Trophic Ecology of Intertidal Harpacticoid Copepods, with Emphasis on their Interactions with Bacteria

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Trophic Ecology of Intertidal Harpacticoid Copepods, with Emphasis on their Interactions with Bacteria

Trofische ecologie van intertidale harpacticoide copepoden, met de nadruk op hun interacties met bacteriën

Clio Cnudde

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SUMMARY

In the era of rapidly changing natural environments, there is an urgent need for understanding biodiversity-ecosystem functioning. Trophic interactions play a key role in structuring the ecosystem. In marine systems, the feeding ecology of the larger-sized species i.e. (top) predators of food webs, is fairly well documented, yet at the basal level of food webs, the complexity of grazer interactions is much higher and trophic linkages are potentially still missing. In this PhD thesis, the focus is on trophic interactions between harpacticoid copepods and bacteria.

The meiofauna (organism size between 38 µm and 1 mm), and in particular harpacticoid copepods (the second most abundant group of the meiofauna, after nematodes), transfer microphytobenthos to higher trophic levels, predominantly larval and juvenile fish. Harpacticoids have highly complex feeding habits. They are able to consume a wide range of food sources, e.g. microalgae, cyanobacteria, flagellates, ciliates, mucoid substances, fungi and heterotrophic bacteria. They are therefore sometimes called 'indiscriminate feeders'. However, laboratory feeding experiments and recently obtained *in situ* data, demonstrate their highly selective behaviour and their species-specific food preferences. Under experimental conditions, harpacticoids discriminated among substrates with poor- versus well-developed bacterial films although copepods are considered to be morphologically incapable of consuming small-sized particles such as bacteria. Based on their potential interaction with a broad range of food sources, the general importance of their herbivorous feeding strategy is being questioned and the recent development of dietary tracers such as stable isotopes and fatty acid analysis, now allows to investigate direct assimilation of bacterial matter or that of other food sources. Interest into the pathways of transfer of benthic bacterial matter upwards the food web, for instance with harpacticoids as intermediates, is due to the lack of insight into the general fate of the extensive benthic bacterial biomass and potential pathways of transfer to higher trophic levels are still unknown. A proportion of energy flows back to the microbial-detrital pool as copepod fecal pellets which are efficiently degraded and remineralized. Beside the upward fluxes of biomass and energy, the degradation and remineriliza tion of waste products resulting from grazing activity is crucial for the recycling of biomass and thus the efficiency of ecosystem functioning.

We gained insight into food utilization by harpacticoids in a heterogeneous estuarine intertidal ecosystem, into species-specific feeding patterns and into the trophic importance of bacteria for harpacticoids. Feeding ecology was studied based on both field data of the harpacticoid species assemblages and on experimental laboratory data using individual species. The process of fecal pellet degradation was investigated with focus on the contribution of internal and external fecal pellet bacteria during early degradation and remineralisation of fecal pellets. In an attempt to gain information on the presence of internal bacteria and their position in the fecal pellet, Atomic Force Microscopy and Laser Scanning Confocal Microscopy (AFM-LSCM), a high-resolution imaging tool, was applied. It offers new prospects for studying microbial degradation of copepod fecal pellets.

Harpacticoid assemblages for chapters 2 and 3 were studied at the Paulina intertidal area (Schelde estuary, The Netherlands) encompassing a gradient from tidal flat to salt marsh, from seasonal periods at five stations differing in sediment characteristics, tidal exposure, presence of vegetation, etc.
To address the structural role of environmental factors, including abiotic sediment characteristics and food-related factor, for harpacticoid assemblages and disclose resource partitioning within harpacticoid assemblages, **chapter 2** presents a field study into spatial-temporal heterogeneity in resource availability and in intertidal copepod assemblage structure (density, diversity and composition). An in-depth analysis of the most influential factors for species distribution added relevant information to the autoecology of intertidal harpacticoid species. Spatio-temporal harpacticoid assemblage variation was assigned to variables relating to total organic matter, microphytobenthic biofilms (characterised by pigments and their degradation products), differences in detrital origin and NH_4^+, suggesting a primary influence of food

availability and quality. Nevertheless, harpacticoid assemblages of tidal flats were seemingly more structured by abiotic factors (granulometry and tidal exposure) and especially the harpacticoid assemblage of the sand flat (station H2, species *Paraleptastacus spinicauda*, *Asellopsis intermedia*) which was highly station-specific and constant over time. Assemblages of salt marsh stations are considerably similar in copepod family composition despite of differences in salt marsh granulometry, suggesting a primary influence of food availability and food quality. Variability in *Microarthridion littorale* abundances is related to microphytobenthos biomass. For Ectinosomatidae and *Tachidius dis*cipes, the limited number and low correlations for all biotic and abiotic factors indicates a generalistic occurrence. For some species, linkages between habitat characteristics and species distributions were little decisive (e.g. *Platychelipus littoralis*, *Paronychocamptus nanus*, *Amphiascus* sp. 1). Overall, high intercorrelation between a broad range of environmental factors hinders us to draw strong conclusions about the main regulating factors for harpacticoids distribution.

Analysis of spatio-temporal variability in resource utilization by harpacticoid copepods, by means of copepod carbon isotopic profiles and fatty acid profiles (**chapter 3**), proved that the majority of intertidal harpacticoid species relied strongly on microphytobenthos (MPB) as a food source, with potentially fine-scaled selectivity among diatom species or other MPB components. In addition, harpacticoids spanned two trophic levels suggesting also an indirect pathway of MPB transfer to harpacticoids. Furthermore, intertidal harpacticoids showed dietary differences and species-specific spatio-temporal variability in food utilization with contributions of suspended particulate organic matter (*Paronychocamptus nanus*, *Amphiascus* sp. 1, *Microartridion littorale*), flagellates (*M. littorale*), and bacteria (*Delavalia palustris*) but not of *Spartina* detritus. Resource partitioning by harpacticoid assemblages occurred in all stations but was especially clear in the sand flat, comprising a diatom feeder (*Asellopsis intermedia*), a diatom feeder with temporal reliance on dinoflagellates (*Tachidius discipes*) and a bacterial feeder (*Paraleptastacus spinicauda*). In the muddy salt marsh, a trophic role of bacteria was found for *Delavalia palustris* and Cletodidae, the latter being unique by the utilization of a chemoautotrophic food source.
In **chapter 4,** results from a microcosm feeding experiment using ^{13}C-labelled bacteria, showed that bacterial feeding is linked to diatom grazing and that overall assimilation of bacterial carbon is low for all tested harpacticoid species. In contrast to the bacterivorous copepod *Delavalia palustris,* non-bacterivorous harpacticoid species (*Nannopus palustris*, *Microarthridion littorale*, *Platychelipus littoralis*) responded negatively on bacterial feeding, as deduced from their mortality and PUFA (polyunsaturated fatty acid) impoverishments. These findings suggest that bacterial biomass may complement feeding on MPB and that an exclusive bacterial diet does not meet copepod nutritional requirements. *Delavalia palustris* was able to biosynthesized PUFA from a bacterial diet but generally, bacteria represent a minor and low-quality food for these intertidal harpacticoid copepods.

In a food-patch choice experiment with ^{13}C-labelled bacterial biofilms (**chapter 5**), the ability of the harpacticoids *Platychelipus littoralis* and *Delavalia palustris* to select between bacterial species with potential different nutritional value (*Gramella* sp., *Jannaschia* sp. and *Photobacterium* sp.) was investigated. In line with chapter 4, bacteria carbon assimilation was low and significant bacterial fatty acid transfer was lacking. A low degree of selectivity was found (preference for *Photobacterium* sp.), and extracellular metabolites rather than biochemical content and bacterial densities are suggested to be the driver of selective feeding behaviour towards bacteria. Furthermore, the energetic cost of differential bacterivory resulted in a negative fatty acid balance for *P. littoralis* while *D. palustris* showed an improved fatty acid profile and thus a positive response to the low-quality bacterial food (similar as in chapter 4).

As the ingested bacterial biomass is of limited use for the majority of harpacticoid species, the largest fraction of ingested bacteria returns to the microbial-detrital food web in the form of fecal pellets. Chapter 6 and 7 demonstrate that these 'internal' fecal pellet bacteria are viable cells with high densities and represent a diverse active community able to significantly participate in fecal pellet degradation and overall recycling of carbon for the grazer food web.

In **chapter 6**, molecular (RNA-based PCR-DGGE) and metabolic profiling (Biolog Ecoplate assay) of freshly egested copepod fecal pellets proved the general presence of internal active bacteria with a broad metabolic potential in fecal pellets of different copepod species and with different fecal pellet content. The strong impact of the food source on the bacterial diversity of the fecal pellet, indicates the direct transfer of ingested food bacteria to the fecal pellets. Furthermore, the colonization of fecal pellets by bacteria from the surrounding water was relatively low. Consequently, internal bacteria diversity was not replaced by a new external bacterial assemblage. About half of the internal fecal pellet bacteria persisted after 60 h of fecal pellet degradation and, hence, internal fecal pellet bacteria deliver a significant contribution to fecal pellet recycling with *Vibrio* sp. as a potential important participant. These findings refute the hypothesis of high bacterial fp colonization, as observed for planktonic fecal pellets.

AFM-LSCM (Atomic Force Microscopy - Laser Scanning Confocal Microscopy, **chapter 7**) imaging confirmed the presence of high densities of viable bacteria packed inside the fecal pellet. Furthermore, AFM—LSCM revealed the fibrillar network structure of the peritrophic membrane from a *Paramphiascella fulvofasciata* fecal pellet, similar to marine polysaccharides and α-chitin and allowed precise measurement of the membrane thickness (0.7-5.9 nm) and bacterial cell volumes (range 0.006-0.117 μm^3, in liquid). This protocol enables high-resolution interrogation of biochemical structural changes and bacterial dynamics within the copepod fecal pellets and other heterogeneous particles such as marine snow under environmental conditions. AFM-LSCM generally allows studying bacterial cell size, cell shape and cell-cell interactions. Here it was applied (1) to visualize the ultra-structure of the peritrophic membrane and (2) to locate and quantify bacterial presence (cell size measurements) both inside and outside the fecal pellet.

In conclusion, it is clear that the majority of intertidal harpacticoid species primarily relies on MPB, in particular diatoms, and their diet can include small contributions of other food sources such as suspended particulate organic matter, protozoa and bacteria. Despite indications that food availability and MPB shape harpacticoid assemblages on the spatio-temporal scale, the 'real' importance of MPB, other food-related factors or physical habitat factors for structuring harpacticoid assemblages remains unclear. Furthermore, bacteria are a nutritional food source for some harpacticoid taxa but overall, transfer of bacterial biomass to harpacticoids seems rather limited and in the intertidal microbial food web, bacteria remain a sink. Although harpacticoids consume predominantly substrate-attached bacteria and often co-incidentally during grazing on a primary food source, there are indications that bacterivorous harpacticoids have special adaptations for consuming a poor-quality food source. Selective feeders *Paraleptastacus spinicauda* and *Cletodidae* proved, the latter consuming chemoautotrophic bacteria, proves harpacticoids ability to discriminate for bacteria and for bacterial groups but more fine-scale selectivity for bacterial species or for bacterial nutritional content, remains unclear.
Although bacterial biomass is (passively) ingested by harpacticoids, the majority will be channeled back to the microbial-detrital food web in the form of fecal pellets. As a consequence of the relative higher contribution of 'internal' fecal pellet bacteria compared to external bacteria, the process of microbial degradation of benthic fecal pellets deviates from degradation of planktonic fecal pellets. This may imply that the functioning of the benthic microbial-detrital loop is not necessarily similar to the plantonic microbial-detrital loop.

SAMENVATTING

In het huidige tijdperk waarin natuurlijke systemen sterk onderhevig zijn aan veranderingen, heerst er hoge nood aan goede kennis van de koppeling tussen biodiversiteit en ecosysteem functionering. Trophische interacties spelen een voorname rol in het structureren van een ecosysteem. In mariene systemen, is de voedingsecologie van de grotere organismen, met name de (top)predatoren van het voedselweb, goed bescheven. De complexiteit van grazerinteracties in de onderste niveaus van voedselwebben zijn veel complexer en een aantal trofische linken is vermoedelijk nog onbekend. Deze thesis legt zich toe op de trofische interacties tussen harpacticoide copepoden en bacteriën.

Meiofauna (organismen van 38 µm tot 1 mm groot) en in het bijzonder harpacticoide copepoden, de tweede meest abundante meiofauna groep naast nematoden (rondwormen), transfereren microphytobenthos (MPB) naar de hogere trofische levels, voornamelijk juveniele vissen. Harpacticoiden bezitten complexe voedingsgewoonten. Ze zijn in staat om een brede range aan voedselbronnen te gebruiken, bv. microalgen, cyanobacteriën, flagellaten, ciliaten, muceuze substanties, fungi en heterotrofe bacteriën, en worden daarom vaak beschouw als niet-kieskeurige grazers. Gegevens uit laboratoriumvoedingsexperimenten en recente veldgegevens tonen echter aan dat harpacticoiden heel selectief gedrag vertonen en dat hun voedselvoorkeur soortsspecifiek is. In experimentele condities, discrimineerden harpacticoiden tussen substraten met arme en deze met rijke bacteriële films en dit terwijl ze geacht worden morfologisch niet in staat te zijn om kleine particles zoals bacteriën, te benutten. Gebaseerd op het gegeven dat harpacticoiden interageren met een brede range aan voedselbronnen, wordt hun herbivore voedingsstategie in twijfel gesteld. Door de recente ontwkkeling van dieettracers, ondermeer stabiele isotopen en vetzuren, is het nu mogelijk om assimilatie van bacteriën of andere voedselbronnen op te volgen. De belangstelling voor de specifieke pathways waarlangs bentische bacteriële materie wordt doorgesluisd, hogerop in het voedselweb, bijvoorbeeld via harpacticoide copeopden, is het gevolg van onwetendheid over wat nu het lot is van deze benthische bacteriële biomassa en deze pathways zijn nog slecht gekend. Een fractie van de energie vloeit terug naar het microbiële en detritusvoedselweb in de vorm van fecal pellets welke vervolgens efficiënt worden afgebroken en geremineraliseerd. Evenzeer als de opwaartse tropfische fluxen in het grazersvoedselweb, zijn de efficiënte afbraak en recyclage van restmateriaal, afkomstig uit het grazersvoedselweb, belangrijk voor het goed functioneren van het ecosysteem.

In deze thesis, verwierven we inzicht in het voedselgebruik van harpacticoiden in een heterogeen, estuarine, intertidal ecosysteem, in de soortsspecifieke voedingspatronen en in het trofische belang van bacteriën voor harpacticoiden. Hun voedingsecologie werd bestudeerd op basis van veldgegevens van harpacticoide soortengemeenschappen en van laboratorium-experimentele data van individueel getestte soorten. Degradatie van fecale pellets werd onderzocht met nadruk op de rol van interne en externe fecale pelletbacteriën tijdens het vroege degradatie- en remineralisatieproces. Informatie omtrent de aanwezigheid van interne bacteriën en hun positie in de fecale pellet, werd bekomen met Atomic Force Microscopy - Laser Scanning Confocal Microscopy (AFM-LSCM). Dit is een visualisatie techniek met hoge resolutie en deze biedt goede perspectieven voor het verdere onderzoek naar microbiele degradatie van fecale pellets.

De bestudeerde harpacticoide gemeenschappen van hoofdstukken 2 en 3, zijn afkomstig uit het Paulina intertidale gebied (Schelde-estuarium) welke een habitatgradiënt bevat van intertidale platen naar een sterk begroeid schorregebied. De gemeenschappen werden éénmalig bemonsterd in elk van de vier seizoenen en op vijf staalname punten (stations) welke van elkaar verschillen inzake fysische sedimentkenmerken (zoals korrelgrootte), de duur van getij-blootstelling, de aanwezigheid van vegetatie, enz.

Voor de studie van de structurerende rol van omgevingsfactoren, inclusief abiotische sedimentkenmerken en voedselgerelateerde factoren, op harpacticoide gemeenschappen en het onderzoek naar de voedselverdeling ('resource partitioning') binnen deze harpacticoide gemeenschappen, werd een veldstudie verricht in **hoofdstuk 2** naar de spatio-temporele heterogeniteit in voedselbeschikbaarheid en in de harpacticoide gemeenschapsstructuur (densiteit, diversiteit en samenstelling). Een grondige analyse van de meest beïnvloedende factoren voor de verspreiding van soorten bracht relevante informatie op omtrent de auto-ecologie van harpacticoide soorten. De spatio-temporele variatie in harpacticoidengemeenschappen werd toegeschreven aan factoren die verband houden met totaal organisch materiaal, MPB biofilms (gekenmerkt door pigmenten en bijhorende degradatieproducten), verschillen in de origine van detritus en ammoniumconcentraties. Dit duidt op een voornaam belang van voedselbeschikbaarheid en -kwaliteit. Toch werden harpacticoidengemeenschappen van de intertidale platen ogenschijnlijk meer gestructureerd door abiotische factoren (granulometrie en getij-blootstelling) en dit geldt voornamelijk voor de harpacticoide gemeenschap uit de zandplaat (station H2, soorten *Paraleptastacus spinicauda*, *Asellopsis intermedia*) welke heel specifiek was voor dit station en constant over de tijd. Gemeenschappen van het schorregebied zijn behoorlijk gelijkaardig in samenstelling van copepoden families, ondankts verschillen in de granulometrie van deze stations. Dit wijst op een primair belang van voedselbeschikaarheid en –kwaliteit. Variatie in de abundanties van *Microarthridion littorale* hielden verband met de MPB biomassa. Voor Ectinosomatidae and *Tachidius dis*cipes, de weinge aantallen en lage correlaties met biotiosche en voedsel-gerelateerde factoren geven aan dat deze een eerder algemeen voorkomen hebben. Voor enkele soorten, kon er weinig afgeleid worden uit de habitatkenmerken en de soorten distributies (vb. *Platychelipus littoralis*, *Paronychocamptus nanus*, *Amphiascus* sp. 1). Als gevolg van hoge intercorrelaties tussen een brede waaier aan omgevingsfactoren, was het niet mogelijk om sterke conclusies te trekken omtrent de bepalende factoren van harpacticoide verspreiding.

Analyse van spatio-temporele variatie in het voedselgebruik van harpacticoide copepoden, door middel van hun koolstofisotopensignalen en vetzuur profielen (**hoofdstuk 3**), gaf aan dat de meerderheid van de intertidale harpacticoide soorten sterk afhankelijk was van MPB als voedselbron, met mogelijks een fijne selectiviteit voor diatomeeënsoorten of andere MPB componenten. Daarnaast overbruggen harpacticoiden twee trofische niveaus, wat erop wijst dat MPB ook langs een indirecte weg naar harpacticoiden wordt getransfereerd. Bovendien vertoonden harpacticoiden verschillen in hun diet en een soortsspecifieke spatio-temporele variabiliteit in voedelgebruik, met daarin een aandeel gesuspendeerd particulair organisch materiaal (*Paronychocamptus nanus*, *Amphiascus* sp. 1, *Microartridion littorale*), flagellaten (*M. littorale*), en bacteriën (*Delavalia palustris*) maar geen noemenswaardig aandeel van S*partina*-detritus. Voedselverdeling (resource partitioning) binnen de harpacticoide gemeenschappen was aanwezig in alle stations maar was in het bijzonder heel duidelijk voor de zandplaat. De zandplaat bevatte een diatomeeëngrazer (*Asellopsis intermedia*), een diatomeeëngrazer welke zich temporeel voedt met dinoflagellaten (*Tachidius discipes*) en een bacteriegrazer (*Paraleptastacus spinicauda*). In het slibbige schorregebied werd het trofisch belang van bacteriën aangetoond voor *Delavalia palustris* en de familie Cletodidae. Cletodidae zijn uniek vanwege het gebruik van chemoautotroof voedsel.

In **hoofdstuk 4**, toonden de resultaten van een microcosmvoedingsexperiment met ^{13}C-gelabelled bacteriën aan dat bacteriële opname gelinkt is met het grazen op diatomeeën en dat assimilatie van bacteriële koolstof laag is voor alle getestte copepodensoorten. In tegenstelling tot de bacterivore copepode *Delavalia palustris*, werd bij niet-bacterivore copepodensoorten (*Nannopus palustris*, *Microarthridion littorale*, *Platychelipus littoralis*) een negatief respons op het begrazen op bacteriën waargenomen, met name een verhoogde mortaliteit en een sterke verarming van de copepoden in hun PUFAs (polyunsaturated fatty acid). Deze bevindingen tonen aan dat de opname van bacteriële biomassa complementair kan zijn met een MPB dieet en dat voor de meeste copepodensoorten een exclusief bacterieel dieet niet voldoet aan hun nutritionele vereisten. *Delavalia palustris* vertoonde PUFA-

biosynthese bij een puur bacterieel diet, maar in het algemeen, voor harpacticoiden zij bacteriën een voedselbron van lage nutritionele waarde .

Met een 'food-patch choice' experiment met ^{13}C-gelabelled bacteriële biofilms (**hoofdstuk 5**), werd onderzocht of harpacticoiden *Platychelipus littoralis* and *Delavalia palustris* selectief gedrag vertonen naar bacteriële soorten (*Gramella* sp., *Jannaschia* sp. and *Photobacterium* sp.) met een mogelijks verschillende nutritionele waarde. Gelijkaardig aan hoofdstuk 4, was de assimilatie van bacteriële koolstof laag en vond er geen significante overdracht van bacteriële vetzuren plaats. Selectiviteit werd in een beperkte mate waargenomen (met voorkeur voor *Photobacterium* sp.). Als oorzaak van het selectieve voedingsgedrag werd vooropgesteld de aanwezigheid van extracellulaire metabolieten eerder dan de biochemische inhoud van de bacteriesoorten en bacteriële densiteit. De energetische kost van het selectieve voedingsgedrag was als dusdanig hoog dat dit leidde tot een negatieve vetzuurbalans for *P. littoralis.* In tegenstelliing, het vetzuurprofiel van *D. palustris* verbeterde en hier deed zich een positieve respons voor op deze laag-kwalitatieve voedselbron (similar as in chapter 4).

