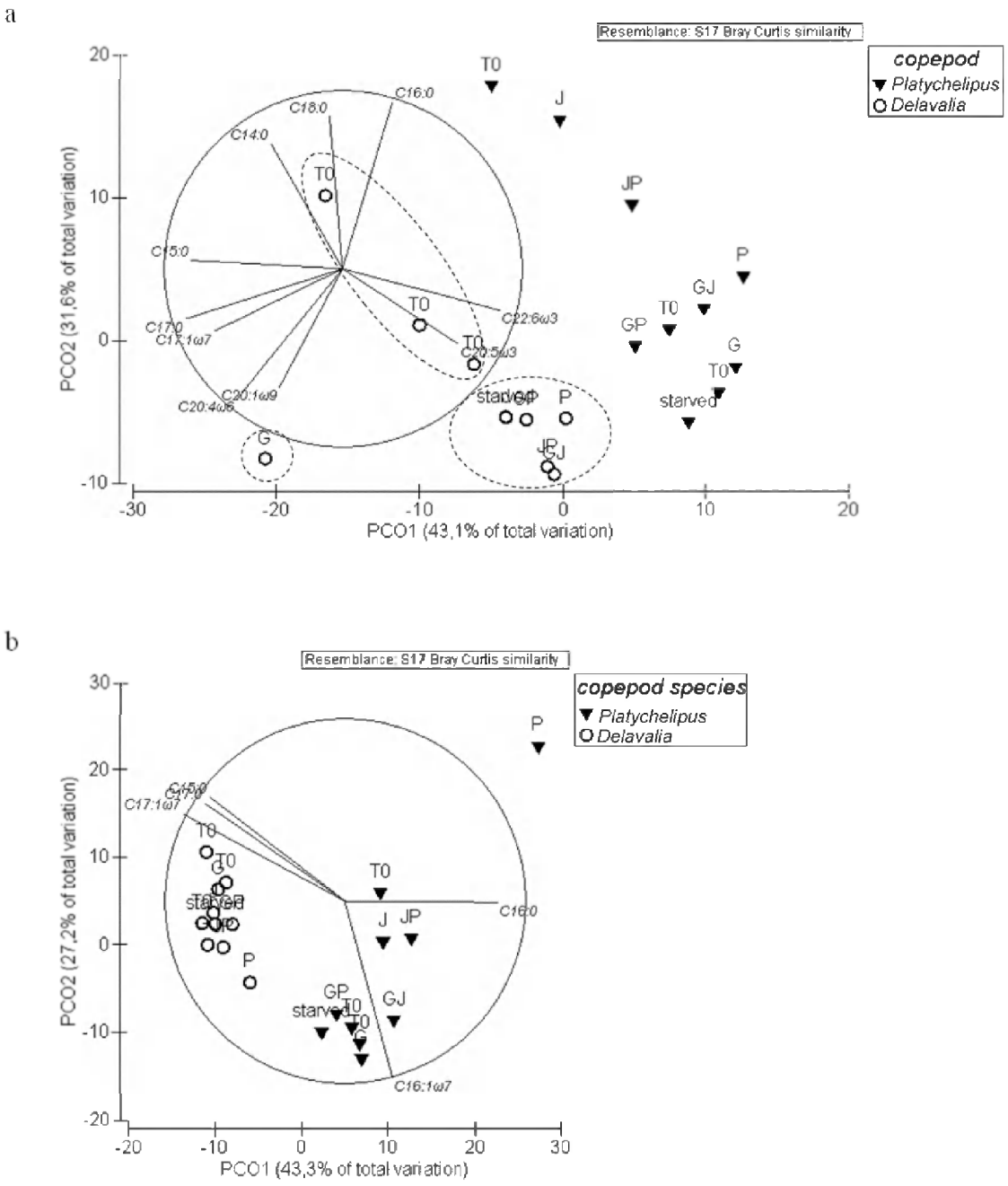

Fig. 4. Principal Coordinate (PCO) analysis of *Platychelipus* (▼) and *Delavalia* (○) FA profiles at the beginning of the experiment (T0) and after 9 days of incubation in the bacterial treatments, based on (a) relative total FA and on (b) relative copepod FA without PUFA. The vectors represent individual fatty acids with a Spearman-Rank correlation of > 70% to one of the first two PCO axes. (G = *Gramella*, J = *Jannaschia*, P = *Photobacterium*, GJ = *Gramella-Jannaschia*, GP = *Gramella-Photobacterium*, JP = *Jannaschia-Photobacterium*).

Selective feeding by harpacticoids on bacteria

No trend of differential carbon assimilation in combination treatments can be observed for *Delavalia* due to the limited bacterial uptake. For *Platychelipus* however, differences in assimilation rates were found between combination treatments, but they were difficult to interpret. *Photobacterium*, for instance, was the most assimilated strain among monotreatments, but in combination treatments it was only

significantly consumed when in the presence of *Gramella* (GP treatment; Tukey HSD test, $p < 0.001$) but not in combination with *Jannaschia*. On the other hand, *Jannaschia* was not significantly assimilated by *Platychelipus* in monotreatments, but it was when offered together with *Gramella* (treatment GJ*, $p < 0.05$) but not when offered with *Photobacterium* (J*P treatment). Carbon assimilation of copepods feeding on *Gramella* was consistently low and never significantly above control (treatments G*J and G*P, $p > 0.05$). Hence, assimilation of the three strains in combination treatments was not necessarily a reflection of the preferential assimilation of *Platychelipus* as found in the monotreatments, yielding an equivocal conclusion about whether or not bacterial grazing by these two copepod species is selective: no strong selection for a bacterial strain was observed when copepods were given a direct choice, although both copepod species tended to consume more *Photobacterium*.