Aangezien de opgenomen bacteriële biomassa van weinig trofisch belang is voor de meerderheid van de harpacticoide soorten, wordt een grote fractie van de opgegeten bacteriën terug naar het microbiële-detritus voedselweb gesluist in de vorm van fecale pellets. Hoofdstukken 6 en 7 geven weer dat deze 'interne' fecale pelletbacteriën levensvatbare cellen zijn welke in hoge densiteiten teruggevonden werden. Deze fecale pellet bacteriën zijn dus een diverse, actieve gemeenschap welke significant kan bijdragen aan de degradatie van de fecale pellet en algemeen aan de koolstofrecyclage van het grazersvoedselweb.
Aan de hand van moleculaire (PCR-DGGE gebaseerd op RNA) en metabolische fingerprints (Biolog Ecoplate assay) van versgeproduceerde fecale pellets, werd in **hoofdstuk 6** het algemeen voorkomen van 'interne' actieve bacteriën met een breed metabolisch potentieel aangetoond, dit voor fecale pellets van verschillende copepodensoorten en met een verschillende samenstelling. De voedselbron had een sterke invloed op de bacteriële diversiteit van de fecale pellet wat impliceert dat opgenomen bacteriën direct naar de fecale pellets worden doorgevoerd. Bovendien was de kolonisatie van de fecale pellet door bacteriën uit het omgevende zeewater laag. Bijgevolg hield de interne bacteriële diversiteit goed stand en werd deze dus niet vervangen door de externe, coloniserende gemeenschap. Ongeveer de helft van de interne fecale pelletbacteriën persisteerden tot aan 60 h degradatie. Aldus leveren de interne bacteriën een significante bijdrage aan de recyclage, met *Vibrio* sp. als één van de belangrijke deelnemers. Onze bevindingen spreken het phenomeen van sterke bacteriële kolonisatie, zoals dit voor planktonische fecale pellets werd beschreven, tegen.

Visualisatie van de fecale pellet met AFM-LSCM (**hoofdstuk 7**) bevestigde de aanwezigheid van hoge densiteiten aan levensvatbare bacteriecellen in de fecale pellet. Met AFM—LSCM kon bovendien de fibrillaire netwerkstructuur van het peritrofe membraan van een fecale pellet van *Paramphiascella fulvofasciata* worden weergegeven. Dit netwerk lijkte sprekend op marine polysacchariden en α-chitine. AFM-LSCM liet toe om membraandikte precies te meten (0.7-5.9 nm) and ook de bacteriële celvolumes te bepalen (range 0.006-0.117 μm^3, in vloeistof). Dit protocol laat toe om op hoge resolutie de structurele biochemische veranderingen en bacteriële dynamiek in de fecale pellet na te gaan en is ook een tool die voor andere heterogene partikels zoals 'marine snow' onder omgevingscondities kan worden toegepast. AFM-LSCM laat ook toe om bacteriële celgrootte, celvorm en cel-celinteracties te bekijken. In dit werk, werd deze techniek gebruikt om (1) de ultrastructure van het peritrophe membraan te visualiseren en (2) de locatie en kwantiteit aan bacteriën (celmetingen) aan de buiten-en binnenzijde van de fecale pellet te bestuderen.

We kunnen besluiten, dat het merendeel van de intertidale harpacticoide copepoden afhankelijk is van MPB, voornamelijk diatomeeën, en dat in hun dieet ook kleine hoeveelheden van andere bronnen vervat zit zoals gesuspendeed particulair organisch materiaal, protozoa en bacteriën. Ondanks indicatie dat voedselbeschikbaarheid en MPB de compositie van harpacticoidengemeenschappen bepaald, blijft het onzeker wat het reeële belang van MPB, andere voedselgerelateerde factoren en de physische habitat factoren is voor de spatio-temporele heterogeniteit van harpacticoidengemeenschappen.

Bacteriën hebben een trofische waarde voor enkele harpacticoide taxa maar algemeen gezien, blijft de transfer van bacteriële biomassa naar harpacticoiden beperkt. Zodoende blijven in het intertidale microbiële voedselweb bacteriën een sink. Harpacticoide copepoden consumeren in hoofdzaak aan-substraat-vastgehechte bacterien en dit tijdens het grazen op een andere primaire voedselbron. Er zijn indicaties dat bacterivore harpacticoiden speciale adaptaties hebben die hun toelaten een laag-kwalitatieve voedselbron te bebruiken. Selectieve voeders *Paraleptastacus spinicauda* and *Cletodidae*, deze laatste zich voedend met chemoautotrofe bacteriën, tonen aan dat harpacticoiden kunnen discrimineren voor bacteriën en zelf voor een bepaalde bacteriële groep. Of deze selectiviteit ook tot op het bacterie-soortsniveau gaat blijft onduidelijk. Van de bacteriële biomassa dat (passief) wordt opgenomen door veel copepoden, zal de meerderheid terugvloeien naar het microbiële-detritus voedselweb in de vorm van fecale pellets. Als gevolg van de relatief hoge bijdrage van interne fecale pelletbacteriën ten op zichte van de externe bacteriën, wijkt het proces van microbiële degradatie van benthisch fecale pellets af van deze in het planktonische ecosysteem. Dit kan erop duiden dat het algemeen functioneren van het benthische microbiële-detritus voedselweb toch niet zo gelijkaardig as aan het planktonische microbiële-detritus voedselweb, als vaak wordt gesteld.

Chapter 1

General introduction

Consumer-food source interactions among different organizational levels are one of the most fundamental processes in ecosystems. Trophic interactions represent fluxes of energy throughout the system, from autotrophs to a series of heterotrophs. In addition, trophic interactions play a structuring role in local diversity and composition of species assemblages. As trophic interactions contribute to both biodiversity and ecosystem functioning (Worm et al. 2002), food-web studies are a valuable research field to unravel biodiversity-ecosystem function (BEF) relationships (Loreau et al. 2002). During the last two decades, global awareness of species extinction and of natural habitat losses resulting from socio-economic development (Loreau et al. 2002), e.g. the problem of overfishing and of trawling activity (Polymenakou et al. 2005), has grown far beyond the scientific research field. Along with these phenomena , the scientific public stresses the importance of the conservation of food-web structure (Pinnegar et al. 2000). Hence, for the conservation and restoration of biodiversity and ecosystem processes and for predicting the impact of antropogenic effects, it remains of high priority to gain more insight into trophic interactions that shape community structure and into the role of species in mediating these interactions, both directly and indirectly. Species that fulfill an important role are not necessarily dominant species (Mills et al. 1993, Piraino et al. 2002). The impact of a species on assemblages is determined by the strength of its interaction with other species, e.g. through trophic cascades or competition for food (Wootton 1993, Menge 1995).

In marine food-web ecology, the energy fluxes between and within the lower trophic levels of the benthos (meio-[1] and microfauna[2] and bacteria) have remained largely unstudied until the '80s. This is primarily due to a late recognition of the significant roles of the most minute organisms in marine sediments, i.e. bacteria and protozoa (phagotrophic protists: flagellates, ciliates), but also to methodological restrictions, the high diversity of benthic species, feeding types and sizes, and the paucity of interdisciplinary research. Bacteria and protozoa are key decomposers of detrital matter from any trophic level of the grazer food web (e.g. faeces, decaying plant/animal material) and are responsible for the nutrient recycling (Stout 1980, Nixon 1982). Recognition of their functional role in marine pelagic systems started three decades ago with a keystone paper on the microbial loop and its feedback link to the grazer food web through feeding interactions between bacteria and metazoan grazers, often with protozoans as intermediates (Azam et al. 1983) (see below). The significance of the microbial loop and feedbacks in benthic systems was recognized much later. Lower food web interactions are considered of high importance for an efficient energy flow because of the considerably high biomass production of meiofauna and microfauna, especially in intertidal flats and estuarine systems (Heip et al. 1995, Schmidt et al. 1998).

In the traditional view, the grazer food web is driven by primary production and meiofauna are among the main herbivores, transferring phototrophic resources to higher trophic levels; this is henceforth referred to as the **classic food web**. With increasing observations of meiofaunal detritivory, however, the perception of herbivory-driven food webs is changing to that of omnivory-driven food webs, wherein an omnivorous meiofaunal component transfers energy from both the classic food web and the microbial loop, through feeding on primary producers and detritus covered with dense microbial biofilms, respectively. In the pelagic, the bacterial fraction (< 2 µm) of these microbial biofilms is mostly available to

[1] Meiofauna: benthic invertebrates that pass through a 1-mm sieve but are retained on a 32-µm sieve

[2] Microfauna: 'animal'like organisms smaller than meiofauna, mainly Protozoa

copepods (Gonzalez et al. 2004). However, there is still high uncertainty about the nutritional value of bacteria associated with detrital matter for benthic meiofauna.

For nematodes, a close interaction with bacteria is apparent, with observations of bottom-up (e.g. Moens & Vincx 1997, Moens 1999, dos Santos et al. 2008) and top-down interactions (e.g. Moens et al. 2005b, Hubas et al. 2010). The first aim of this PhD thesis was to gain insight into the dietary importance of bacteria for harpacticoid copepods, which are often the second most abundant meiofaunal taxon in marine sediments, next to nematodes (Hicks & Coull 1983). In combination with the second objective, i.e. assessing the mechanism of microbial degradation of copepod fecal pellets, this thesis covered the link between the classic and the microbial food web in both directions (Fig. 1).

The Paulina intertidal area, encompassing a salt marsh and tidal flat zone in the polyhaline reach of the Westerschelde Estuary (SW Netherlands), was used as study area. The area spanned a range of sediment types and other abiotic factors (hydrodynamics, tidal height, pore water content, physico-chemical gradients, shore vegetation etc.), providing several habitats with variable food availability and food quality, different harpacticoid assemblages and thus presumably differences in local food-web interactions. In this way, we attempted to obtain a broader view on the spatio-temporal context of trophic interactions between harpacticoid species and bacteria.

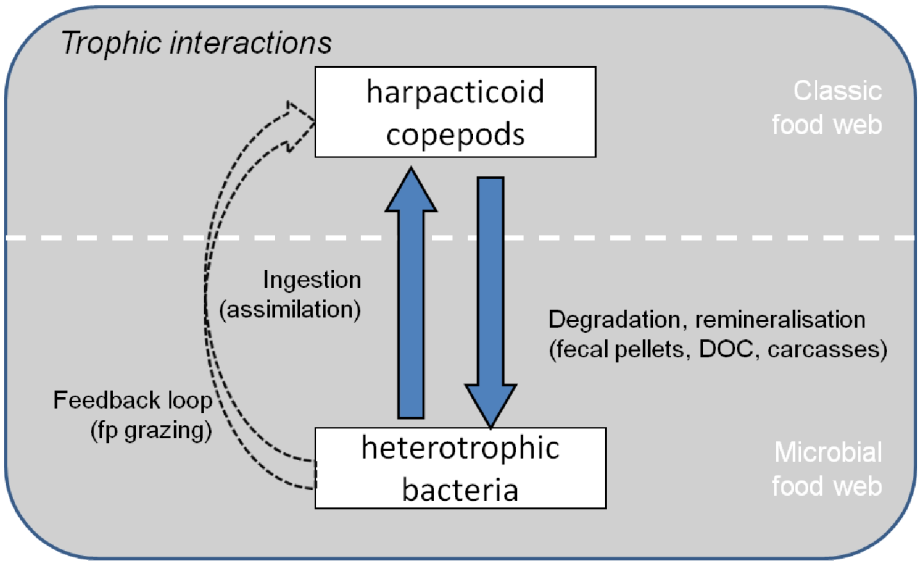

Fig. 1. Trophic interactions between harpacticoid copepods and bacteria, indicating upward fluxes from the microbial food web to the classic (primary production-based) food web and downward fluxes of waste products from the traditional food web to the microbial food web.

In the topics elucidated below concerning food webs and trophic interactions, we have concentrated on specific examples from benthic intertidal and shallow coastal systems. For the introduction on the microbial loop and copepod fecal pellets, however, the only available reference works stem from the planktonic environment.

BENTHIC FOOD WEBS AND LOWER FOOD-WEB INTERACTIONS

Marine temperate intertidal sediments are highly productive ecosystems as a result of high primary production, high nutrient load and high inputs of allochtonous detritus. Intertidal flats receive allochtonous inputs from marine, riverine and terrestrial origin, and in salt marshes and nearby sediments, from exported salt marsh vegetation. Microphytobenthos (MPB) and mainly diatoms contribute predominantly to the total primary production (McIntyre 1969, Underwood & Kromkamp 1999) and form the basis of the **classic food web** where carbon and energy are transferred to herbivores such as non-pigmented microflagellates, ciliates, nematodes and harpacticoid copepods (Montagna et al. 1995, Epstein 1997), and these in turn to higher trophic levels such as macrofauna and larval and juvenile fish (Gee 1987) (Fig. 2).

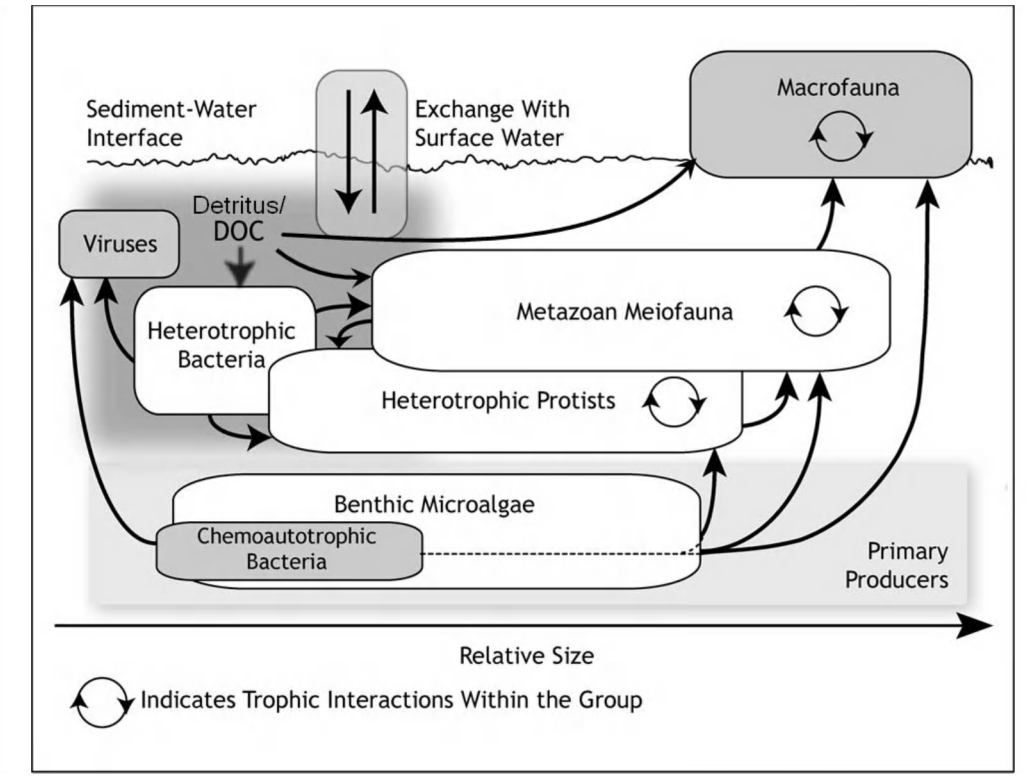

Fig. 2. Simplified conceptual model of the benthic microbial and classic marine food web in shallow-water marine sediments. Arrows indicate carbon flow. The shaded area represents the dissolved organic carbon (DOC) based microbial loop. Slightly modified from First (2008).

In a partly separate **detritus pathway**, the detritus/DOC pool supplied by the herbivorous food web (dead MPB, animal carcasses and fecal matter) is a nutrient source for heterotrophic bacteria (Fig. 2.). A fraction of the benthic bacterial production is consumed by bacterivorous protists and nematodes and was estimated to vary largely, from < 1% to 528 % of bacterial production (Hondeveld et al. 1995, Epstein 1997). The latter studies clearly demonstrated that the removed fraction of bacterial production strongly relates to spatio-temporal dynamics in bacterial grazing rates and bacterial production, owing to e.g. sediment type and temperature conditions. They in turn are preyed upon by non-bacterivorous protists, harpacticoid copepods or macrofauna (Azam et al. 1983, Rieper 1985, Coull 1990, Reiss & Schmid-Araya 2011). Nevertheless, for example in Westerschelde intertidal sediments, the largest fraction of bacterial biomass does not flow back to the grazer food web (Herman et al. 2001) but is probably lost by viral-induced bacterial cell lysis (Danovaro et al. 2008). The detrital food chain also includes macro- and

microdetritivores, terms referring to detritus-feeding macro- and meiofauna. However, for macrofauna which actually ingest detrital matter (Tenore 1977, Leduc & Probert 2009), the term 'detritivore' is more applicable than for the smaller meiofaunal organisms which may consume detritus-associated bacteria rather than the actual detrital matter itself.

Bacteria and protists, the smallest-sized component of the marine biota (< 20 µm), are an integral part of the lower trophic levels of the classic and detrital food webs. In early conceptual models of pelagic food webs and carbon flows, these organisms were ignored, based on the assumption that primary production is transferred to higher trophic levels predominantly in the particle phase and not as DOM. As a consequence, bacterial populations were assumed to be too sparse and their activity too low to play a significant role in organic matter flow (Azam 1998). The **microbial loop** concept of Azam et al. (1983) introduced microbes into marine food-web ecology (Fig. 3). The microbial food web predominantly comprised heterotrophic DOM-consuming bacteria, involved in organic matter degradation and nutrient remineralisation, and bacterivorous flagellates and larger-sized ciliates. These organisms are unique in their ability to recuperate DOM originating from algae, sloppy feeding, virus-induced cell lysis, etc., thus reducing energy loss from the classic food chain. A smaller component of the microbial loop are the most minute autotrophic phytoplankton organisms and herbivorous flagellates (photosynthetic picoplankton) feeding on them. Later, the role of viruses, with abundances often exceeding those of bacteria (i.e. 10^{10} per liter compared to 10^{9} per liter in open waters), became incorporated into the concept (Azam 1998, Azam & Worden 2004) (Fig. 3). Viruses are particularly abundant in nutrient-rich waters and in sediment pore water rather than in overlaying waters. With bacteria being the main host of viruses, virus-induced bacterial mortality (10 - 50% of bacterial mortality) (Cochlan et al. 1993) forms an additional input of DOC which is recycled by other heterotrophic bacteria. Viruses are considered a sink as bacterial biomass is made unavailable for the grazer food chain and an increased bacterial respiration associated with this enlarged DOC pool implies a loss in efficiency of the microbial loop.

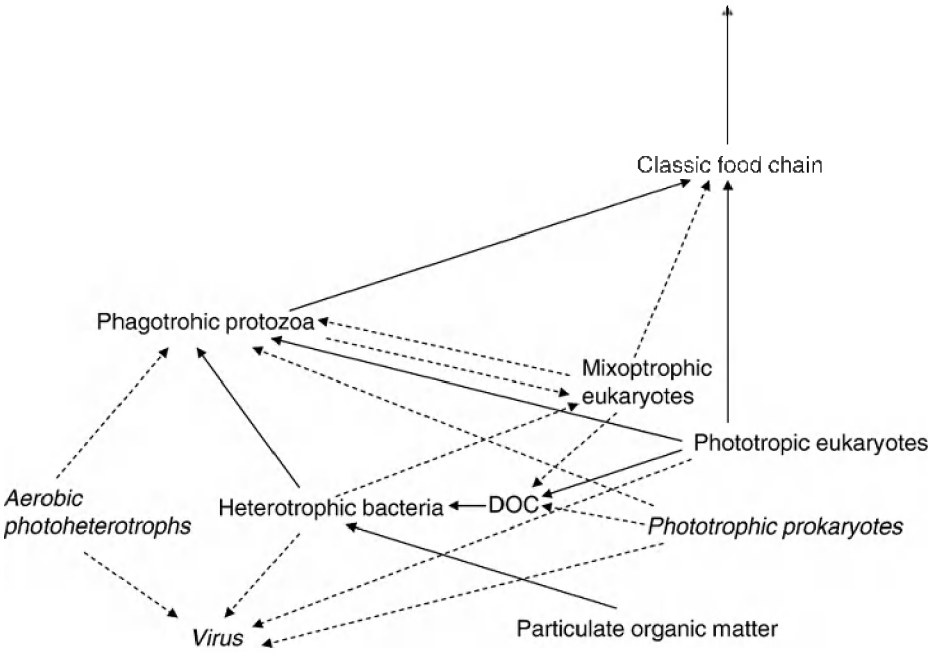

Fig. 3. The 'original' microbial loop, as described in Azam et al. (1983) (solid arrows), and with later additions (dotted arrows). DOC: dissolved organic matter. From: Fenchel (2008)

The transferable fraction of heterotrophic microbial biomass associated with the detritus pool is shunted in multiple ways to the classic food web: (1) from bacterivorous protozoa to protozoan grazers (e.g.

harpacticoid copepods, as mentioned before), and (2) through direct grazing on detritus-associated bacteria by microdetritivores. In addition, and of specific importance in the benthic system, heterotrophic microbes are living associated with fresh algae and consume algal-derived DOC and extracellular polymeric substances (EPS) (Grossart & Ploug 2001, van Oevelen et al. 2006b), which comprise ca. 40-70 % of algal productivity (Goto et al. 1999). Hence, the 'classic' meiofaunal microphytobenthos grazing and co-ingestion of bacteria is potentially an underestimated pathway of microbial energy transfer. Detailed benthic food-web schemes are scarce but one of the most detailed schemes integrating components of the classic, the detrital and the microbial pathway and reporting on the carbon flows among those components, is from Van Oevelen et al. (2006b) on the Molenplaat intertidal flat (Schelde estuary, Belgium-The Netherlands) (Fig. 4). This food web can be considered representative for the Paulina food web. In the food web of the Molenplaat, the trophic significance of bacterial carbon for the different benthic taxa was low and similar for macro- and meiobenthos, with < 1% contribution to the organisms' carbon requirements (van Oevelen et al. 2006a). The macro- en meiobenthos depended mainly on phytodetritus and microphytobenthos (classic pathway), the microbenthos on detritus and DOC (detrital pathway), and bacterial biomass largely formed a sink. Other studies have confirmed the limited role of bacteria for meiofauna (Sundback et al. 1996, Moens & Vincx 1997) and macrofauna (Cammen 1980, Kemp 1987, Andresen & Kristensen 2002), the latter with an upper limit of 10% of the carbon requirements met by bacterial carbon consumption. Among meiobenthos taxa, some nematodes and ciliates showed higher bacterial carbon consumption than harpacticoid copepods and foraminiferans (Epstein & Shiaris 1992, van Oevelen et al. 2006a). Nevertheless, bacteria may deliver some trace elements, such as vitamins and the high bacterial ingestion rates compared to ingestion in case of indiscriminate feeding suggest selective uptake of bacteria by macro- and meiofauna (Lopez & Levinton 1987, van Oevelen et al. 2006a). In the Molenplaat and Paulina area, the microphytobenthos is of high importance to all heterotrophic levels, including bacteria, nematodes, foraminifera and macrofauna (Herman et al. 2000, Middelburg et al. 2000, Moodley et al. 2000). Furthermore, nematodes represent a second trophic level as predators (Gallucci et al. 2005, Moens et al. 2005a). Harpacticoid copepods were not included in any of these studies and their trophic niche is yet unexplored.

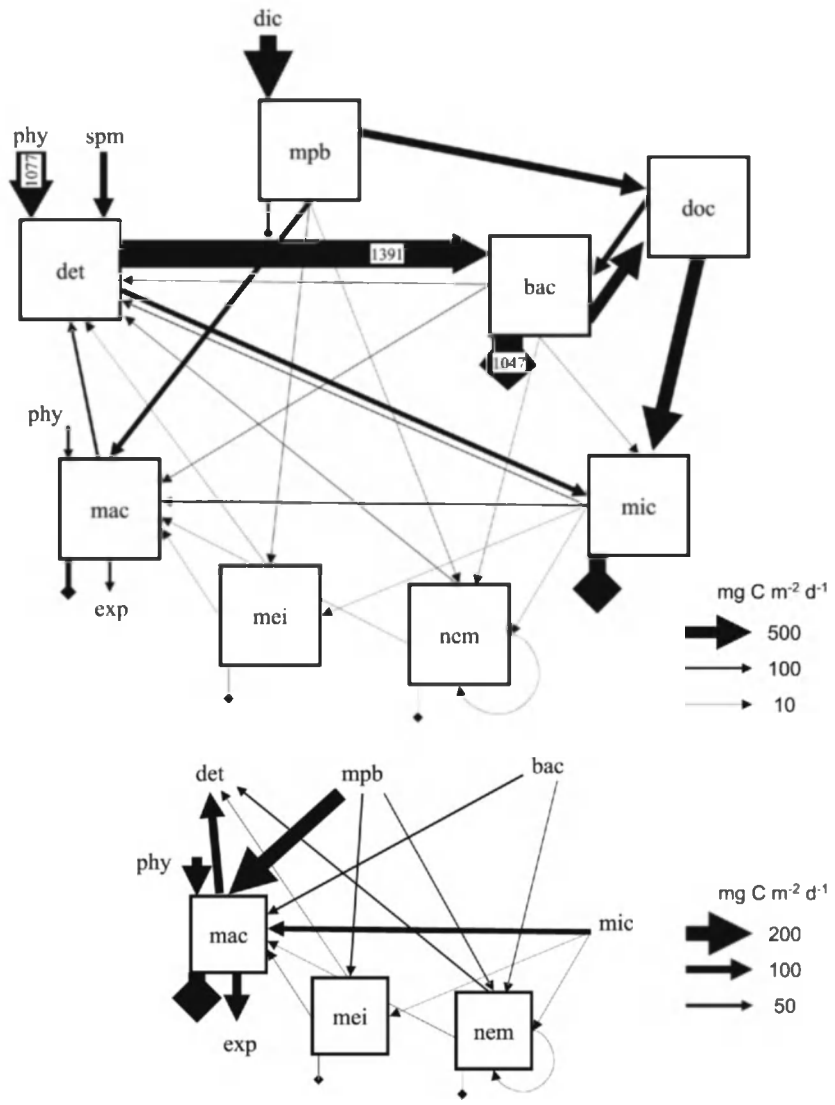

Fig. 4. The intertidal food web. Carbon inputs are primary production by microphytobenthos, macrobenthic suspension feeding on phytoplankton, phytoplankton and suspended particulate matter deposition. DOC is produced through EPS excretion by microphytobenthos and bacteria and consumed by bacteria and microbenthos. Detritus is consumed and produced (death and faeces production) by all heterotrophic compartments. Microphytobenthos and bacteria are grazed by nematodes, meiobenthos and macrobenthos, nematodes are grazed by predatory nematodes and macrobenthos, and meiobenthosis grazed by macrobenthos.
Carbon outflows are respiration (diamond head arrows), macrobenthic export (e.g. consumption by fish or birds) and bacterial burial. Only non-zero flows are pictured. The arrows with indicated values are not scaled, because their dominance would otherwise mask the thickness differences among other arrows. The lower panel shows nematodes, meiobenthos and macrobenthos on a different scale to better indicate the flow structure. Abbreviations – mpb: microphytobenthos, bac: bacteria, mic: microbenthos, mac: macrobenthos, doc: dissolved organic matter, det: detritus, phy: phytoplankton, spm: suspended particulate matter, dic: dissolved organic carbon and exp: export from the system. From: Van Oevelen et al. (2006)

HARPACTICOID COPEPODS: ECOLOGY AND SPATIO-TEMPORAL DISTRIBUTION

Harpacticoid copepods are small crustaceans within the size range of 0.2 to 2.5 mm and belong to the Order Harpacticoida of the subclass Copepoda (systematic position see addendum II). Harpacticoida comprise 52 families and well over 3000 species, and are essentially free-living, benthic copepods (Boxshall & Halsey 2004). They are cosmopolites in the marine environment, occurring from marine to brackish water, from the intertidal zone to the deep sea (review by Hicks & Coull 1983, Van Gaever et al. 2009). Harpacticoid copepods are the second most abundant meiofaunal taxon in marine sediments,

second to nematodes. Maximum densities in intertidal sediments are 10 to 100 ind. per cm^2 (Boxshall & Halsey 2004). Their body morphology is highly diverse and characteristic for their life mode, i.e. interstitial, burrowing or epibenthic. Harpacticoid copepods may form an important link in benthic food webs by channeling energy to higher trophic levels. In addition, harpacticoids, as part of the meiofauna, may facilitate biomineralisation of organic matter, enhance nutrient regeneration and may be ecosystem engineers, shaping the habitat in such a way that resources become more easily available for other organisms (Coull 1999, and references herein).

Harpacticoid copepods are rarely the dominant meiofaunal group in marine soft sediments (dominance of nematodes). Their occasional dominance in coarse sediments as well as in muddy salt marsh sediments (Hicks and Coull, 1983 and references therein) and during certain periods of the year, indicates that their distribution patterns are very habitat-specific and determined by a set of interacting biotic and abiotic variables and by their (re)colonization success (Giere 2009).

Small-scale spatial zonation patterns of harpacticoid copepods (from cm to ha) are regulated by physico-chemical gradients and other abiotic factors (temperature and salinity, grain size, redox potential discontinuity layer, oxygen, pore water content) (Findlay 1981). The distribution of harpacticoids is also determined by the patchiness of microbial food sources, as shown from laboratory experiments (Gray 1968, Lee et al. 1977, Ravenel & Thistle 1981, Decho & Castenholz 1986) as well as spatial auto-correlation studies (Findlay 1981, Pinckney & Sandulli 1990, Blanchard 1991, Sandulli & Pinckney 1999). In turn, microalgal patchiness tends to be higher in siltier relative to sandy sediments, pointing towards a close link between food source distribution and sediment type (Sandulli & Pinckney 1999).

The few available studies on temporal fluctuations suggest that harpacticoid species distribution may be regulated by temperature, food supply, predation pressure and trophic competition with other meiofaunal groups. Possible examples of competition are the inverse relationship between the harpacticoid *Tisbe* sp. and nematodes, and between *Amphiascus limicola* and Foraminifera in a mud flat (Hicks and Coull, 1983). Trophic competition among harpacticoid species has not been demonstrated directly, but indirect or correlative observations and biochemical content of copepods suggest resource partitioning (Pace & Carman 1996, Guisande et al. 2002, De Troch et al. 2005). Vanden Berghe and Bergmans (1981) suggested that the differential exploitation of photoautotrophic (herbivore) and bacterial food sources (bacterivore) among some co-occuring *Tisbe* sibling species can drive coexistence. By means of water-borne cues, harpacticoid copepods can locate food patches at distances of many body lengths (Seifried & Dürbaum 2000, Fechter et al. 2004). Whether trophic niche separation reduces resource competition and contributes to the co-existence of harpacticoid species is still unclear.

POTENTIAL FOOD SOURCES AND POSITION OF HARPACTICOID COPEPODS IN THE BENTHIC FOOD WEB

Meiofauna may play a significant trophic role in benthic energetics (Pinckney et al. 2003) and are involved in biomineralisation, nutrient regeneration andecosystem engeneering. Some of these ecosystem functions relate to the use of food sources and feeding rates of harpacticoid copepods. Harpacticoida are able to consume a wide range of natural food sources e.g. microalgae, cyanobacteria, flagellates, ciliates, mucoid substances, fungi, yeasts, bacteria as well as artificial food sources, e.g. fish flakes (Hicks and Coull 1983 and references herein). The natural food sources that will be discussed in this thesis are presented in fig. 5. These copepod-food source interactions were mainly concluded from laboratory feeding studies and indirect observations, using field distribution patterns, gut analyses, etc. Currently, direct evidence of food source assimilation from the field is obtained from tracing molecular dietary markers such as stable isotopes and fatty acids (see further).

Harpacticoid copepods in intertidal and shallow coastal sediments are primarily considered grazers on microphytobenthos (MPB) (herbivorous), predominantly diatoms (Buffan-Dubau et al. 1996) (fig. 5), and as consumers of detritus with its associated microbiota (detritivorous) (Danovaro 1996) . A high reliance of meiofauna on diatoms is generally expected owing to the constant availability of diatoms in shallow-water ecosystems (Gall & Blanchard 1995) and to their high nutritional value because of their high content of essential amino acids and fatty acids (Brown et al. 1997), compared to the temporal fluxes and low nutrient quality of *Spartina* sp. (cordgrass) and other vascular plant detritus in salt marshes or adjacent sediments. Moreover, harpacticoid copepods are able to discriminate between diatom species, different diatom sizes or different diatom growth phases (Lee et al. 1977, Azovsky et al. 2005, De Troch et al. 2006, De Troch et al. 2012b). Due to such a highly selective feeding, harpacticoid copepods may (down- or up-) regulate MPB diversity without changing overall MPB stock biomass (Azovsky et al. 2005). Detritivorous meiofaunal organisms, feeding on detrital particles and associated bacterial epibionts, have generally been thought to derive nutrients from the bacterial component (Giere 2009), unlike in real detritivores such as the polychaete *Capitella capitata*, which, depending on detritus quality, can derive a major portion of nitrogen from the detrital substrate itself (Tenore 1981, Findlay & Tenore 1982). There is still a strong uncertainty about dietary contributions of non-algal food sources to harpacticoid diets. Observations of flagellate and ciliate predation or other carnivorous feeding by harpacticoid copepods are relatively scant, e.g. predation on ciliates (Rieper 1985, Reiss & Schmid-Araya 2011), on their own offspring (Dahms & Qian 2006) and on nematodes (Lehman & Reid 1992), etc. In contrast, detritivory and **bacterivory** among harpacticoid copepods have often been suggested, but little concrete evidence exists. On the other hand, indirect trophic reliance of estuarine intertidal nematodes on detrital carbon through bacterial feeding has been documented for a number of species (e.g. Moens & Vincx 1997, Moens et al. 1999a, Hamels et al. 2001, De Mesel et al. 2004)

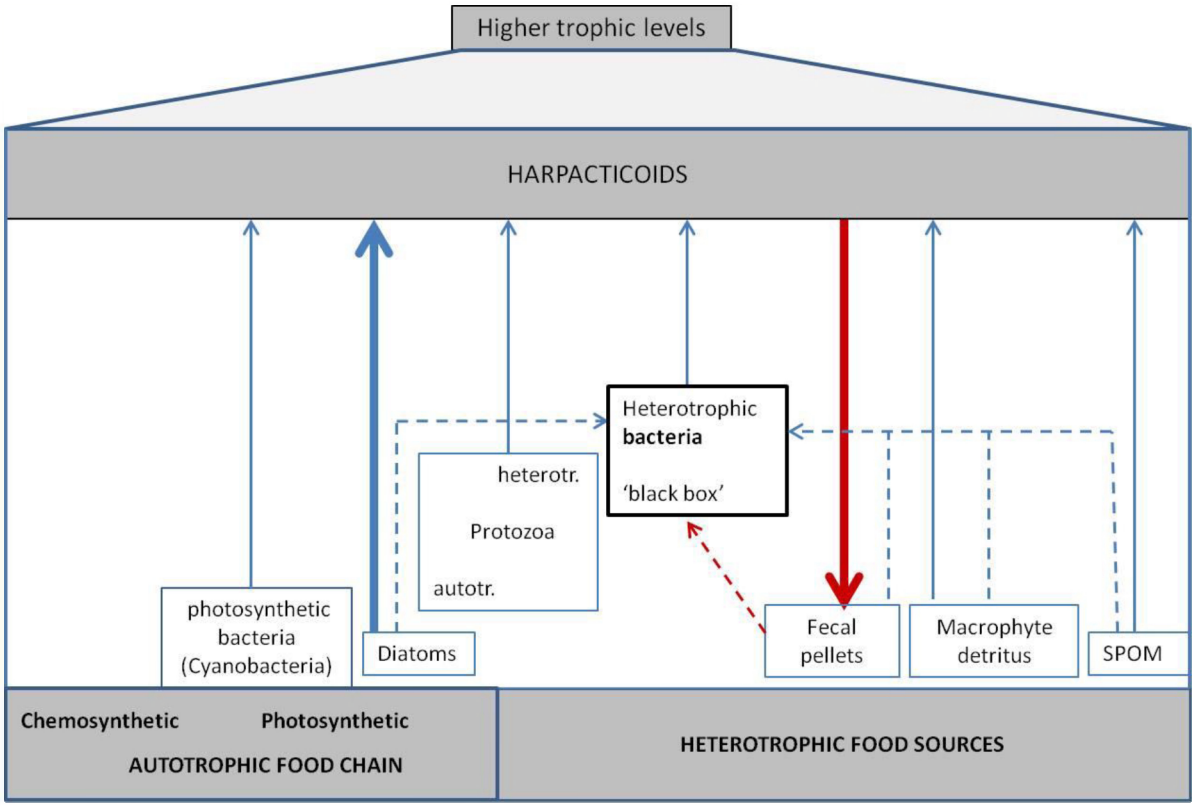

Fig. 5. Schematic overview of the different microbial and non-microbial food sources which will be dealt with in this thesis. Blue arrows represent reported interactions in literature. Red arrows are indicative for the reverse or top-down copepod-bacteria link. Arrow thickness is an indication of the significance/strength of the interaction. Dotted lines are indirect copepod-bacteria interactions.

Bacterivory

Bacterial densities in estuarine sediments are often in the order of 10^9 cells ml^{-1} porewater, which is two orders of magnitude higher than in the pelagic, and are fairly constant (Schmidt et al. 1998). However, their fate is under debate. Heterotrophic protists are the main bacterivores, but bacterial carbon transfer to higher trophic levels is often rather limited. Nevertheless, among meiofaunal taxa, bacterivorous nematodes are well recognized (Moens & Vincx 1997, Moens 1999), e.g. in salt marshes, where they may play a role in the decomposition processes of *Spartina* sp. detritus through their top-down effect on the bacterial assemblages (De Mesel et al. 2003, De Mesel et al. 2004). However, no straightforward statements on 'bacterivory by harpacticoid copepods' have been made. Yet, several laboratory studies have proven (1) the importance of associated bacterial biofilms for harpacticoid substrate selection (Gray 1968, Hicks 1977), (2) harpacticoid preferential feeding on bacteria while a more nutritional food source is available, (3) the ability of harpacticoid copepods to selectively choose certain bacterial groups (Rieper 1978, Vandenberghe & Bergmans 1981, Rieper 1982, Carman & Thistle 1985, Montagna et al. 1995, Dahms et al. 2007), and (4) assimilation of bacterial carbon by harpacticoid copepods (Brown 1977, Decho & Castenholz 1986, Montagna & Bauer 1988). Observations are, however, too scattered and limited to reveal patterns in the occurrence and prominence of harpacticoid bacterivory, in relation to harpacticoid taxonomy, habitat or bacterial groups. Furthermore, a trophic dependence on bacteria is thought to be specific for some mucus-producing copepods, e.g. the encysting copepods *Diarthrodes nobilis* (Hicks & Grahame 1979) and *Heteropsyllus nunni* (Coull & Grant 1981), where the mucus provides an easily degradable substrate for bacteria and may function for microbial gardening, as also suggested for nematodes and a few macrofaunal species (Riemann & Schrage 1987, Riemann & Helmke 2002). The few indications of bacterial carbon assimilation by harpacticoid copepods are related to grazing on autotrophic food sources such as diatoms, resulting in a combined uptake and assimilation of diatom and bacterial carbon (Decho & Fleeger 1988) (fig. 5), or in an elevated or even exclusive assimilation of bacterial carbon relative to diatom carbon (Brown 1977, Decho & Castenholz 1986). Also, the copepod *Paramphiascella fulvofasciata* responded to the removal of its fecal pellets by an increased production of fresh fecal pellets. In combination with other observations, this suggests that this harpacticoid copepod grazes upon its fecal pellets and more specifically on the bacteria associated with the pellets (De Troch et al. 2009) (Fig. 1).

Since bacteria (and detrital matter) are comparatively poor-quality food sources, typically lacking polyunsaturated fatty acids (Chen et al. 2012) which are essential to harpacticoid copepods, the nutritional contribution of bacteria to consumers is thought to be restricted to some trace elements, such as vitamins. In contrast, De Troch et al. (2012a) demonstrated that copepods assimilate bacterial fatty acids and may bioconvert these to essential poly-unsaturated fatty acids which are lacking in bacteria. Copepod gut absorption is suggested to be evolutionarily adapted to the absorption of these essential nutrients (Mayzaud et al. 1998, Thor et al., 2008).

APPLIED MOLECULAR TECHNIQUES TO STUDY HARPACTICOID FEEDING ECOLOGY

Investigation of meiofaunal trophic interactions has been greatly advanced by analyzing the molecular composition of consumer and food sources (Leduc et al. 2009). Stable isotopes ($^{13}C/^{12}C$, $^{15}N/^{14}N$) have been proven to be powerful tools to assign consumer diet in the field and species trophic position in the food web, or to quantify diet consumption in isotope enrichment experiments (review Boschker & Middelburg 2002, Boecklen et al. 2011). With each assimilation step in the food web, there is a stepwise enrichment ('fractionation') of the organism in the heavier isotope (^{13}C, ^{15}N), owing to a metabolic loss of the lighter isotope (^{12}C, ^{14}N) during food assimilation and growth. Fractionation of carbon isotopes between consumer and resource is ca. 0.5 ‰, and thus carbon isotopic signature of the consumer closely resembles that of its diet, and fractionation of nitrogen is ca. 3.4 ‰ (Post 2002, McCutchan et al. 2003).

Hence, dual stable isotope signatures of field-caught copepods reveal information on their energy sources and their trophic position. A minimal of 5 µg C and N is required for analysis and this corresponds to around twenty and sixty harpacticoid copepods, respectively. Consequently, stable isotope ratios of harpacticoid copepods have mostly been reported at the community level and especially nitrogen isotopic signatures of copepods are rare.

Given that harpacticoid copepod diets may be complex (multiple food sources) and variable in time, their 'mixed' natural isotopic signatures may still give a false indication of food source utilization. Harpacticoid feeding ecology has been extensively studied under simplified feeding conditions in laboratory microcosms. In laboratory feeding experiments, harpacticoid copepods are fed with ^{13}C-enriched food sources, such as diatoms and bacteria which have been ^{13}C-labelled during laboratory culturing with ^{13}C-bicarbonate and ^{13}C-glucose, respectively (e.g. De Troch et al. 2008, Ingels et al. 2010). Isotope-enriched food sources allow precise tracing of selective food uptake and this dietary information from both experimental and natural isotopic signatures can be implemented in linear mixing models to determine the relative importance of resources and quantify energy transfer in marine benthic food webs (e.g. van Oevelen et al. 2006a, 2006b).

Diet can also be inferred from the consumers' fatty acid composition and the concentrations of individual fatty acids. Fatty acids are essential for the functioning of cell membranes (phospholipids) and for energy storage (triacylglycerols, wax esters). Certain individual fatty acids are characteristic for a group of organisms and when transferred to the consumer without further modification, these are good dietary tracers or biomarkers (review Kelly & Scheibling 2012). Biomarker fatty acids applied in marine studies originate for instance from microphytobenthos (20:5ω3, 22:6ω3) and bacteria (15:0, 17:0, 15:1, 17:1, 18:1ω7) (Kelly & Scheibling 2012, and references therein). In comparison to stable isotopes, the fatty acid approach potentially has a higher trophic resolution as a mixed diet may be revealed by the presence of different biomarkers in the consumers' fatty acid pool. Analogous to the fractionation-issue in stable isotope approaches, the trophic transfer of fatty acids is not absolute since many incorporated food-related fatty acids are modified by the consumer. Optimization of the fatty acid approach for meiofaunal ecology research is still ongoing, for example, by the search for more diagnostic fatty acids and by attempts to unravel the pathways of fatty acid bioconversion. Consequently, stable isotopes and fatty acids data can complement each other, circumventing the limitations of the individual approaches bu even this dual approach may not allow to differentiate feeding on diverse microphytobenthos taxa (Leduc et al. 2009).