All treatments contained two food patches of the same size and thus encounter probability for any given strain was twice as high in the monobacterial treatments than in the combination treatments, since the former contained two patches of the same strain per microcosm versus one in the latter. This could have affected the comparison of food uptake in mono- vs combination treatments, especially for the less motile, non-swimming copepod *Platychelipus*. This copepod tends to stay close to the spot of inoculation, so its chance of feeding on, for instance, *Jannaschia* would be twice as high in the monotreatment than in the combination treatments. However, *Platychelipus* consumed three times more *Jannaschia* in the combination treatment '*Gramella-Jannaschia*' than in the monotreatment. Also, uptake of *Photobacterium* in one combination treatment ('*Jannaschia-Photobacterium*') equalled uptake in the monotreatment with *Photobacterium*, suggesting that this factor did not bias our results.

So far, any selectivity by harpacticoids towards bacteria was mostly inferred from behavioural responses to substrates such as algae and sediment (Ravenel & Thistle 1981, Hicks & Coull 1983) or to pure bacterial treatments (Rieper 1982, Dahms et al. 2007), but the underlying mechanism of selection has not been properly addressed. Rieper (1982) noticed a selection against a Gram-positive bacterium, but this cell wall characteristic was not considered important by Dahms et al. (2007). The latter linked selective colonization to demographic performance in the harpacticoid *Schizopera*. They showed that copepods did not select for the bacteria delivering the most benefits: a clear behavioral preference of copepods for a natural mixed-species biofilm compared to monobacterial films did not translate into a better demographic performance on the former. A similar phenomenon has also been observed in bacterial-feeding nematodes (Salinas et al. 2007).