RELEVANCE OF COPEPODS FOR THE MICROBIAL LOOP

Apart from their potential trophic interaction with bacteria, copepods have feedback links to the microbial loop. Firstly, copepods contribute to the organic and inorganic inputs of the microbial loop through the production of dissolved organic matter (DOM) and particulate organic matter (POM) in the form of fecal pellets (fig. 5), and the excretion of inorganic nutrients (NH_4^+, urea) (Møller et al. 2003, 2011, Saba et al. 2011). Secondly, through fecal pellet production, copepods continuously deliver new physical substrates for bacterial attachment and subsequent cell division. Hence, copepod feeding activity enhances bacterial production and biomass (Eppley 1981, Roman et al. 1988, Peduzzi & Herndl 1992, Vargas et al. 2007), and bacterial activity (Richardot et al. 2001). The copepod body itself, especially the mouth and anal region, is also a favored substrate for bacterial attachment (Carman & Dobbs 1997, Maran et al. 2007).

DOM

In the pelagic, the release of extracellular material by primary producers may provide about 50 % of carbon requirements of heterotrophic bacteria (Baines & Pace 1991), suggesting that all heterotrophic

bacteria partly depend on allochtonous carbon from the detrital pathway. Copepods lose about 50-70 % of grazed carbon, primarily in the form of DOM rather than POM (Møller & Nielsen 2001). DOM production is mainly due to sloppy feeding and to fecal pellet leaching. Rates of DOM production relate to copepod grazing activity and assimilation efficiency, both of which are in turn influenced by the size ratio copepod-food source and by food availability (Møller & Nielsen 2001, Møller 2007)

POM

POM production of planktonic copepods (Calanoida) mainly comprises fecal pellets enclosed by a peritrophic membrane (Fig. 6). Planktonic copepods channel about one third of ingested carbon to fecal pellets (Fig. 7). In addition, dispersed amorphous small particles lacking a membrane are released and these may even reach up to 66% of total fecal POM (Olesen et al. 2005). It is unknown whether harpacticoid copepods also excrete such large fraction of amorphous matter in addition to the larger-sized fecal pellets.

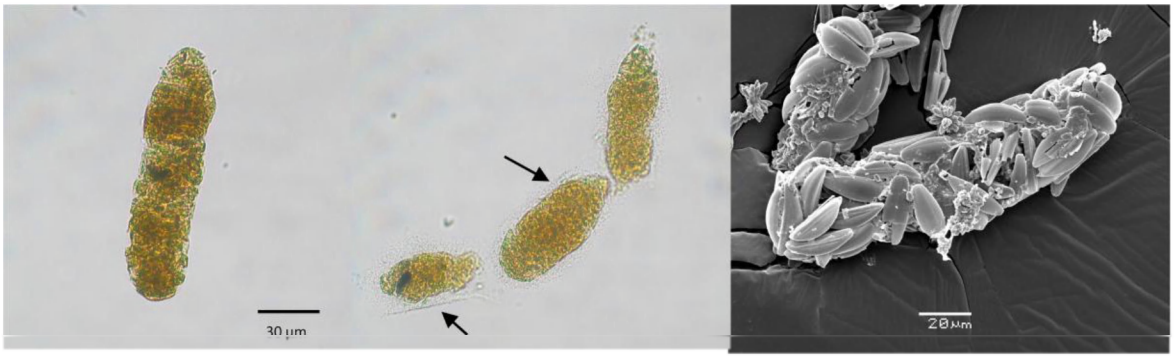

Fig. 6. Harpacticoid fecal pellets resulting from diatom feeding in the laboratory: a) light microscopic image of fecal pellets from the harpacticoid *Paramphiascella fulvofasciata*, enclosed by a peritrophic membrane (arrows) (De Troch et al. 2009), and b) SEM image of fecal pellets from *Platychelipus littoralis.*

Fecal pellet decomposition – trophic upgrading

Through efficient degradation of sinking fecal pellets by heterotrophic bacteria and zooplankton, 'lost' energy is shunted back to the grazer food web. As such, copepod fecal pellets contribute significantly to the energy flow and nutrient cycling in marine ecosystems. Studies on the degradation and fate of benthic fecal pellets are not available, but a similar process of fecal pellet recycling could be expected, whereby fecal pellets are reworked by bacteria and potentially through fecal pellet-grazing harpacticoid copepods. Rapid degradation of planktonic fecal pellets is concluded from the low fecal pellet abundances retrieved from sediment traps in coastal environments. Over 60% of fecal pellet production appears to be recycled in spite of the fast sinking rates of copepod fecal pellets compared to phytoplankton (Olesen et al. 2005). No studies on degradation rates for benthic fecal pellets exist.

Firstly, DOM-leaching soon after fecal pellet egestion stimulates bacterial fecal pellet colonization and thus the rate of bacterial conversion of fecal POM to DOM. Planktonic copepod fecal pellets are bacterial hot spots in terms of activity and abundance (Jacobsen & Azam 1984, Tang et al. 2001, Thor et al. 2003), and a similar situation may be observed for benthic fecal pellets in view of their close contact with fecal pellets in the sediment (De Troch et al. 2010). Fecal pellet degradation rates can be highly variable, depending on a range of factors such as temperature, pellet density (Hansen et al. 1996), surface:volume ratios (Hansen and Bech, 1996), etc. Also, the precise mechanism of bacterial degradation is still uncertain. The

contribution of external versus internal bacteria to fecal pellet degradation remains unknown and the functional groups of active bacteria (anaerobic or facultative aerobic bacteria) are as yet unidentified.

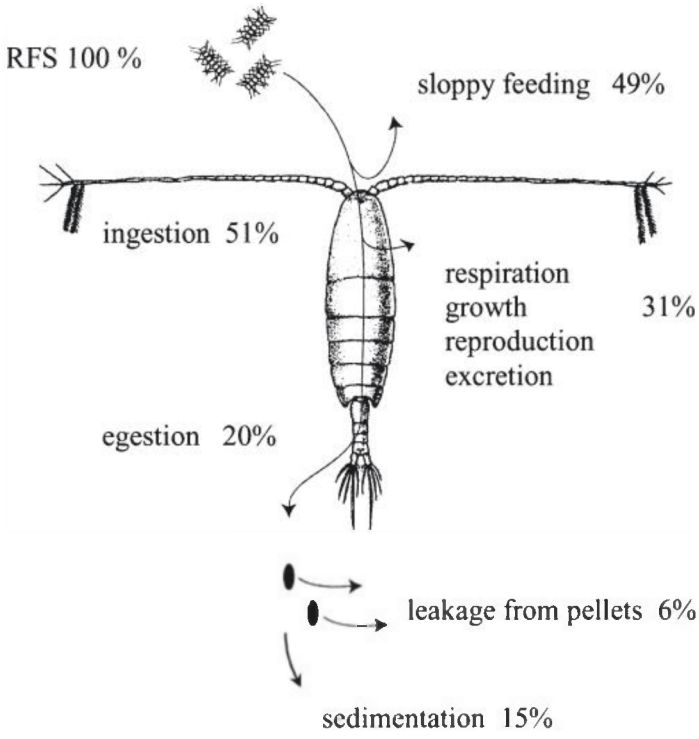

Fig 7.. Carbon fluxes of grazing copepods (*Calanus* spp.) during spring bloom in Disko Bay, western Greenland, showing the percentage of carbon removed from suspension (RFS) that ends up in the carbon DOC and POC pools. From: Møller et al., 2003.

Secondly, fecal pellets may be consumed by marine detritivorous Metazoa. Filter-feeding planktonic copepods capture fecal pellets and re-process them through coprorhexy (fragmentation of fecal matter) and potentially coprophagy (ingestion of fecal matter) (Poulsen & Kiørboe 2005, Iversen & Poulsen 2007), thus facilitating fecal pellet retention in the water column (review by Turner 2002). Fecal pellet matter is generally considered a poor-quality food source and there are two explanations for the interaction between zooplankton and their fecal pellets. Fecal pellet reworking by zooplankton is suggested to be a passive mechanism, resulting from unintentional capturing of these particles during filter-feeding. Alternatively, fecal pellets are of some trophic value and are actively consumed by zooplankton. Fecal pellets can contain high proportions of undigested, protein-rich matter. For example, during excessive food availability (diatom blooms) when food ingestion rates are high and gut transit time is short, food digestion is less efficient and viable diatom cells are packed within the fecal pellet. On the other hand, fecal pellets may be biochemically or trophically upgraded (gaining in trophic value) by bacteria whereby the extensive bacterial biomass associated with the fecal pellet and not the fecal matter itself serves as a source of proteins. During bacterial degradation of the fecal pellet, heterotrophic bacteria incorporate nitrogen from the water and use energy from nitrogen-poor organic compounds from the fecal pellet to biosynthesize new proteins (Johannes & Satomi 1966). De Troch et al. (2009) illustrated that also in the benthic food web, a similar interaction among copepods and fecal pellets exists, with bacterial decomposers as potential intermediates. The harpacticoid copepod *Paramphiascella fulvofasciata* responded to the removal of its fecal pellets by increasing its fecal pellet production in terms of number of pellets (but not net production) resulting in more but smaller fecal pellets (De Troch et al. 2009). This points towards the need for fecal pellets in its vicinity and a potential trophic role of fecal matter. Copepods are among the group of detritivores that re-use fecal pellets and in particular the associated

bacterial biofilm, albeit evidence for benthic species is still scarce (Koski et al. 2005, De Troch et al. 2009, Møller et al. 2011). Fecal pellets can be a favourable food source during periods of low diatom availability (Johannes & Satomi 1966, Morales 1987). Yet, unequivocal evidence of the trophic role of fecal pellets and its heterotrophic bacteria is lacking.

RESEARCH OBJECTIVES AND THESIS OUTLINE

The overall aim of this work was to gain insight into food source utilization by harpacticoid copepods from a heterogeneous estuarine intertidal ecosystem, focusing on species-specific responses to varying food availability, food quality and physical sediment characteristics and with particular interest in the trophic importance of bacteria for harpacticoid copepods. Hereby, this thesis dealt with the upwards link (energy flow) between the microbial loop and the basal grazers of the intertidal benthic classic food web. For this purpose, both field data **(chapter 2 and 3)** of harpacticoid species with regard to their spatio-temporal distribution and dietary pattern were collected and experimental feeding studies **(chapter 4 and 5)** with individual species were carried out. The studied harpacticoid species originated from the Paulina intertidal area. In the second part, the reverse or top-down link is studied, which involves the role of copepod fecal pellets as a substratum for bacteria and the bacterial degradation process of copepod fecal pellets, hereby focusing on the contribution of internal and external fecal pellet bacteria during early degradation **(chapter 6)**. Bacterial cell abundances, biomass and the (state of) peritrophic membrane were also visualized by Atomic Force Microscopy and Laser Scanning Confocal Microscopy (AFM-LSCM), an innovative high-resolution imaging tool which offers new prospects for studying microbial degradation of copepod fecal pellets **(chapter 7)**.

All chapters from this thesis, apart from the general introduction and discussion, and addendum I represent stand-alone research papers, which are published or in press (chapter 4, 5, 6; addendum I), submitted (chapter 7) or in preparation for submission (chapter 2, 3). Consequently, there is some overlap in the material-and-methods sections and in the description of the study area.

Here, in the general introduction (**chapter 1),** the scientific setting of this PhD thesis was outlined. It is important to note that the order of the following chapters does not necessarily reflect the order in which these research aspects were performed, let alone completed. A logical first step when addressing the importance of (variability in) food availability for, and resource partitioning within, harpacticoid copepod assemblages, is to perform a field study into both spatial and temporal heterogeneity in resource availability and copepod abundance and assemblage structure in a field situation, taking into account not only variability in resources but also in other potential, mainly abiotic, drivers of assemblage structure and abundance. Hence, **chapter 2** focused on the heterogeneity and structural characteristics of intertidal harpacticoid assemblages (density, diversity and composition) from five habitats in the Paulina intertidal area differing in sediment characteristics, tidal height, presence of vegetation, etc. These were sampled during 4 sampling campaigns. The environmental factors, comprising both abiotic factors and food-source related factors regulating the spatio-temporal variation in harpacticoid assemblage composition, were determined. For the most abundant species, a more in-depth analysis of the most influential factors for species distribution was performed, adding relevant information to their autoecology.

Chapter 3, then, used natural (mainly) carbon isotopic signatures as well as fatty acid biomarker patterns and abundances to infer field resource use of, and resource partitioning among, the most abundant copepod taxa from chapter 2, covering exactly the same spatial and temporal variability as in chapter 2 and therefore allowing maximal linkage between both chapters. This approach allowed us to get a general idea of the *in situ* trophic importance of bacteria, MPB and detritus as food for copepods and provided insights into (1) the overall trophic diversity of harpacticoid species in the study area, comprising different habitats, and (2) trophic diversity within habitat types, as well as into (3) spatio-temporal

dietary variability of some of the most abundant species. It also allowed a first assessment of the extent of bacterivory in harpacticoid copepods from this tidal flat-salt marsh system.

In chapters 4 and 5, we used microcosm experiments to evaluate some aspects of harpacticoid bacterivory using ^{13}C-labelled bacteria as food. Two to four co-occuring harpacticoid species originating from the Paulina area were selected for these experiments**.** Experiments were performed while the sampling campaign for chapters 2 and 3 was still ongoing and we did not yet have adequate information on the *in situ* feeding strategy. Our selection of species, therefore, aimed to incorporate representatives of different putative feeding types/habits (Hicks & Coull 1983). Since feeding behavior of harpacticoid copepods is often linked to morphology and/or general behavior, e.g. epi- vs endobenthic life style, motility, tube-building, species to be used in the laboratory experiments were selected based on morphological and behavioral characteristics and habitat preference (epi/endobenthic) and on field abundances. In the feeding experiment of **chapter 4**, species-specific bacterial uptake and the mechanism of uptake were examined by means of a ^{13}C-enriched bacterial inoculum. Bacterial uptake might be selective considering the highly selective feeding behavior of harpacticoid copepods towards other food sources such as diatom cells. Additionally, harpacticoid copepods generally co-ingest bacteria during diatom grazing, but it is unclear whether bacterial uptake can be an independent feeding strategy, i.e. independent from feeding on another food source and independent of the presence of a grazing substratum in general. We specifically addressed the following questions: can copepods target bacteria in absence of another food source? Or in other words, can harpacticoid copepods consume bacteria associated with a purely physical grazing substratum without nutritional value (i.e. sediment grains). We also assessed whether harpacticoid copepods are physically able of consuming bacteria in absence of such a grazing substratum. In addition, copepod fatty acid content and polyunsaturated fatty acid content (PUFA) were screened to assess copepods' general condition after 4 days of incubation with a bacterial food source, and to identify the potential nutritional contribution of feeding on bacteria in terms of fatty acids (next to the bulk carbon transfer assessed by the ^{13}C assimilation). In a subsequent food selection experiment (**chapter 5**), the ability of harpacticoid copepods to select between two bacterial species was tested, using three bacterial strains offered as 1x1 combinations. Selected bacterial strains were chosen based on their differences in fatty acid and protein composition and thus potential nutritional value, a characteristic that might drive harpacticoid selective feeding and/or assimilation.

The remaining two regular chapters (chapters 6 and 7) focused on the role of bacteria as colonizers and decomposers of copepod fecal pellets. In particular, in **chapter 6** we attempted to evaluate the relative importance of internal versus external active fecal pellet bacteria in the early degradation process (up to 60 h incubation in natural seawater) through genetic and metabolic community profiling of active bacterial communities on fecal pellets of different age. We hypothesized that active external bacteria, which are expected to rapidly colonize the fecal pellet, would dominate over internal active bacteria and therefore dominate the decomposition process. The presence of internal and external bacteria, as well as the peritrophic membrane surrounding the fecal pellet were visualized using the high-resolution imaging technique Atomic Force Microscopy - Laser Scanning Confocal Microscopy (AFM-LSCM) **(chapter 7)** by means of an optimized protocol**.** AFM generally allows studying bacterial cell size, cell shape and cell-cell interactions. Here it was applied (1) to visualize the ultra-structure and measure the thickness of the peritrophic membrane and (2) to locate and quantify bacterial presence (cell size measurements) both inside and outside the fecal pellet.

The main conclusions of this work are summarized and discussed in **chapter 8**.

This work has been supported by a Ph.D. grant from the agency for Innovation by science and Technology (IWT) (data from October 1th 2008 onwards). During the year prior to attaining IWT financing, an experimental study was performed with focus on harpacticoid microalgal feeding and the suitability of a preserved food source (**addendum I**). Diatom assimilation was lower on the preserved food source compared to freshly-cultured living diatom cells. The reduced palatability of preserved diatom cells could be related to reduced chemical signaling (absence of diatom EPS) interfering with copepod grazing

activity and diatom ingestion. On the other hand, assimilation of preserved diatoms could be hampered by a reduced (active) bacterial film attached to the diatom cells, which in turn has affected copepod gut flora and fecal pellet bacteria.

Addenda II to VI comprise supplementary material to the chapters 2 to 6.

Chapter 2

Spatio-temporal variation in harpacticoid copepod assemblages and of their food sources in an estuarine intertidal zone

In preparation:

Clio Cnudde, Willem Stock, Anne Willems, Annelien Rigaux, Marleen De Troch and Tom Moens. Spatio-temporal variation in harpacticoid copepod assemblages and of their food sources in an estuarine intertidal zone

ABSTRACT

Spatio-temporal patterns in harpacticoid assemblage composition and in individual abundances of harpacticoid species were studied in an estuarine intertidal area with high habitat heterogeneity and in relation to spatio-temporal patterns in environmental variables including sediment abiotic characteristics (granulometry, nutrient concentrations), organic matter availability, organic matter quality (chlorophyll a and pheophytine, protein content, lipid content), composition of microphytobenthos biofilms (carotenoid pigments) and sediment bacterial abundances. The tidal flat (a sand flat and a mud flat) and the salt marsh region (a *Spartina*-dominated sandy sediment, a *Spartina*-dominated muddy sediment and a muddy gully) were sampled over 4 sampling events. The two tidal flat stations have distinct harpacticoid assemblages while the harpacticoid assemblages of salt marsh stations were composed of the same abundant harpacticoid families. The influence of abiotic habitat characteristics (e.g. granulometry, inorganic nutrients) and biotic characteristics relating to food source availability and quality (total organic matter, microphytobenthic biofilms characterised by pigments and their degradation products, differences in detrital origin) on harpacticoid assemblage structure was determined and species-specific responses to environmental factors were revealed. Spatial harpacticoid assemblage variation was assigned to five variables: ammonium concentrations, total organic matter, abundance and composition of microbial biofilms (chlorophyll a proportion of total organic matter; proportion of diatoxanthin in the microphytobenthos) and abundance of detritus (pheophytine over chlorophyll a). However, harpacticoid assemblages of tidal flats were seemingly structured by abiotic factors (granulometry and tidal height) and especially copepod species from the sand flat (*Paraleptastacus spinicauda*, *Asellopsis intermedia*) were highly specific in space and constant over time. High intercorrelations between variables and especially granulometry presumably masked the role of granulometry. In contrast, the high resemblance among salt marsh harpacticoid assemblages is in spite of differences in salt marsh granulometry, and points towards a primary influence of food availability and food quality. Variability in *Microarthridion littorale* abundances related to microphytobenthos abundance. For Ectinosomatidae and *Tachidius discipes*, the low densities and low correlations over all environmental factors points towards a low affinity with the sediment surface and a generalistic occurrence, respectively. For some species, linkages between habitat characteristics and species distributions were little decisive (e.g. *Platychelipus littoralis*, *Paronychocamptus nanus*, *Amphiascus* sp. 1). Overall, it is not possible to denote with certainty the main variables regulating harpacticoid species distributions due to complex intercorrelations between environmental variables, including abiotic and food-related variables

INTRODUCTION

In many marine ecosystems, harpacticoids (Crustacea, Copepoda) are among the most abundant meiobenthic taxa, only outnumbered by nematodes. Their taxonomic diversity has been studied from intertidal and shallow waters (Chertoprud et al. 2013) to the deep sea (Gregg et al. 2010), and their large-scale distribution is reasonably well characterized (Chertoprud et al. 2010, Veit-Köhler et al. 2010). On the local scale, however, harpacticoid distributions reveal a high spatial-temporal variability, even at decimeter scales (≤ 10 cm^2)(Azovsky et al. 2004). The ecological mechanisms structuring local species diversity and patchiness remain insufficiently understood. Many studies have discussed the influence of habitat characteristics such as sediment type and heterogeneity, physico-chemical characteristics and hydrological conditions, food availability and biotic interactions on harpacticoid and other meiofaunal taxa abundances (e.g. Huys et al. 1992, Kotwicki et al. 2005, Rubal et al. 2012) and to a very limited extent on harpacticoid assemblage composition (Veit-Kohler 2005, Stringer et al. 2012).

Depending on their association with the sediment, harpacticoid species are divided into ecotypes. Interstitial copepods are small species living in the interstitial spaces between sand grains; epi- and endobenthic species live on top or in the upper centimeters of the sediment, respectively; and free-living species are swimming but remain in close contact with the sediment. The distribution of harpacticoid ecotypes strongly depends on the median sediment grain size and sediment sorting (Hulings & Gray 1976, Rybnikov et al. 2003, Giere 2009), which in turn is intricately linked to local hydrodynamics and tidal flat morphology. Besides a direct structuring role of grain size on the meiobenthos, sediment type also affects food availability and quality. Sediment organic matter content, density and diversity of microbial biofilms, and other biotic environmental variables are often correlated with grain size (e.g. Decho & Castenholz 1986, Stal & de Brouwer 2003). However, in sediments with similar granulometry, the structuring role of food resources becomes apparent: within a habitat type, harpacticoid distributions are conform the patterns of microbial food sources such as diatoms, ciliates and purple sulfur bacteria (Decho & Castenholz 1986, Azovsky et al. 2004). *Enhydrosoma littorale* had differential abundances in two adjacent tidal habitats with different granulometry but showed no preferences for either granulometry under laboratory conditions. However, the species responded only to sediment particles coated with microbial epigrowth (Ravenel & Thistle 1981). The relative importance of these different factors for harpacticoid distribution and community structure remains poorly understood. Distinct spatial heterogeneity and temporal dynamics of meiobenthos and harpacticoid assemblages are prominent in estuaries (Hicks & Coull 1983, Heip et al. 1985, Chertoprud et al. 2007). Estuarine sediments are often characterized by a mosaic of habitat types (Davidson et al. 1991) and, especially in intertidal areas, are subject to short-term fluctuations in interstital water content and temperature, salinity, dissolved nutrients, grain size, (Thomson-Becker & Luoma 1985) and resource availability, e.g. microphytobenthic biofilm production, organic matter deposition, etc. (Blanchard et al. 2002, Chen et al. 2005). Data on the simultaneous dynamics of environmental variables and multiple harpacticoid species from the same harpacticoid assemblage are scant (Veit-Kohler 2005, Stringer et al. 2012). In both cited studies, species distributions were predominantly impacted by physical factors (mainly grain size, water depth or tidal height) and by pH.

The present study investigated the spatio-temporal patterns of harpacticoid assemblages in an estuarine intertidal area composed of a range of habitats, in relation to variability in environmental factors. For this purpose, 5 habitats were sampled in a tidal flat-salt marsh area within an area of ca. 0.3 km^2 and at 4 sampling periods within one year. These habitats differed in sediment granulometry, tidal height, and vicinity and type of vegetation. The following questions were investigated: (1) Which environmental variables structure the horizontal distribution of harpacticoid assemblages in an estuarine intertidal area (between-habitat heterogeneity) and which variables account for temporal fluctuations in harpacticoid assemblages (within-habitat heterogeneity)? (2) Which environmental variables correlate with variability in abundances of individual harpacticoid species within and among habitats? We hypothesized that spatial harpacticoid heterogeneity would be mainly governed by granulometry and/or large differences in total

organic matter content due to presence of vegetation, and expected to observe differences in harpacticoid assemblages between habitats in the tidal flat and in the salt marsh ('vegetation-effect'), as well as between the habitats in the tidal flat (grain size-effect) and in the salt marsh (grain size-effect and effect of type and prominence of vegetation).

MATERIALS AND METHODS

Study area

The Paulina intertidal area is located along the southern shore of the polyhaline zone of the Westerschelde estuary (SW Netherlands, 51°20'55.4''N, 3°43'20.4''E) (Fig 1a). Mean tidal range is 3.8 m (low) (Claessens & Meyvis 1994) and hydrodynamic energy is relatively low. Five sampling stations were chosen, covering different intertidal habitats (Fig. 1b, c) in terms of, among other things, tidal height, granulometry and presence/absence of vegetation (Table 1; Fig. 1). These five stations were geographically oriented over an east-west distance range of approximately 750 m and a north-south distance range of approximately 350 m. Two stations (H1 and H2) are situated in the tidal flat area. Station H1 is located at the lower intertidal and exhibited a temporally variable granulometry, while station H2 is located in the mid-intertidal and was characterized by fine sandy sediment with a negligible silt fraction throughout the year. The other three stations H3, H4 and H5 are situated in or at the edge of the salt marsh. Station H3 is a bare sediment patch positioned at the mid to high intertidal amidst *Spartina anglica* vegetation. Samples were collected within less than half a meter of *Spartina* vegetation, in sediment dominated by fine sand and with a variable mud fraction (0 to 25 %). Station H4 is located in the high intertidal, near *Spartina* vegetation and bordering a small area with stones covered by *Fucus vesiculosus*. Samples were collected at about 1 m from the *Fucus* vegetation. Station H5 is positioned in a major drainage gully in the salt marsh, which cuts through dense vegetation which, at the level of station H5, is dominated by a combination of *Spartina anglica*, *Aster tripolium* and *Atriplex portulacoides*. Samples were collected intermediate of the bed and the flank of the gully, on exposed horizontal sediment surfaces. Since stations H3, H4, and H5 were in close proximity of salt marsh vegetation, we henceforth refer to these as 'salt marsh' stations, while H1 and H2 are referred to as 'tidal flat' stations or more specific the 'mud flat' and 'sand flat', respectively.

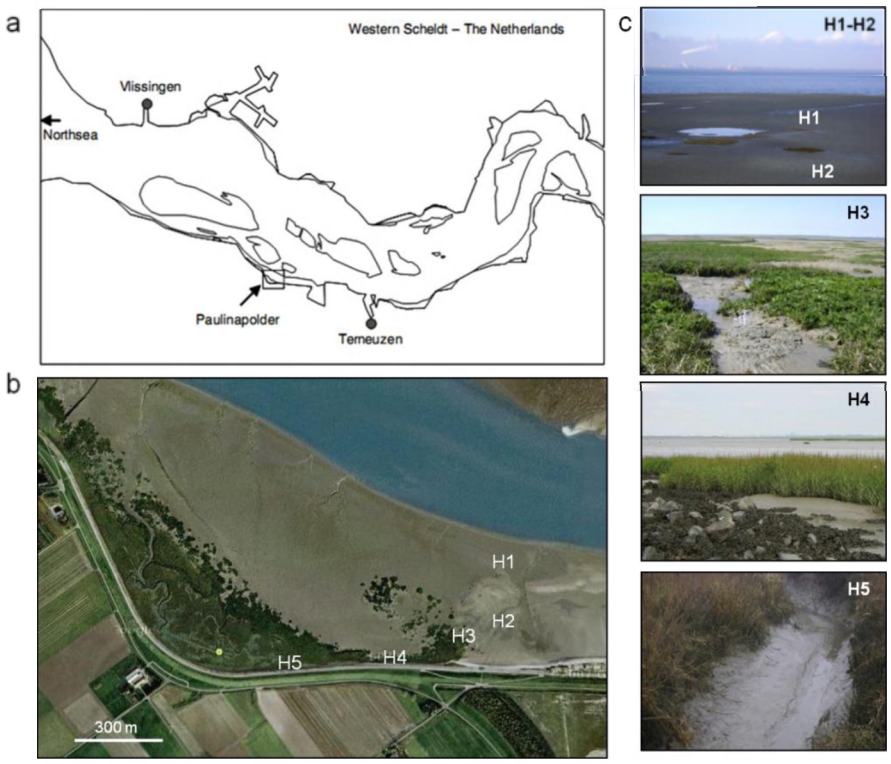

Fig. 1. Location of a) the Paulina intertidal area and b) sampling stations H1 to H5. The diversity of sampled habitats is shown in c). Coordinates of stations are given in Table 1.

Table 1. Geografic location and visual characteristics of the five sampling stations. (For detailed data on sediment characteristics, see table 3)

Station	Location	Tidal height* (m)	Tidal exposure* (% of tidal cycle)	Habitat type	Sediment type	Vegetation
H1	N 51°21'06.8" E 03°43'53.2"	-21	47	Tidal flat	Mud (though variable)	none
H2	N 51°21'00.7" E 03°43'52.2"	119	71	Tidal flat	Sand	none
H3	N 51°20'57.6" E 3°43'49.1"	237	93	Salt marsh	Sand (though variable)	*Spartina anglica*
H4	N 51°20'56.1" E 03°43'34.2"	143	78	Salt marsh	Mud	*Spartina anglica* and macroalgae
H5	N 51°20'55.7" E 03°43'30.6"	230	97	Salt marsh	Mud	Salt marsh vegetation

* data from 2008 (data source: Rijkswaterstaat Servicedesk

Sampling procedure

Four sampling campaigns were carried out in the year 2010-2011 with 3-month intervals, covering the four calendar seasons: 2-3 June 2010 (spring), 31 August - 1 September 2010 (summer), 29-30 November 2010 (autumn) and 7-8 February 2011 (winter). Sediments of the five stations were sampled at low tide for harpacticoid assemblage analysis and for analyses of environmental biotic and abiotic sediment characteristics, by means of plexiglass cores with inner diameter = 3.6 cm (surface = 10 cm^2), except for samples for nutrient and bacterial analyses, which were collected with larger cores (i.d. = 6.2 cm, surface = 30.2 cm^2) and syringes (i.d. = 2.0 cm, surface = 3.1 cm^2), respectively. For each type of analysis, four replicate sediment cores were sampled within a surface of ca 1 m^2, replicate cores for abiotic and biotic

sediment analyses matching replicate cores for harpacticoid assemblage analysis. Exceptions are station H3 where no homogeneous horizontal sample area of 1 m^2 was available and replicates were hence taken over a slightly larger sampling area, and station H5, where sediments were sampled along the exposed sides of the gully bed over a distance of ca. 10 m.

Cores were sliced into sediment layers 0-0.5 cm, 0.5-1 cm and 1-3 cm. Sediments for nutrient analysis were sliced, after removal of any water layer on top of the sediment surface, in 0-1 cm and 1-3 cm, because insufficient pore water could be obtained from slices of half a cm thick. Sediment slices for bacterial analyses were (vertically) bisected to provide one subsample for bacterial cell counting and one matching subsample for genetic bacterial assemblage analysis. Samples for protein and lipid analysis were subdivided in the same way. All samples were cooled on ice during the sampling campaign, except for lipid and pigment samples which were frozen using dry ice; the latter were wrapped in aluminium foil to avoid photodegradation. For long-term storage, copepod samples were preserved in a 4% formaldehyde solution, lipid and pigment samples were stored at -80°C and other samples at -20°C.
Interstitial water temperature and salinity were measured at each station using a field electrode. Water temperature ranged from 1 °C to 27.5 °C (in November and June, respectively). Salinity varied from 20.7 to 27.9 (in February and June, respectively).

Harpacticoid assemblage analysis

Sediment slices were rinsed with tap water over a 38-µm sieve and copepods were extracted from the sediment by flotation with ludox (density 1.18 g/cm^3) using a 1:10 ratio sediment:ludox and centrifugation for 12 min at 3000 rpm. This procedure was repeated three times. After staining with Rose Bengal, copepods were enumerated and manually sorted under a Leica MZ stereomicroscope (125 x magnification) using an eyed needle. Only adult specimens were identified; they were mounted on glass slides in a drop of glycerin. From samples with a high number of copepods, only the first 100 randomly-picked adult specimens were identified; for samples with fewer than 100 specimens, all were identified. Harpacticoids were identified to species level using Lang (1948) and Boxshall and Halsey (2004). A taxonomic list of identified harpacticoid taxa is presented in addendum II Table S1. Data were obtained from three replicates.

Environmental variables

Sediment granulometry was analysed with a Malvern Hydro 2000G particle size analyser on sediment dried for 24h at 60°C. Grain size fractions (in vol %) were classified according to the Wentworth scale (Wentworth 1922). Characteristics used in this study were median grain size, percentage mud (clay-silt fraction, < 63 µm), sorting coefficient SC (QD_φ) and skewness Sk (Sk_φ). SC and Sk were calculated based on the statistical parameters median grain size (Md), the first (Q1) and the third (Q3) quartile (in mm) using the formula of Giere (2009):

$$QD_\varphi = \frac{(\varphi Q1 - \varphi Q3)}{2}$$

$$Sk_\varphi = \frac{(\varphi Q1 + \varphi Q3)}{2} - \varphi Md$$

with $\varphi = -(\log x / \log 2)$ and x = grain size (in mm)

Concentrations of nitrogenous nutrients (NO_x^-,NH_4^+), phosphate (PO_4^{3-}) and silicium (SiO_2) in the sediment pore water were measured using a SAN^{plus} Segmented Flow Analyser (SKALAR)

Various sediment characteristics were used to estimate food availability and quality: sediment total organic matter content (TOM), absolute and relative phytopigment concentrations, lipid and protein concentrations, and bacterial abundance and diversity.

TOM (total organic matter) was determined as the weight loss of sediment after combustion in a muffle furnace at 550 °C for 2 h. The photosynthetic component of TOM was quantified by means of phytopigment analysis. Pigments of lyophylised and homogenized sediments were extracted in 90% v/v aceton at 4°C in the dark and separated by reverse-phase high-performance liquid chromatography (HPLC Agilent 1100 Series) according to Wright and Jeffrey (1997). Chlorophyll pigments measured were chlorophyll *a* (chla), pheophytin *a* and pheophytin *a*-like (both were summed to a single value, pheo), and chlorophyll c_2 (chlc). Carotenoid pigments measured were fucoxanthin (fuc), zeaxanthin (zea), lutein (lut), diadinoxanthin (diadino), diatoxanthin (diato) and β-carotene (bcar). Pigment concentrations were expressed as micrograms per gram sediment dry weight. The ratio chla:TOM was used as an indication for the proportion of fresh photoautotrophic-derived organic matter in the total organic matter pool. The ratio pheo:chla was considered indicative of the turnover of photoautrotrophic matter (e.g. herbivory leading to high pheo:chla ratio, Cartaxana et al. 2003). Carotenoid pigments (and their ratios to chla) provide information on the taxonomic composition of the microphytobenthos (MPB) (Table 2).

Table 2. Taxa affinity of pigments, based on Barranguet et al. (1997), Lucas and Holligan (1999), and Buchaca and Catalan (2008).

Pigment	Taxa
Chlorophyll c_2	Chrysophytes and diatoms, cryptophytes and dinoflagellates
Fucoxanthin	Diatoms and chrysophytes
Zeaxanthin	Cyanobacteria (and chlorophytes)
Lutein	Chlorophytes
Diadinoxanthin	Diatoms, dinoflagellates, chrysophytes, euglenophytes
Diatoxanthin	Diatoms
β-carotene	Cyanobacteria, eukaryotic algae and vascular plants (or cosmopolitan)

Total proteins were extracted in accordance to Hartree (1972), as modified by Rice (1982) to compensate for phenol interference. Total lipids were extracted through elution with chloroform and methanol in accordance to Bligh and Dyer (1959), as modified by Marsh and Weinstein (1966). Protein (PRT) and lipid (LIP) concentrations, as proxy for organic matter quality, were measured spectrophotometrically and concentrations are expressed as albumin and tripalmitin equivalents, respectively. Protein and lipid data were normalized to sediment dry weight after dessication at 60°C (details see Pusceddu & Danovaro 2009). With PRT as a proxy for organic nitrogen and TOM as a proxy for total organic matter, the ratio PRT:TOM can be indicative of sediment organic matter quality.

Bacterial analysis

Bacterial cell abundances were determined using 4',6-diamidino-2-phenylindole (DAPI) staining and epifluorescence microscopic counting (Porter & Feig 1980). Bacteria were extracted from 0.5 – 2.0 g frozen sediment. Frozen sediments were impregnated overnight with 2 ml of ice cold 0.2-µm pre-filtered and borax buffered ($Na_2B_4O_7.10H_2O$) glutaraldehyde (final conc. 4%, salinity of 27). For detachment of cells from sediment and detritus particles, the sample was incubated for 1h with 0.2 µm pre-filtered tetrasodium pyrophosphate (Na_4P_2O, 10 mM final conc., salinity of 27). Separation of bacterial cells from the sediment was achieved by adding an additional volume of borax buffered seawater (salinity 27), followed by 3 x 30s sonication at 20% amplitude, short centrifugation and supernatant collection (containing the dislodged bacteria). This procedure was repeated 3 times resulting in a final volume of 40 ml of supernatant. 1 ml of supernatant was stained with DAPI 200 µg ml^{-1} (Sigma D9542, final conc w/v)

for 15 min, then filtered on a 0.2 µm black polycarbonate filter (Whatman) following the protocol of Porter and Feig (1980) and counted. At least 10 microscopic fields and a total of 300 bacterial cells were counted. Since bacterial samples were stored at -20°C without fixative, counted bacterial cell densities will be an underestimation of actual bacterial densities and data should be interpreted exclusively in a relative manner, i.e. to illustrate spatio-temporal changes.

Bacterial assemblage structure was analyzed using Denaturing Gradient Gel Electrophoresis (DGGE). Total bacterial DNA was extracted from approximately 3 - 5 g wet sediment following the phenol-based protocol of Muyzer et al. (1993). Prior to cell lysis and DNA extraction, cells were separated from the sediment and extracellular DNA was removed following Corinaldesi et al. (2005). The extracellular DNA pool is by far the largest DNA fraction in marine sediments (Frostegard et al. 1999). Despite natural fragmentation of extracellular 16S rRNA, preserved short sequences of the 16S rRNA gene might still interfere with the PCR-DGGE analysis since this electrophoresis technique specifically targets short DNA sequences (≤ 500 bp). PCR-DGGE of the variable V_3 region of the 16S rDNA and gel staining were performed as described in Cnudde et al. (2013, chapter 6). 800 ng purified PCR-product was loaded on DGGE. Gels were digitally visualized using a charge-coupled device (CCD) camera and the Bio-Rad Quantity One software program. On each DGGE gel, 3 reference lanes were included to allow digital normalization of the fingerprint profiles using the BioNumerics software version 5.10 (Applied Maths, St.-Martens-Latem, Belgium). As a measure of bacterial diversity, number of bands per sample (OTU richness, phylotype richness) was counted. The reference for DGGE analysis was composed of selected cultured bacterial strains originating from the Paulina tidal flat and salt marsh (addendum II, appendix 1). Bacterial abundance and diversity data were obtained from two replicates only.

Data analysis

Data from the depth layer of 1-3 cm were excluded from the data analysis because of very low copepod abundances or even complete absence beneath the top one cm. Additionally, since separate measurements of 0 - 0.5 cm and 0.5 – 1 cm could not be obtained for nutrients, data on copepods and other variables from the 0 - 0.5 cm and 0.5 – 1 cm slices were combined by averaging (for concentration data, e.g. nutrient and pigment concentrations) or by summation (for abundance data, e.g. copepod species and bacterial abundance).

Spatial heterogeneity and temporal fluctuations in environmental variables and in harpacticoid assemblage structure were analysed separately by multivariate ordination of all sampled stations over all four sampling campaigns. The environmental data matrix was composed of 22 variables (see Table 3, indicated by asterisks) with N = 4. Variables omitted from multivariate analysis were (i) bacterial abundances and diversity (number of OTU) because of N = 2 and (ii) highly collinear or redundant variables (see also results). Collinearity between variables was tested using Pearson pairwise correlations, applying a threshold of 90 % collinearity. Furthermore, carotenoid pigment ratios were used instead of concentrations. Preliminary testing showed that using individual pigment concentrations instead of ratios did not strongly affect the ordination (1.9 % increase in percent explained variation) and both concentrations and ratios contained no collinearities of > 90 % . All environmental variables were log(X+1) transformed, reducing the right-skewness of data for many variables, and the normalized matrix was analysed by Principal Component Analysis (PCA). The matrix of harpacticoid species abundances was standardized to relative abundances, overall square-root transformed and subjected to Principal Coordinates Analysis (PCO). The null hypothesis of 'no spatio-temporal differentiation' for environmental data and harpacticoid data was tested with a two-factorial, fully crossed Permutational ANOVA (PERMANOVA) with the factors station (St) and month (Mo). The significance level was set at 5% and p-values > 0.05 were marked as 'insignificant' (ns). Monte Carlo p-values (p_{MC}) were interpreted in case of low number of permutations (< 10). When the assumption of homogeneity of dispersion, as tested with PERMDISP, was rejected ($p < 0.05$), we report the exact p-value to indicate that PERMANOVA results

should be interpreted with caution. Differences in harpacticoid assemblage structure were further analysed by Hill's diversity indices (N_0, N_1, N_2, N_{inf}) (Hill 1973). As for total copepod abundance, these univariate data were analysed using crossed 2-way PERMANOVA based on Euclidian resemblance matrices. SIMPER analysis on the transformed relative copepod abundance matrix was conducted to identify the copepod species characteristic for each station. In addition, nMDS was performed on absolute species counts (with zero-adjusted Bray-Curtis matrix) and, by means of species bubble plots, distribution patterns of most abundant species in the Paulina intertidal area are visualized.

To determine the environmental variables which best explain the similarities between environmental and copepod assemblage patterns, BEST-BVSTEP analysis was used (Clarke & Warwick 2001). The combination of variables with maximum rank correlation coefficient (rho) is the subset of variables which best explain the observed copepod assemblage differences. The significance of the rho value was confirmed using the BEST permutation test using 999 permutations and applying a significance level of 1 %.

Relationships between environmental variables and harpacticoid abundances were analyzed for the most characterstic species, as previously determined by SIMPER, and using Spearman rank correlation analysis (correlation coeffient $-1 < r_s < 1$) and similarly, pairwise correlations between species were analysed. Only species-environmental variable interactions and species-species interactions with significant correlation coefficients were reported.

STATISTICA 7.0 (Microsoft, StatSoft ver. 7.0) was used for Spearman rank correlation analyses and Primer 6.0 for calculating diversity indices and for the uni- and multivariate analyses.

Table 3. Environmental variables for the top 1 cm sediment layer. Averaged values (N = 4 unless indicated otherwise) with standard deviations between brackets. *: variables included in multivariate statistical analysis. Abbreviations: MGS: median grain size; SC: sorting coefficient, Sk: skewness; LIP: lipids; PRT: proteins; TOM: total organic matter; Bact A: bacterial abundance; Bact D: bacterial diversity.