In our study, the considerable difference in assimilation by *Platychelipus* between *Jannaschia* and *Photobacterium*, two strains with fairly similar FA, further suggests that FA content of bacteria, located in the cell membranes, is not driving copepod selectivity. Moreover, while *Photobacterium* was the best assimilated bacterial strain, copepod survival in this treatment was comparatively poor (see further). Copepod selective feeding (or the lack thereof) could also be affected by a difference in bacterial cell abundance between strains, but the best assimilated strain in our study (*Photobacterium*) had nearly 20 times lower cell densities than the secondbest strain (*Gramella*). Selective feeding on bacteria may further be affected by strain-specific EPS production. *Jannaschia* is a slow-growing bacterium and formed many circular, minute colonies, resulting in high cell abundances but probably low EPS. *Photobacterium* can cover the surface of an agar plate very fast and produces copious extracellular metabolites (Cnudde C., pers. obs.). Bonet et al.(1994) reported that *P. damselae* subsp. *piscicida* produced capsular polysaccharides especially when grown in a glucose-enriched medium. It is plausible that in our experiment the water-soluble fraction of the EPS did not remain restricted to the patch where it was produced but spread out over the entire microcosm, thereby stimulating copepod feeding activity in the other bacterial patch in combination treatments, and thus potentially clouding the preferences observed in the monospecific treatments. Mixing between the two different bacterial patches over a 9-day incubation, and particularly of secreted metabolites, could have occurred and could have interfered with efficient chemosensory detection, an important mechanism for copepods to discriminate between food sources (Fechter et al. 2004). Finally, although the bacteria used in the present study covered a wide range

of phylogeny and biochemical characteristics, it is possible that the three strains did not include any suitable strain for these copepod species, thus providing a biased picture on the importance of bacterivory.

Copepod species-specific responses

A pronounced copepod species-specific pattern was found for bacterial feeding in general (1) with *Platychelipus* consuming more of the bacterial food source than *Delavalia,* (2) *Platychelipus* showing a differential uptake of the strains, and (3) bacterial feeding differentially influencing FA content of these two copepod species. The first point confirms our previous results (Cnudde et al. 2012). Copepod mortality was not different between both copepod species (copepod species: $p > 0.05$, treatment: $p < 0.05$, copepod species x treatment: $p > 0.05$). For both copepods elevated mortalities were found in the monotreatment and combination treatments with *Photobacterium* (23.5 ± 17.3% and 25.7 ± 20.6%, $p < 0.05$). *Photobacterium* species are generally encountered on the exoskeleton or in the intestinal tract of copepods (Heidelberg et al. 2002). It has hydrolytic activity towards chitobiose (Honda et al. 2011), which could have contributed to copepod mortality. In addition, *Platychelipus* tend to have a higher mortality in all bacterial treatments compared to the starvation treatment, in contrast to *Delavalia*, where bacterial feeding resulted in slightly lower mortalities (apart from treatments containing *Photobacterium*) than in complete absence of food. The generally better survival of *Delavalia* may be related to its tube-dwelling ecology (Nehring 1993): this genus lives in close vicinity to mucous tube walls on which microbial films can flourish and is used to being exposed to high bacterial densities. Under starvation, both copepod species maintained their FA, suggesting that harpacticoids are able to protect their FA content under food limitation. In *Delavalia*, FA seemed to be even actively produced (PUFA biosynthesis). On the other hand, *Platychelipus* did not manage to maintain its nutritional status (reduction in FA and PUFA) when feeding on bacteria, which in turn is reflected in its elevated mortality. This leads to the conclusion that harpacticoid copepods exhibited a different strategy in energy budgeting as a response to the lack of food or of food of suitable nutritional quality: *Delavalia* invested little energy in consumption of the poor-quality bacterial food but instead allocated its energy to extensive FA-production. *Platychelipus* spent more energy on keeping up its grazing activity, at the expense of its FA reserves and survival, the 'cost of grazing' (Mayzaud 1973, Kiørboe et al. 1985) then exceeding the acquired energy of this poor-quality food. Since *Delavalia* can maintain its nutritional status, it is more flexible towards episodes of food quality stress. Different survival strategies within one copepod community may promote niche segregation and thus co-occurrence and finally a higher diversity.

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