	Sediment				Nutrients				Bacteria					
	MGS* (µm)	Mud (%)	SC*	Sk*	NO_x* (µg l^{-1})	NH_4* (µg l^{-1})	PO_4* (µg l^{-1})	Si* (µg l^{-1})	Bact A (10^8 cells g^{-1} DW)	Bact D (# OTUs)	TOM* (%)	LIP* (mg g^{-1})	PRT* (mg g^{-1})	PRT:TOM*
H1 June	77.8 (3.2)	41.7 (1.7)	0.959 (0.045)	0.096 (0.019)	1263.8 (464.4)	7312.5 (936.1)	1737.8 (538.2)	2253.8 (114.3)	3.606 (1.236)	24 (0)	3.32 (1.14)	0.936 (0.317)	3.759 (0.884)	0.119 (0.038)
Aug	125.7 (22.6)	22.6 (8.4)	0.732 (0.053)	0.060 (0.015)	618.5 (341.5)	2306.5 (378.5)	541.3 (157.2)	2598.3 (505.7)	2.280 (0.382)	22 (4)	1.59 (0.36)	0.235 (0.149)	2.844 (1.202)	0.194 (0.123)
Nov	105.4 (20.4)	30.7 (8.7)	0.889 (0.116)	0.089 (0.027)	734.3 (56.2)	1625.8 (290.2)	495.0 (105.1)	2004.8 (536.6)	1.115 (0.178)	22 (4)	1.86 (0.30)	0.290 (0.143)	2.275 (0.810)	0.123 (0.038)
Febr	187.2 (38.5)	13.2(11.9)	0.514 (0.192)	0.034 (0.029)	889.0 (260.9)	1510.0 (570.1)	557.0 (357.4)	2395.5 (874.1)	0.848 (0.034)	24 (2)	1.40 (0.77)	0.157 (0.048)	3.310 (0.833)	0.284 (0.144)
H2 June	227.0 (3.2)	0.0 (0.0)	0.314 (0.001)	0.000 (0.000)	1239.5 (733.4)	3862.5 (960.7)	2389.0 (370.2)	888.8 (424.8)	2.779 (1.246)	21 (1)	0.74 (0.24)	0.111 (0.012)	0.605 (0.057)	0.086 (0.017)
Aug	221.3 (4.6)	0.4 (0.8)	0.330 (0.025)	0.001 (0.002)	971.8 (388.9)	10869.5 (11847.0)	1494.8 (439.6)	143.5 (92.0)	2.088 (0.531)	21 (0)	0.64 (0.04)	0.159 (0.083)	0.637 (0.334)	0.100 (0.053)
Nov	228.2 (1.0)	0.0 (0.0)	0.311 (0.002)	0.000 (0.000)	2190.0 (490.1)	2425.0 (209.1)	1961.5 (277.0)	1182.3 (354.7)	0.909 (0.128)	19 (1)	1.48 (0.74)	0.345 (0.199)	0.246 (0.073)	0.020 (0.010)
Febr	230.0 (2.8)	0.0 (0.0)	0.310 (0.008)	0.000 (0.000)	1881.8 (607.5)	1124.0 (55.9)	628.5 (110.8)	1754.3 (835.4)	0.860 (0.148)	20 (2)	0.46 (0.02)	0.076 (0.031)	0.876 (0.309)	0.190 (0.070)
H3 June	203.8 (37.9)	14.1 (9.4)	0.567 (0.227)	0.052 (0.046)	607.0 (314.8)	7230.0 (2190.8)	1921.5 (462.7)	2218.3 (164.5)	2.936 (1.627)	20 (5)	1.36 (0.54)	0.363 (0.126)	2.917 (1.825)	0.199 (0.056)
Aug	188.8 (7.5)	18.6 (2.6)	0.697 (0.102)	0.169 (0.077)	420.8 (356.2)	3689.0 (815.4)	732.0 (387.5)	2751.0 (503.0)	2.469 (0.951)	17 (1)	1.46 (0.23)	0.223 (0.119)	3.166 (1.886)	0.211 (0.112)
Nov	211.3 (14.6)	12.9 (4.1)	0.530 (0.098)	0.067 (0.045)	988.5 (518.2)	3988.8 (1110.5)	1176.0 (660.4)	2186.3 (1073.2)	1.360 (0.264)	20 (1)	2.16 (0.49)	0.340 (0.045)	2.259 (0.512)	0.105 (0.012)
Febr	205.0 (36.7)	6.9 (8.2)	0.465 (0.175)	0.039 (0.063)	426.5 (197.1)	3775.3 (1420.5)	1390.5 (480.4)	2991.8 (697.9)	1.101 (0.197)	19 (5)	2.01 (1.65)	0.310 (0.116)	2.823 (1.496)	0.175 (0.071)
H4 June	43.9 (4.1)	66.4 (4.6)	0.937 (0.067)	0.048 (0.021)	68.3 (75.3)	5870.0 (1937.1)	2069.5 (1190.5)	2573.3 (433.0)	3.681 (1.554)	23 (0)	5.55 (1.63)	0.919 (0.151)	5.923 (1.212)	0.114 (0.042)
Aug	48.8 (4.3)	66.0 (4.5)	0.642 (0.070)	0.052 (0.034)	1003.5 (1199.6)	6950.5 (1255.5)	825.8 (34.1)	2489.8 (915.7)	2.549 (0.014)	22 (2)	3.58 (1.14)	0.680 (0.261)	6.014 (2.340)	0.163 (0.074)
Nov	47.8 (1.9)	66.9 (2.1)	0.655 (0.019)	0.035 (0.012)	1070.5 (922.3)	4729.3 (538.9)	1106.8 (286.6)	1717.3 (135.9)	2.049 (0.013)	27 (0)	4.75 (0.91)	0.829 (0.189)	3.225 (0.432)	0.070 (0.019)
Febr	47.5 (2.8)	65.5 (3.3)	0.757 (0.008)	0.068 (0.015)	643.0 (205.2)	4049.0 (902.8)	2405.5 (2367.8)	3996.0 (1361.6)	1.754 (0.656)	26 (0)	3.11 (0.22)	0.797 (0.166)	8.030 (1.760)	0.261 (0.066)
H5 June	62.2 (12.3)	51.6 (5.1)	1.290 (0.128)	0.051 (0.120)	37.8 (28.7)	4571.0 (1147.3)	2242.0 (1400.0)	2735.8 (220.9)	3.396 (1.033)	23 (1)	7.07 (2.66)	1.058 (0.599)	7.877 (0.889)	0.123 (0.048)
Aug	126.1 (19.1)	30.4 (6.0)	1.064 (0.176)	0.385 (0.071)	645.3 (250.6)	3889.8 (496.1)	919.8 (221.1)	3474.5 (287.6)	2.213 (0.460)	22 (0)	3.67 (1.89)	0.604 (0.291)	5.688 (4.256)	0.144 (0.068)
Nov	90.6 (18.3)	42.2 (5.6)	1.367 (0.103)	0.378 (0.098)	1602.0 (553.3)	3068.0 (355.7)	681.3 (103.5)	3242.3 (942.0)	1.401 (0.071)	25 (1)	6.26 (0.77)	1.129 (0.571)	4.532 (1.528)	0.072 (0.020)
Febr	60.3 (13.4)	52.6 (6.6)	1.399 (0.108)	0.130 (0.069)	837.5 (162.8)	2320.8 (773.5)	451.0 (191.1)	4438.3 (375.2)	2.095 (0.986)	23 (0)	6.90 (2.80)	0.909 (0.268)	9.680 (7.068)	0.133 (0.049)
									N = 2	N = 2				

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		Chlorophyll			Carotene						Pigment ratios								
		Chla*	c2	Pheo*	fuc	diadino	diato	zea	lut	b-car	chla:TOM*	pheo:chla*	chlc:chla*	fuc:chla*	diato:chla*	diadino:chla*	zea:chla*	lut:chla*	bcar:chla*
		($\mu g\ g^{-1}$)	($\mu g\ g^{-1}$)	($\mu g\ g^{-1}$)	($\mu g\ g^{-1}$)	($\mu g\ g^{-1}$)	($\mu g\ g^{-1}$)	($\mu g\ g^{-1}$)	($\mu g\ g^{-1}$)	($\mu g\ g^{-1}$)									
H1	**June**	15.62 (4.93)	1.17 (0.26)	1.35 (0.55)	7.24 (2.53)	1.48 (0.53)	0.50 (0.07)	0.176 (0.096)	0.052 (0.030)	0.65 (0.19)	0.481 (0.127)	0.085 (0.012)	0.0784 (0.0178)	0.459 (0.020)	0.431 (0.115)	0.0939 (0.0053)	0.0106 (0.0028)	0.0039 (0.0028)	0.0417 (0.0017)
	Aug	5.72 (1.39)	0.50 (0.06)	0.31 (0.05)	1.72 (0.39)	0.47 (0.18)	0.10 (0.02)	0.017 (0.023)	0.000 (0.000)	0.17 (0.04)	0.384 (0.170)	0.056 (0.008)	0.0898 (0.0116)	0.302 (0.008)	0.609 (0.233)	0.0802 (0.0169)	0.0025 (0.0031)	0.0000 (0.0000)	0.0292 (0.0032)
	Nov	7.65 (0.81)	2.35 (2.81)	0.48 (0.16)	2.72 (0.19)	0.71 (0.09)	0.21 (0.07)	0.041 (0.015)	0.064 (0.028)	0.35 (0.06)	0.419 (0.070)	0.064 (0.026)	0.2847 (0.3083)	0.356 (0.014)	0.744 (0.062)	0.0925 (0.0027)	0.0055 (0.0024)	0.0087 (0.0043)	0.0472 (0.0123)
	Febr	13.82 (0.87)	1.04 (0.14)	0.29 (0.03)	3.20 (0.30)	1.23 (0.13)	0.11 (0.12)	0.007 (0.006)	0.001 (0.002)	0.31 (0.04)	1.167 (0.472)	0.021 (0.001)	0.0749 (0.0073)	0.232 (0.014)	0.537 (0.610)	0.0889 (0.0069)	0.0005 (0.0004)	0.0001 (0.0002)	0.0226 (0.0021)
H2	**June**	5.63 (1.08)	0.49 (0.05)	0.10 (0.04)	1.63 (0.27)	0.59 (0.07)	0.05 (0.01)	0.188 (0.041)	0.091 (0.181)	0.22 (0.04)	0.808 (0.255)	0.019 (0.006)	0.0910 (0.0261)	0.306 (0.116)	0.466 (0.056)	0.1092 (0.0341)	0.0350 (0.0140)	0.0133 (0.0265)	0.0407 (0.0155)
	Aug	9.98 (1.31)	0.78 (0.25)	0.23 (0.06)	2.11 (1.94)	0.91 (0.48)	0.09 (0.04)	0.054 (0.074)	0.027 (0.025)	0.27 (0.08)	1.554 (0.196)	0.023 (0.003)	0.0770 (0.0188)	0.225 (0.220)	0.793 (0.445)	0.0951 (0.0583)	0.0060 (0.0081)	0.0026 (0.0024)	0.0272 (0.0100)
	Nov	11.74 (2.10)	2.09 (0.39)	0.13 (0.07)	3.86 (0.64)	1.33 (0.19)	0.06 (0.02)	0.057 (0.029)	0.000 (0.000)	0.35 (0.06)	0.935 (0.396)	0.011 (0.004)	0.1781 (0.0134)	0.330 (0.007)	0.713 (0.409)	0.1139 (0.0042)	0.0046 (0.0019)	0.0000 (0.0000)	0.0295 (0.0006)
	Febr	7.87 (0.65)	0.71 (0.12)	0.17 (0.00)	2.13 (0.27)	0.86 (0.09)	0.06 (0.00)	0.021 (0.002)	0.000 (0.000)	0.25 (0.02)	1.701 (0.105)	0.021 (0.002)	0.0903 (0.0129)	0.269 (0.016)	0.646 (0.048)	0.1088 (0.0044)	0.0027 (0.0005)	0.0000 (0.0000)	0.0313 (0.0012)
H3	**June**	4.18 (2.12)	0.22 (0.10)	0.70 (0.69)	1.11 (0.64)	0.28 (0.17)	0.21 (0.10)	0.145 (0.076)	0.355 (0.224)	0.29 (0.13)	0.348 (0.248)	0.165 (0.100)	0.0603 (0.0215)	0.256 (0.044)	0.511 (0.298)	0.0644 (0.0166)	0.0465 (0.0349)	0.1116 (0.0825)	0.0840 (0.0487)
	Aug	7.26 (2.73)	0.43 (0.22)	1.03 (0.08)	1.83 (0.69)	0.33 (0.07)	0.20 (0.05)	0.125 (0.125)	0.512 (0.420)	0.42 (0.10)	0.490 (0.117)	0.152 (0.037)	0.0571 (0.0085)	0.252 (0.007)	0.310 (0.089)	0.0495 (0.0170)	0.0155 (0.0156)	0.0873 (0.0784)	0.0631 (0.0257)
	Nov	11.42 (1.93)	1.54 (0.75)	1.76 (0.99)	3.61 (0.78)	0.97 (0.19)	0.31 (0.17)	0.143 (0.070)	0.592 (0.414)	0.65 (0.27)	0.542 (0.126)	0.146 (0.076)	0.1294 (0.0507)	0.315 (0.028)	0.320 (0.118)	0.0853 (0.0063)	0.0119 (0.0048)	0.0489 (0.0119)	0.0558 (0.0183)
	Febr	13.15 (2.19)	0.82 (0.16)	0.57 (0.25)	3.44 (0.60)	1.41 (0.28)	0.10 (0.02)	0.050 (0.012)	0.090 (0.024)	0.43 (0.08)	0.985 (0.642)	0.043 (0.015)	0.0626 (0.0105)	0.262 (0.013)	0.262 (0.074)	0.1065 (0.0058)	0.0038 (0.0004)	0.0068 (0.0019)	0.0325 (0.0014)
H4	**June**	13.94 (1.65)	0.61 (0.21)	3.49 (0.27)	3.53 (0.74)	0.82 (0.20)	0.79 (0.24)	0.576 (0.104)	0.335 (0.128)	0.68 (0.22)	0.269 (0.085)	0.252 (0.018)	0.0427 (0.0110)	0.254 (0.047)	0.250 (0.060)	0.0590 (0.0124)	0.0418 (0.0091)	0.0238 (0.0073)	0.0492 (0.0152)
	Aug	16.71 (5.65)	1.01 (0.27)	2.87 (1.52)	4.49 (2.66)	1.01 (0.42)	0.32 (0.18)	0.243 (0.133)	0.186 (0.062)	0.60 (0.25)	0.476 (0.129)	0.165 (0.046)	0.0617 (0.0079)	0.258 (0.100)	0.163 (0.035)	0.0618 (0.0203)	0.0137 (0.0042)	0.0122 (0.0067)	0.0350 (0.0039)
	Nov	22.51 (6.15)	2.39 (0.79)	4.08 (0.56)	7.49 (2.08)	2.14 (0.80)	0.72 (0.15)	0.400 (0.018)	0.368 (0.094)	1.02 (0.20)	0.505 (0.247)	0.188 (0.039)	0.1056 (0.0109)	0.333 (0.010)	0.232 (0.029)	0.0931 (0.0102)	0.0186 (0.0041)	0.0171 (0.0056)	0.0459 (0.0039)
	Febr	14.87 (2.61)	0.79 (0.08)	3.00 (0.94)	3.92 (0.64)	1.36 (0.12)	0.58 (0.20)	0.245 (0.044)	0.343 (0.119)	0.88 (0.23)	0.480 (0.093)	0.199 (0.042)	0.0545 (0.0102)	0.264 (0.021)	0.273 (0.055)	0.0926 (0.0102)	0.0166 (0.0025)	0.0226 (0.0053)	0.0586 (0.0076)
H5	**June**	20.44 (5.30)	0.78 (0.23)	5.97 (1.88)	5.43 (2.98)	1.21 (0.73)	0.94 (0.50)	1.021 (0.682)	1.066 (0.635)	1.20 (0.36)	0.309 (0.085)	0.291 (0.051)	0.0385 (0.0062)	0.247 (0.100)	0.182 (0.066)	0.0541 (0.0253)	0.0459 (0.0246)	0.0501 (0.0297)	0.0586 (0.0117)
	Aug	16.36 (12.84)	0.71 (0.32)	5.33 (4.77)	3.18 (1.00)	0.27 (0.19)	0.25 (0.28)	0.740 (0.630)	0.614 (0.640)	1.17 (1.23)	0.410 (0.097)	0.308 (0.041)	0.0504 (0.0122)	0.240 (0.085)	0.107 (0.107)	0.0280 (0.0203)	0.0440 (0.0234)	0.0327 (0.0147)	0.0625 (0.0233)
	Nov	11.45 (3.09)	1.41 (0.65)	3.29 (0.75)	4.40 (1.29)	0.94 (0.43)	0.45 (0.14)	0.179 (0.026)	0.463 (0.063)	0.63 (0.10)	0.189 (0.072)	0.291 (0.023)	0.1191 (0.0316)	0.383 (0.035)	0.201 (0.115)	0.0789 (0.0181)	0.0161 (0.0026)	0.0418 (0.0078)	0.0565 (0.0085)
	Febr	12.85 (2.23)	0.75 (0.11)	3.26 (0.99)	3.68 (0.44)	1.10 (0.11)	0.31 (0.09)	0.137 (0.043)	0.383 (0.186)	0.57 (0.15)	0.198 (0.042)	0.249 (0.043)	0.0583 (0.0014)	0.289 (0.019)	0.129 (0.013)	0.0865 (0.0087)	0.0105 (0.0019)	0.0286 (0.0097)	0.0436 (0.0045)

RESULTS

Environmental variables

Mutually redundant variables were (1) mud fraction and median grain size (negatively correlated) and (2) total nutrients, inorganic N and NH_4^+. From the intercorrelated variables, only median grain size and NH_4^+ were retained in the environmental data matrix.

The five stations clearly differed in sediment characteristics. In the PCA, where the first two axes explained 52.0 % of the total variation, samples grouped primarily according to location rather than time (Fig. 2). Nevertheless, both spatial and temporal differences were significant (PERMANOVA $p < 0.001$ for St, Mo and St x Mo; PERMDISP St x Mo: $p = 0.015$; Addendum II Table S2). Stations were consistently differentiated throughout the year (pairwise tests $p < 0.05$; addendum II Table S2), with few exceptions ($p > 0.05$ for H2-H3 and H4-H5 in June and H1-H3 in February; addendum II Table S2). Station differentiation was mainly located along PC1 which explained 39.1 % of the variation, with H4-H5 positioned on the negative side, H1-H3 around zero and H2 on the positive side of the axis. Ten variables correlated strongly with PC1 with $r_s > 0.75$ (Fig. 2). The prime three variables (highest correlation coefficients) contributing to station differentiation related to general OM availability (TOM, $r_s = - 0.88$) and to the amounts and proportions of photoautotrophic matter i.e. pheo ($r_s = - 0.90$), and pheo:chla ($r_s = - 0.94$); all were higher in stations H4 and H5. TOM ranged from 3 - 7 % at H5 to < 1 % at H2 (Table 3), and the pheo:chla ratio was five to ten times higher in the marsh stations H4 and H5 compared to tidal flat stations H2 and H1. The contributions of fresh photoautotroph-derived organic matter to the total organic matter pool (chla:TOM, $r_s = 0.76$) were consistently highest for H2 (≥ 0.81) and lowest for H4 (≤ 0.48) and H5 (≤ 0.41) (Fig. 2, Table 3). Nevertheless, stations H4 and H5 harboured higher concentrations of chlorophyll *a* (chla, Table 3). For lipids and proteins ($r_s = -0.78$ and $r_s = -0.85$), highest concentrations were measured in muddy marsh stations H4 and H5 (LIP = 0.60 – 1.13 mg g^{-1}, PRT = 3.22 – 9.68 mg g^{-1}) than in tidal flat stations and the sandy salt marsh station (H1-H2-H3: LIP = 0.08 – 0.36 mg g^{-1}, PRT = 0.25 – 3.31 mg g^{-1}), except for high values at station H1 in June (LIP = 0.94 mg g^{-1}; PRT = 3.76 mg g^{-1}).

There was no evidence of pronounced variability in sedimentary bacterial abundance among stations, but a temporal variability was apparent. Cell abundances were lower during November-February, ranging from 0.85×10^8 to 2.10×10^8 compared to 2.09×10^8 to 3.68×10^8 in June-August (Table 3). Bacterial diversity, in terms of number of phylotypes, did not show any spatial or temporal differences (Table 3).

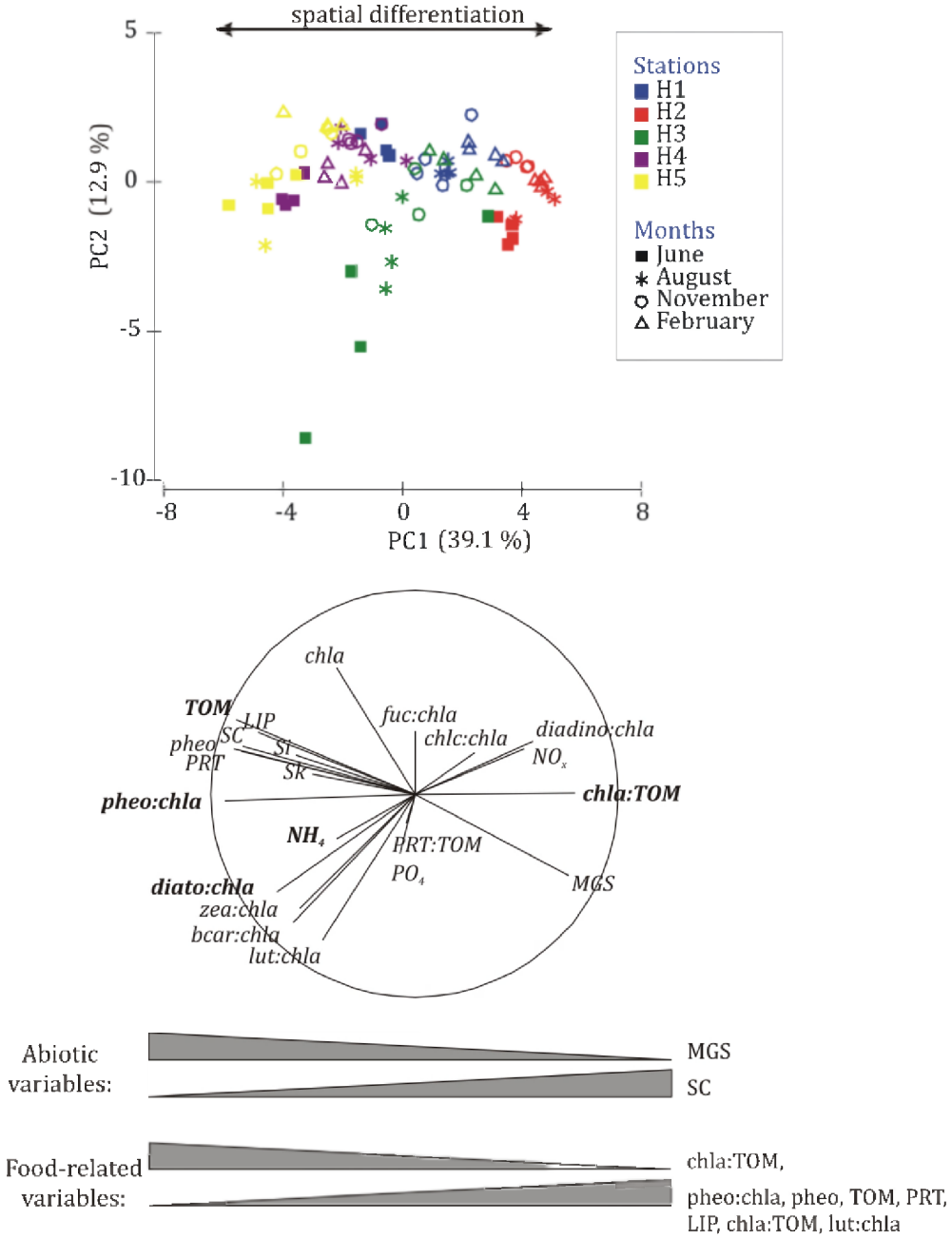

Fig 2. Principle component analysis (PCA) of normalized, log-transformed environmental variables from stations H1 to H5 over four sampling occasions (June, August, November, February). Underneath the PCA, eigenvectors of variables are presented, followed by the presentation of those variables with highest correlations to PC1 axis (r_s > |0.75|). The five vectors in bold are the BEST explanatory variables of spatial harpacticoid assemblage differentiation. Abbreviations: MGS: median grain size; SC: sorting coefficient, Sk: skewness; LIP: lipids; PRT: proteins; TOM: total organic matter.

Abiotic variables contributing to station differentiation were median grain size (r_s = 0.79) and the sorting coefficient (r_s = - 0.83). Median grain size ranged from silt in station H4 to fine sand in station H2 (from 44 to 221 µm, respectively) with corresponding mud fractions of 66 % in H4 and 0 % in H2 (Table 3). Silty sediments were moderately (0.64 ≤ SC ≤ 0.94, for H4) to poorly sorted (1.06 ≤ SC ≤ 1.40, for H5), while coarser sediment was very well sorted (SC ≤ 0.33, for H2). Furthermore, from the nutrients only NO_x^- and Si contributed to station differentiation (r_s = 0.54 and r_s = - 0.58, respectively).

In general, with the exception of bacterial abundance, temporal fluctuations in environmental variables in the Paulina area were limited relative to spatial heterogeneity, and were more located along axis PC2.

Only station H3 showed a large temporal variability. The variable most strongly correlated to PC2 was chla (r_s = - 0.51). However, chla concentrations showed temporal variability in all stations but not according to a uniform pattern (Fig. 3).

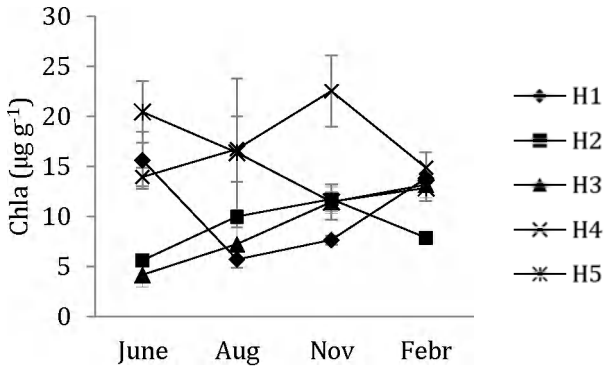

Fig.3. Temporal fluctuations in chl *a* concentrations (mean ± SE, N = 4) in Paulina stations (H1 to H5), from June to February.

Fucoxanthin, primarily indicative of diatoms, was consistently the main pigment in terms of absolute concentrations as well as proportions (Fig. 4 a, b), followed by chlc and diadinoxanthin, both with elevated concentrations in November (Fig. 5). The pigments with overall lower concentrations and ratios were zeaxanthin, lutein and diatoxanthin (which were nearly absent from stations H1 and H2) (fig. 4, 5). Concentrations of all pigments were higher in H4 and H5 compared to H2 and H3 (Fig. 4). At stations H1 and H5, fluctuations in fucoxanthin concentrations correlated significantly with fluctuations in chlc and diatoxanthin (Fig. 5; for H1: r_s = 0.70 and: r_s = 0.55; for H5: r_s = 0.79 and 0.54), which is consistent with their prominent presence in diatoms. However, in other stations, fucoxanthin and diatoxanthin concentrations did not correlate.

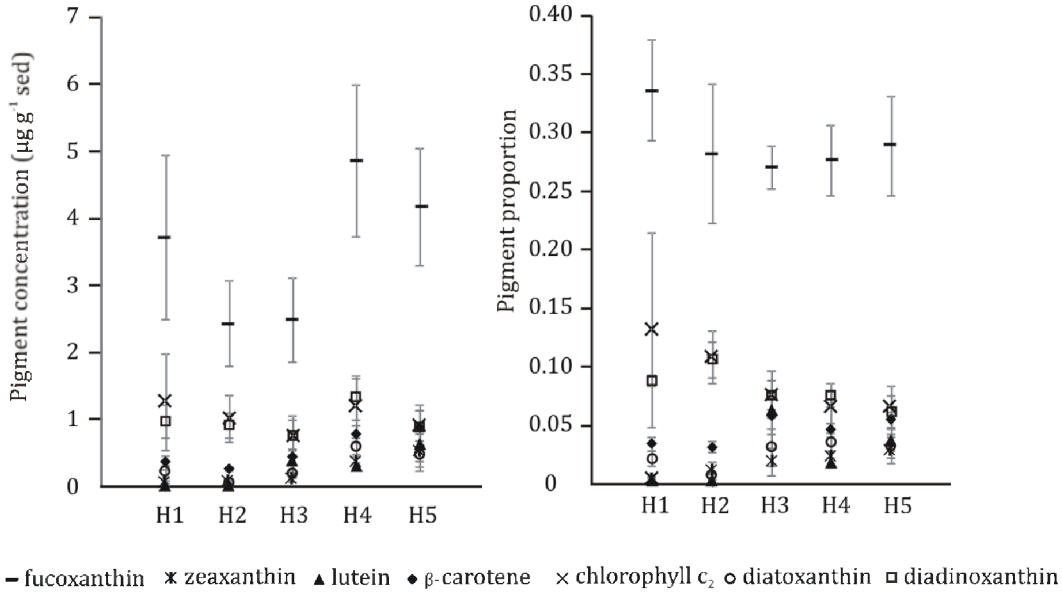

Fig. 4. Carotenoid pigments in each station (means over months ± SD, N = 4): (a) as absolute pigment concentrations and (b) as proportional (pigment concentration divided by chla concentration). Absolute concentrations are a proxy for taxon density of photoautotrophs, pigment proportions are a proxy for relative taxon abundance of photoautotrophs.

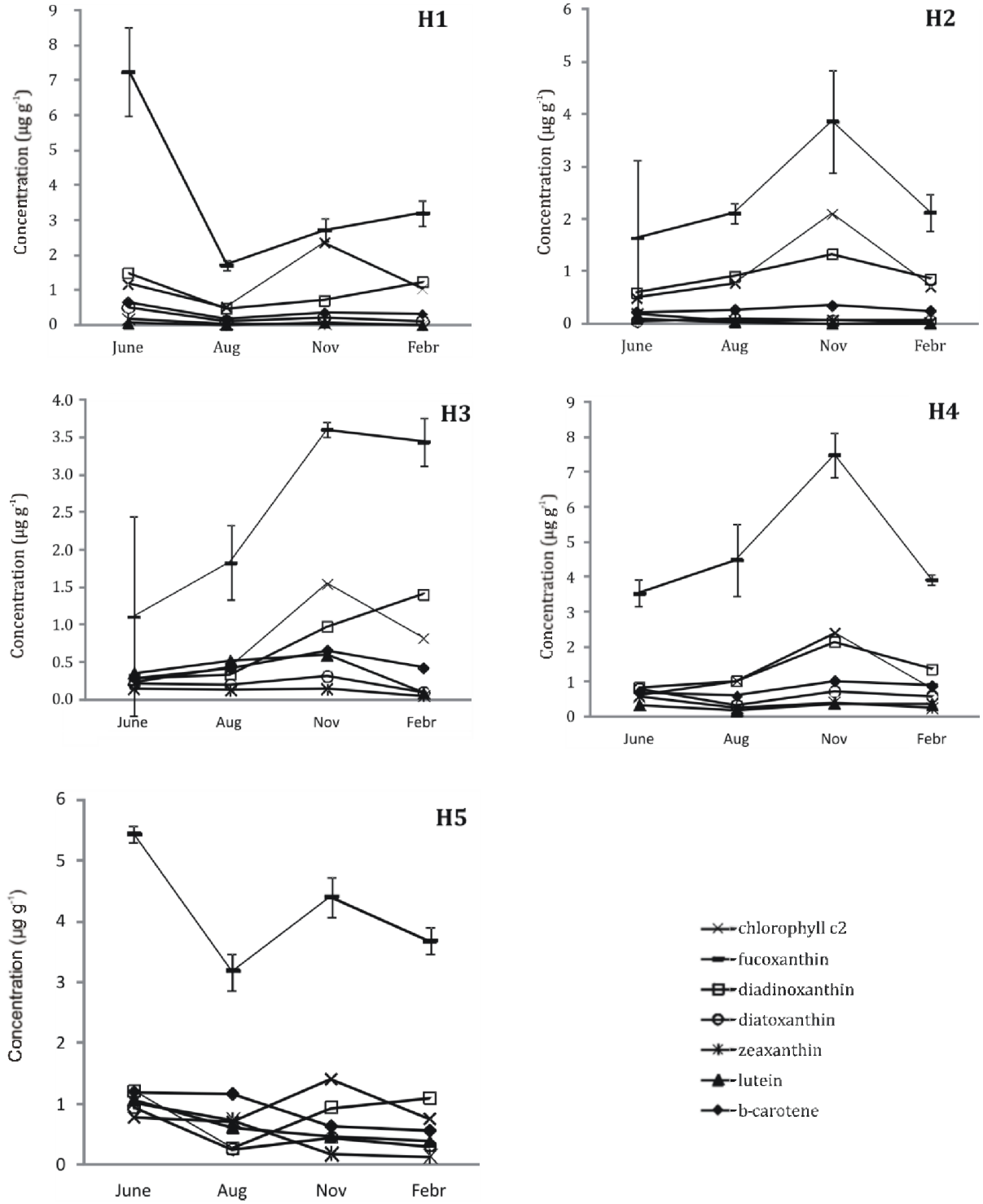

Fig. 5. Temporal fluctuations in carotenoid pigments per each station. Mean ± SE (N = 4). Only SE of fucoxanthin are presented. SE of other pigments are too small to present.

Harpacticoid copepods

Harpacticoid abundance

Total copepod densities in the top 3 cm ranged from 0 to more than 700 ind. 10 cm^{-2} (Addendum II Table S3). Densities varied among stations but not among seasons (PERMANOVA, St: p<0.01, Mo: ns, St x Mo: ns; addendum II Table S3). Harpacticoid densities were 171 ± 222, 230 ± 194, 161 ± 57 ind. 10 cm^{-2} (average ± SD, N = 12) in H3, H4 and H5, respectively, and were significantly higher in those three stations than at H1 (24 ± 21 ind. 10 cm^{-2}, p ranging from <0.05 to <0.001; addendum II Table S3) and H2 (68 ± 60 ind. 10 cm^{-2}, p<0.05; addendum II Table S3). With the exception of H5, copepod abundances were highly variable among replicates.. Copepods were largely confined to the upper 1 cm. The proportion of copepods in the 1 – 3 cm slice ranged from 1 - 3 % for stations H1, H3, H4 to 11 - 12 % for stations H2 and H5. Community analyses are based on adult specimens only and on the top-1 cm of sediment.

Harpacticoid assemblage structure and diversity

Identified adults over all stations and seasons comprised a total of 20 species from 16 genera and belonging to 8 harpacticoid families (addendum II, Table S1). An overview of absolute and relative species abundances is provided in Addendum II Table 5 and 6. The PCO plot, the first two axes of which explained 64.4 % of the variation, indicated strong spatial differentiation in harpacticoid assemblage structure (PERMANOVA: St, Mo, St x Mo, all p < 0.001, PERMDISP St x Mo: p = 0.03, addendum Table S4). This is in agreement with the observed spatial differences in environmental characteristics (Fig. 5, Fig. 6). Tidal flat stations H1 and H2, positioned at the positive side of PCO1, grouped separately, from each other (pairwise, p < 0.05; addendum II Table S4) and from salt marsh stations H3, H4 and H5 (pairwise, p < 0.05; addendum II Table S4). Harpacticoid assemblages of H3, H4 and H5 were similar to each other throughout the year (p > 0.05), except in November, when H3 separated from H4 and H5 (pairwise, both p < 0.05; addendum Table S4) (Fig. 6). Overall, the five stations comprised three different harpacticoid assemblages, i.e. the assemblage of H1, of H2 and of H3-4-5 (Fig. 6, encircled).

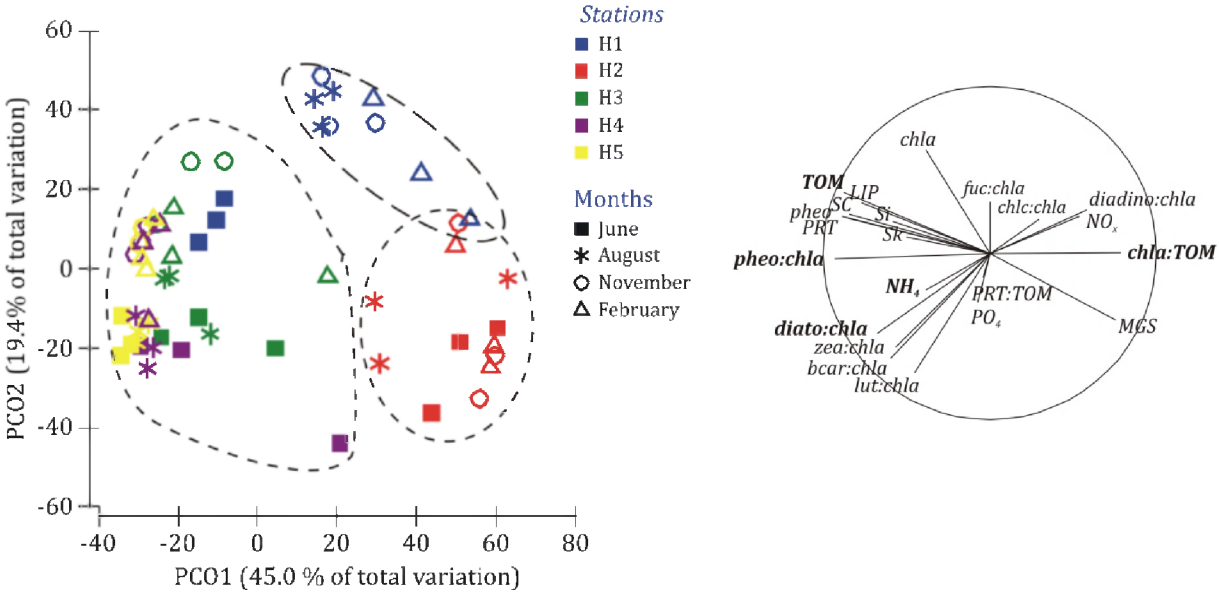

Fig. 6. Principle coordinates analysis (PCO) of harpacticoid species assemblages based on relative, square-root transformed species abundances from all stations over four sampling occasions. Environmental vectors from fig. 2 are repeatedly shown here for the ease of comparison between harpacitcoid data and environmental data.

Temporal variability was less obvious than spatial variability, but still significant (PERMANOVA, see above) (Fig. 6), again much like for the environmental variables. In salt marsh stations (H3, H4, H5), major changes in assemblage composition occurred between August and November (A-N, $p_{MC} < 0.05$; addendum II Table S4); November was the only moment where the copepod assemblage of H3 significantly differed from those of H4 and H5 (pairwise, $p_{MC} < 0.05$; addendum II Table S4). The post-hoc test revealed that the harpacticoid assemblage composition of H2 did not vary during the year (all $p_{MC} > 0.05$). However, the two most abundant and characteristic species, *Paraleptastacus spinic*auda and *Asellopsis intermedia*, showed clear variation in abundance (fig. 7). The low-tidal mud flat assemblage (station H1) showed major seasonal changes in composition between June and August and between November and February (pairwise, both $p_{MC} < 0.05$; addendum II Table S4). These patterns were equally observed using copepod family data as when using species data.

Hill's diversity indices also demonstrate structural differences in copepod communities in terms of richness and diversity (N_0, N_1) as well as evenness and dominance (N_2, N_{inf}). N_0 and N_1 fluctuated both spatially and temporally (for both N_0 and N_1, PERMANOVA St x Mo: $p < 0.05$; addendum II table S7). Tidal flat stations were characterized by lower copepod richness than salt marsh stations (pairwise, most $p < 0.05$; Addendum II Table S5) (Table 4). Temporal variability in species richness was present at H1 and H5 (pairwise, most $p < 0.05$; Addendum II Table S7). In H1, number of species was highest in June and lowest in February. In site 5, number of species was slightly higher in August and November. Evenness/dominance did not vary over time but differed between locations (for both N_2 and N_{inf}, PERMANOVA St: $p < 0.001$, Mo: ns, St x Mo: ns; Addendum II Table S7): the sandy sediment harpacticoid assemblage (H2) showed low evenness and high dominance compared to all other stations (pair wise, all $p < 0.05$; Addendum II Table S7).

Table 4. Copepod assemblage structure of the top cm (0-1 cm) presented by univariate Hill's diversity measures, averaged over time (N=12, standard deviations between brackets)

	N_0	N_1	N_2	N_{inf}
H1	5.08 (2.71)	3.91 (1.78)	3.41 (1.50)	2.50 (1.03)
H2	3.08 (1.38)	1.92 (1.05)	1.64 (0.86)	1.36 (0.45)
H3	8.27 (2.69)	4.33 (2.05)	3.38 (1.89)	2.33 (1.20)
H4	8.67 (2.23)	4.43 (0.96)	3.34 (1.00)	2.29 (0.70)
H5	8.83 (1.40)	5.30 (1.21)	4.16 (1.25)	2.70 (0.88)

Copepod species contributing to differentiation among stations, as indicated by their correlation to PCO1 (Fig. 6; threshold $r_s > |0.70|$) were *Microarthridion littorale* ($r_s = -0.86$), *Nannopus palustris* ($r_s = -80.0$) and *Enhydrosoma sp.* ($r_s = -0.78$) which were most abundant in stations H4 and H5 (and few individuals in H3), and *Paraleptastacus spinicauda* ($r_s = 0.72$) which was unique for H2. The species with the strongest correlation to PCO2 was *Paronychocamptus nanus* ($r_s = 0.68$), mainly present in H3, H4, and H5 and occurring in higher densities in November-February.

The copepod community of station H1 was characterized by the general presence of Laophontidae, Ectinosomatidae and Miraciidae, albeit with temporally variable abundances. Some families significantly contributed to the assemblage during one season only (Tachidiidae, Harpacticidae, Leptastacidae). The most characteristic species of station H1 was *Asellopsis intermedia* (SIMPER, 42 % contribution to similarities within station H1; see Fig. 7). However, similar abundances of this species were also found in

station H2 (Fig. 8). Station H2 was dominated by Leptastacidae, in particular *Paraleptastacus spinicauda* (SIMPER, 82.4 %; Fig. 8), and by considerable abundances of Laophontidae (species *A. intermedia*, Fig. 8).

All salt marsh stations H3, H4 and H5 primarily hosted the same four main families, i.e. Tachidiidae, Laophontidae, Cletodidae and Miraciidae. Their distribution varied among the stations and even within each station. In station H3, Laophontidae and in particular *Paronychocamptus nanus* dominated the assemblage during the colder period (November and February, Fig. 7, 8). In addition to *P. nanus*, *M. littorale* (SIMPER 17.0%) strongly contributed to the copepod community of station H3 (Fig. 7). In station H4, Tachidiidae were dominant during that period, and Cletodidae and Laophontidae in the other months. The most important species were *M. littorale* (SIMPER, 23.3 %) and *Platychelipus littoralis* (SIMPER, 20.0 %), although both were also abundant in station H5 (Fig. 8). Family composition and dominance of station H5 were largely similar to station H4. Furthermore, station H5 showed a fairly constant presence of Huntemanniidae. The most characteristic species here is also *Microarthridion littorale* (SIMPER, 29.7 %) followed by *Nannopus palustris* (SIMPER, 13.2 %) and *Enhydrosoma sp.*

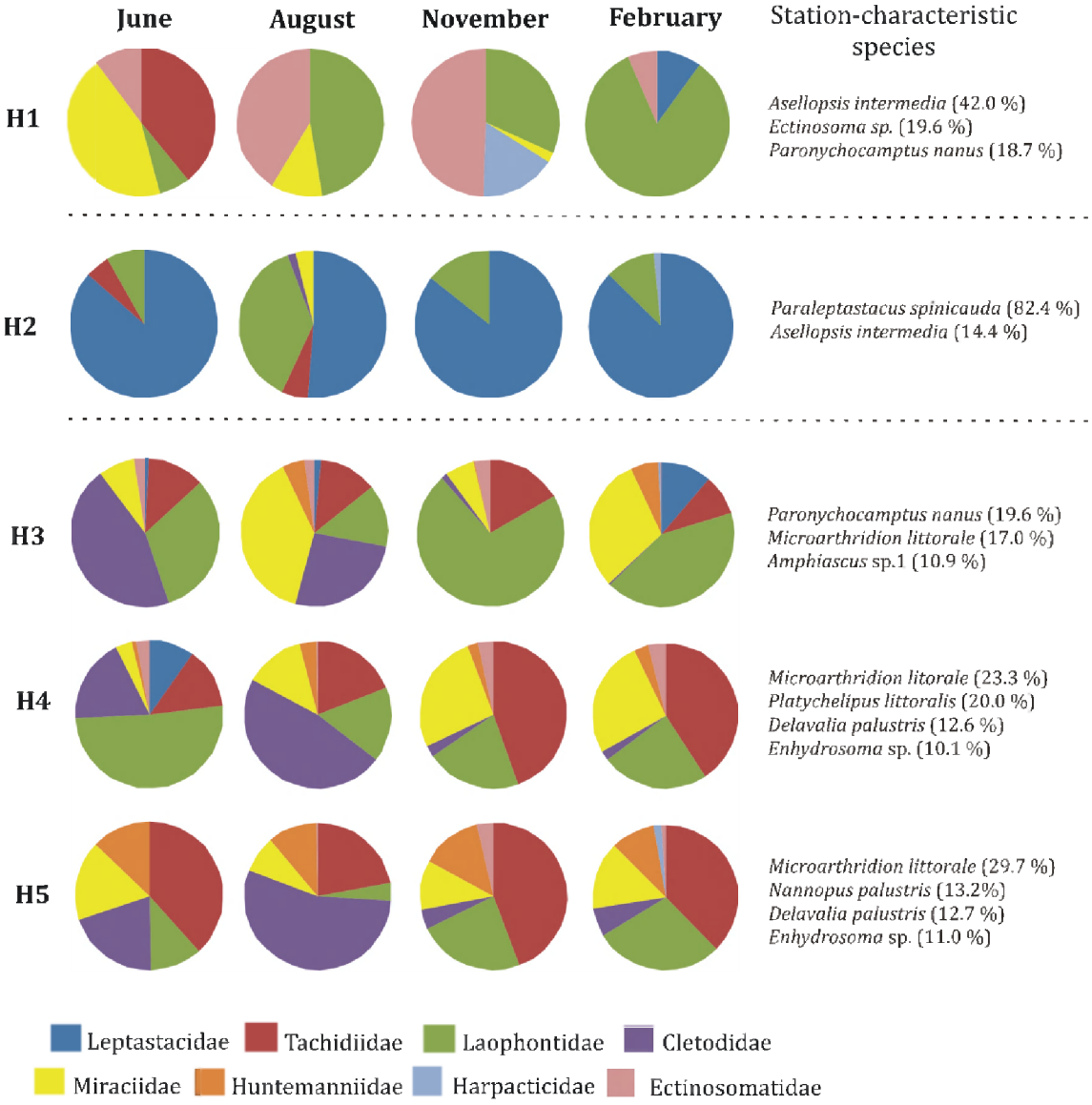

Fig. 7.. Spatio-temporal variability in harpacticoid communities based on relative family abundances, and an overview of the species indicated by PCO as characteristic for a particular station. Species contributions to 'station similarity', presented between brackets, were obtained by SIMPER analysis using a threshold contribution value of 10 %.

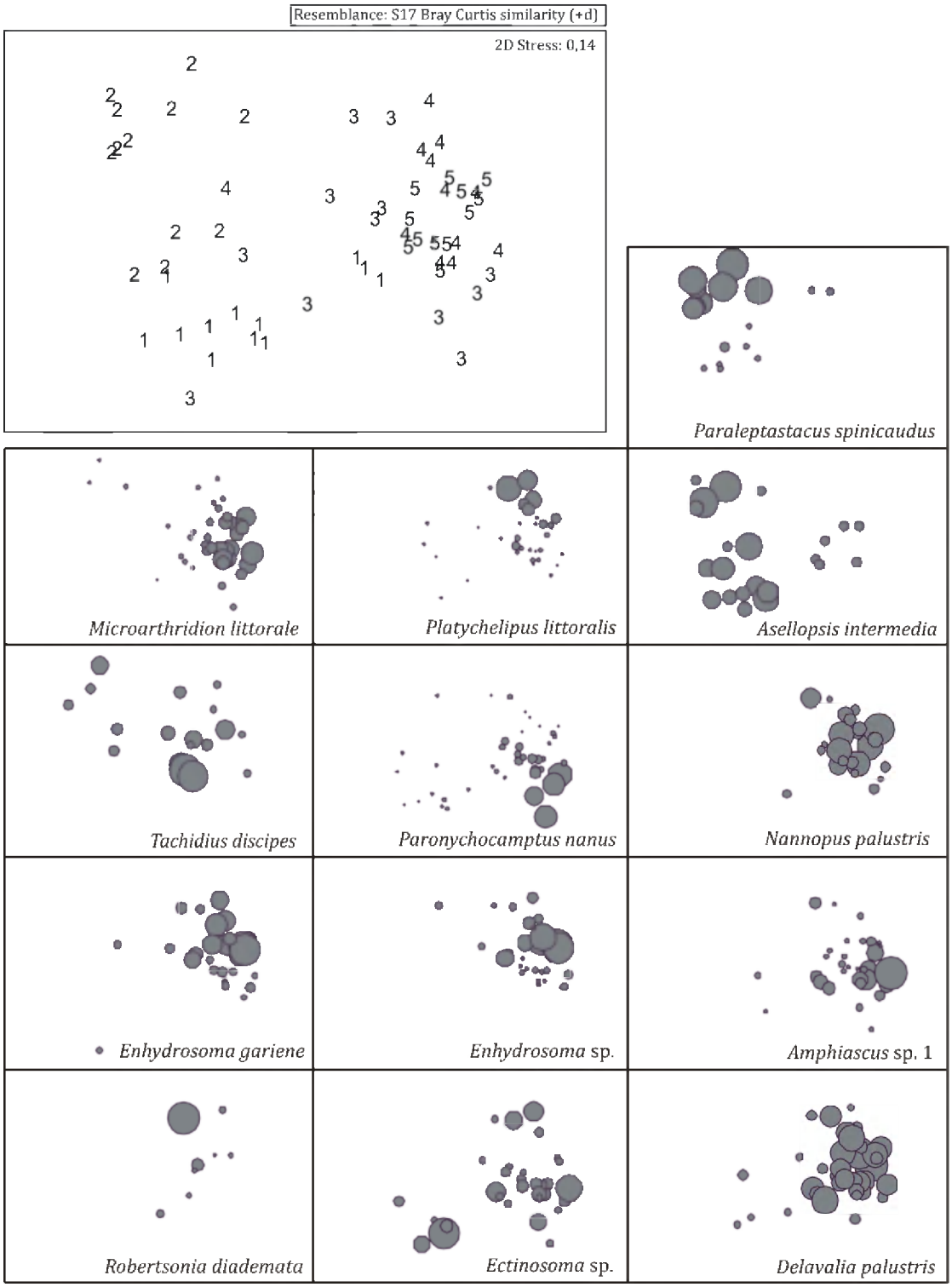

Fig. 8. nMDS presenting differences between harpacticoid communities from stations H1 to H5 (numbers 1 to 5) based on absolute species abundances, and species distributions along the stations.

Environmental variables explaining copepod assemblage differentiation

Since ordination of environmental variables (PCA) and copepod communities (PCO) showed similarities, it was expected that some of the measured environmental variables would contribute to the spatio-temporal variation in copepod assemblage composition. The subset of best explanatory variables, as determined by BV-STEP on the total dataset, was a combination of the following 6 variables (rho = 0.617, significance level < 0.1 %): TOM, NH_4^+, chla:TOM, pheo:chla, diato:chla and PRT. Four of these variables (TOM, pheo:chl a, diato:chla, PRT) were also important for station differentiation by ordination (PCA; Fig. 2).

The subset of environmental variables best explaining temporal shifts in copepod assemblage structure was determined by BV-STEP analysis per station to exclude the dominant spatial effect. Only for stations H1 and H5, a significant match between the copepod and environmental variables matrices was found (p < 1%, Table 4). The change in harpacticoid assemblages in these two stations related to changes in pigment ratios of fucoxanthin, diadinoxanthin and diatoxanthin (Table 4). In the other stations, except H2, carotenoid pigment ratios were also denoted as best explanatory variables, for instance zeaxanthin in station H4 (Table 4). More specifically, in station H1, the reduced copepod species richness in August concurred with reduced ratios of diatoxanthin and fucoxanthin to chl a (diato:chla and fuc:chla, fig. 9). Thus, the pigment diatoxanthin contributed to both spatial and temporal variation,

Table 5. Subsets of best explanatory variables for temporal fluctuation in harpacticoid community composition, with highest correlation coefficient (rho) and lowest number of variables (# var). Note that only for stations H1 and H5 the p values are low (< 1%).

	rho	p value (%)	# var	BEST var
H1	0.763	**0.1**	3	fuc:chla, diadino:chla, diato:chla
H2	0.452	52.0	2	MGS, LIP
H3	0.530	14.7	2	diadino:chla, diato:chla
H4	0.428	21.5	1	zea:chla
H5	0.618	**0.6**	2	diadino:chla, diato:chla

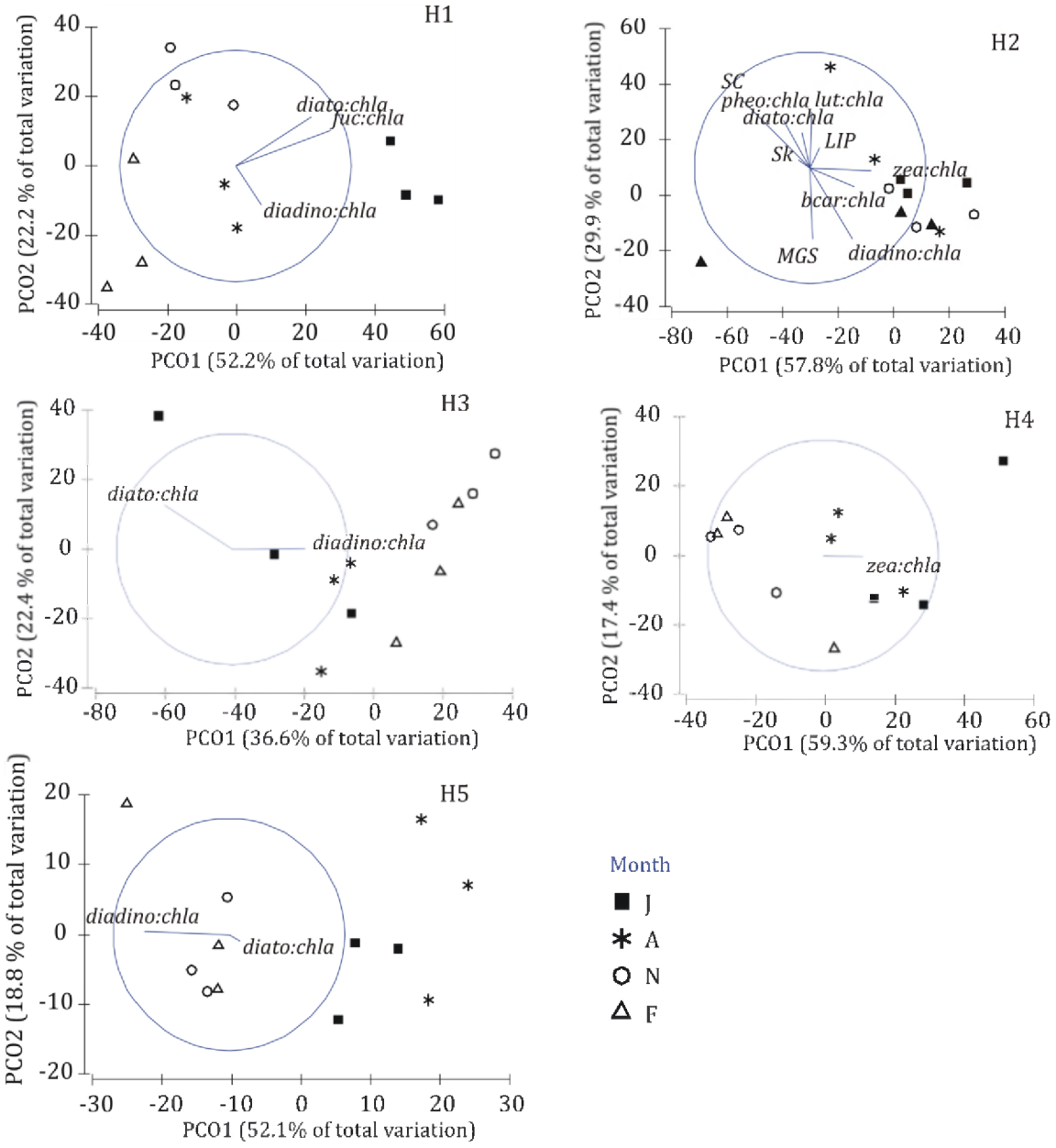

Fig. 9. Temporal variation in harpacticoid assemblages (PCO per station) and the best explanatory environmental variables (see Table 4). For station H2, other variables have been added which originate from other BEST subsets of variables though with equally high explanatory value (correlation coefficient rho). J, A, N, F (June, August, November, February)

Univariate correlation analyses of total copepod abundances with individual environmental variables (Table 6) also pointed at a substantial link with phytopigments. Total copepod abundances of stations H1, H2 and H3 related to fluctuations in pigments and pigment ratios. At the low-tide station H1, copepod abundances also correlated with median grain size and TOM. In contrast, at H4 and H5, copepod abundances did not significantly correlate to any of the environmental variables.

Species occurring in similar habitats, i.e. in salt marsh stations (H3, 4, 5) or tidal flat stations (H1 and H2), generally correlated in the same direction (positive or negative) to variables. Salt marsh species (e.g. *Microarthridion littorale*, *Platychelipus littoralis, Nannopus palustris, Enhydrosoma* sp. 1) correlated relatively strongly and positively with pheo:chla as well as with other pigment ratios. Species of tidal flat

stations and H3 generally showed negative correlations except with chla:TOM, indicative of food quality (fresh photoautotrophic production) and, for H2, also with median grain size.

Furthermore, within each station, the abundances of some species were mutually correlated (data not presented). At station H1, *Amphiascus* sp and *Tachidius discipes* abundances were highly correlated (r_s = 0.90) and additionally, a high abundance of these species alternated with a high abundance of *Asellopsis intermedia* (r_s = -0.72, r_s = -0.76). Also in station H2, *Asellopsis intermedia* abudances were inversely correlated to *Paraleptastacus spinicaudus* (r_s = -0.89). In H4, *Platychelipus littoralis* abundances were at their lowest when both *Amphiascus* sp 1 and *Microarthridion littorale* were at their highest (A-M: r_s = 0.81, P-A: r_s = -0.70, P-M: r_s = -0.67). This is a similar pattern to H1, where there was also a close coupling between a Tachidiidae species and Miraciidae species and a inverse relation of both with a Laophonthidae species. Finally, at H5, a significant correlation was found between *Paronychocamptus nanus* and *Enhydrosoma s*p (r_s = -0.73) and between *Delavalia palustris* and *Nannopus palustris* (r_s = 0.66).

Table 6. Significant spearman rank correlations between total harpacticoid abundances or relative species abundance and each environmental variable. The table includes only species which were highly specific for each habitat, as determined by SIMPER (see Fig. 7) or which were among the three most abundant in the habitat. Correlations between species and environmental variables were made on the largest spatial scale i.e. over all sampled stations where it occurred as well as on the scale of individual habitats. 'All' = between habitats (analysis of complete species dataset), 'H..' = within habitat fluctuation (analysis per station) and relation with environmental variables. Highest significant correlations (r_s > 70 %) are marked grey.

	Copepod abundance						*Paralaptastacus spinicauda*		*Asellopsis intermedia*				*Paronychocamptus nanus*		*Platychelipus littoralis*				*Ectinosoma sp.*		*Delavalia palustris*				*Amphiascus sp. 1*					*Microarthridion littorale*					*Tachidius discipes*		*Nannopus palustris*		*Enhydrosoma sp.*			
	all	H1	H2	H3	H4	H5	all	H2	all	H1H2	H1	H2	all	H3	all	H3H4	H3	H4	all	H1	all	H4H5	H4	H5	all	H1H3H4	H1	H3	H4	all	H3H4H5	H3	H4	H5	all	H1	all	H5	all	H4H5	H4	H5
MGS	-0.30	-0.66					0.65		0.32		0.88				-0.49				-0.29		-0.52		0.72		-0.36		-0.64			-0.56	-0.38					-0.76	-0.41		-0.42	0.45		
mud	0.36	0.65					-0.70		-0.43		-0.85				0.56				0.31		0.53		-0.58		0.43		0.64			0.61	0.38					0.76	0.47		0.47	-0.43		-0.59
SC		0.61																									0.68									0.73				-0.41	-0.21	-0.58
Sk		0.60																																								
NOx				0.75			0.26	0.56		-0.55				0.60	-0.36								0.71				0.72				0.35		0.66			0.76			-0.28			
NH4									-0.42		-0.64			-0.66	0.45						0.49				0.29		0.62			0.26	-0.37				0.47	0.73	0.25		0.28			
PO4							0.35		-0.27	-0.52	-0.61		-0.39						-0.35				-0.80				0.72		-0.70								0.76						
Si							-0.54		-0.49				0.28		0.44						0.39				0.41					0.51							0.58		0.45			
inorg. N	0.27								-0.42		-0.63				0.36						0.43				0.27	0.43	0.67								0.42	0.75						
nutrient	0.27								-0.47						0.42		0.61				0.46				0.33	0.43	0.63			0.33					0.36	0.75	0.27					
TOM	0.41	0.65					-0.65		-0.59		-0.93				0.59						0.59				0.42		0.67			0.73	0.55	0.56		0.63		0.75	0.67		0.50			
chla	0.30		-0.66				-0.30		-0.34					0.66	0.46						0.36				0.38	0.36				0.46	0.40						0.42				
pheo	0.50	0.62	-0.59				-0.67	-0.73	-0.59						0.64						0.58				0.52	0.43	0.83			0.77	0.55	0.60				0.76	0.69		0.64			
chlc		0.65												0.68												0.33					0.50											
fuc	0.32	0.58					-0.33		-0.38				0.31	0.66	0.27						0.36				0.47	0.51	0.63			0.49	0.50					0.75	0.38					
diadino													0.27	0.69												0.34				0.28	0.42									-0.42		
diato	0.40	0.87	-0.64				-0.61	-0.68	-0.53		-0.80				0.52				0.41		0.52		-0.66		0.51	0.39	0.79			0.65	0.43	0.57				0.76	0.50		0.47			
zea	0.57	0.79	0.62				-0.33		-0.68	-0.63	0.68				0.45						0.37		-0.68		0.36	0.36	0.83			0.61						0.76	0.51		0.54			0.76
lut	0.53	0.82					-0.53		-0.71		-0.64				0.56						0.38				0.46	0.35	0.77		0.59	0.74	0.38						0.61		0.64			
hcar	0.49	0.65					-0.50		-0.58						0.52						0.44				0.56	0.55	0.82		0.65	0.72	0.52	0.62				0.75	0.60		0.45			
CPE	0.40		-0.66				-0.38		-0.45					0.69	0.52						0.43				0.43	0.36				0.59	0.49						0.53		0.34			
CPE:TOM							0.58	-0.56	0.40						-0.37						-0.40					0.37		0.65		-0.42							-0.40		-0.34			0.80
chla:TOM							0.60	-0.56	0.48					0.59	-0.44						-0.42							0.79		-0.50			0.59					-0.48		-0.44			0.76
pheo:chl a	0.47	0.63					-0.67		-0.62		-0.78				0.60						0.55		-0.80		0.45		0.79			0.75	0.46					0.59	0.69		0.70			
chlc:chla									0.40						-0.55	-0.47	-0.59			0.62			0.64							-0.275							-0.40		-0.49			
fuc:chla	0.46	0.81		0.83					-0.61	-0.58	0.88		0.31	0.63					0.32						0.28	0.33	0.82				0.43					0.75						
zea:chla	0.46	0.73	0.67						-0.61	-0.58	-0.68				0.34								-0.71				0.84			0.48					0.29	0.75	0.40		0.50			0.82
lut:chla		0.76					-0.50		-0.69						0.46						0.31				0.39		0.62			0.67							0.55		0.62			
diadino:chla							0.38		0.26	-0.42					-0.34															-0.26			0.61		-0.28		-0.36		-0.58	-0.60		-0.78
diato:chla	0.27	0.74					-0.58		-0.44		-0.78				0.39				0.47		0.37		-0.68		0.37		0.75			0.51							0.33		0.43			
bcar:chla	0.40	0.69	0.69				-0.46		-0.54	-0.81	-0.76				0.30										0.42		0.73			0.58							0.41		0.42		-0.69	
LIP	0.38						-0.57		-0.55		-0.59				0.47						0.53				0.41		0.59			0.67	0.49	0.65				0.75	0.47		0.49			
PRT	0.29						-0.66	-0.60	-0.46						0.50						0.46				0.38					0.58							0.56		0.45			
PRT/TOM										0.54	0.72																															
bact-abund									-0.34						0.38						0.45								-0.87				-0.83		0.51			0.34			
bact-div															0.46	0.57			0.40		0.33				0.34					0.42	0.52			0.74					-0.58		
# of samples	40	40	40	40	40	40	40	8	40	16	8	8	40	8	40	16	8	8	40	8	40	16	8	8	40	24	8	8	8	40	24	8	8	8	40	8	40	8	40	16	8	8

DISCUSSION

Overall, the spatial heterogeneity of harpacticoid copepod species in the estuarine intertidal area under study was high, and over the 5 stations, 3 significantly different harpacticoid assemblages were recognized. Differences among copepod assemblages in terms of composition and abundance were most extreme between the tidal flat and the salt marsh stations, but also among the two stations on the tidal flat, which mainly differed in granulometry and tidal height. Harpacticoid variability among the salt marsh habitats (H3, H4, H5) was smaller despite their differences in tidal height, median grain size and prominence of vegetation. Harpacticoid assemblages in the mud and sand flat (H1, H2) were characterized by lower abundances and species richness, and at the sandy station H1 also by a high dominance of *Paraleptastacus spinicauda* and to a lesser extent *Asellopsis intermedia*. Interstitial Leptastacidae are highly competitive in sandy intertidal flats characterized by strong physical impacts and reduced food availability, and they may even occur as a monospecific assemblage (George & Rose 2004). In contrast to epibenthic species, interstitial *Paraleptastacus* sp has high vertical dispersal ability and an extended breeding season which contributes to their steady occurrence in disturbed habitats (Hockin & Ollason 1981). In contrast, *A. intermedia* may have a short breeding season. Lasker (1970) observed only one generation for *A. intermedia* in a sand flat in Scotland, with mating in August and hatching of nauplii from the eggs only after May in the next year (Lasker et al. 1970). This can be an explanation for their increased abundances in August. Moreover, fish predation on *A. intermedia* in sand flats is known to be a down-regulating factor during most of the year, whereas fish predation in muddy sediments is less specific due to the larger range of suitably sized prey (Gee 1987). In the sandy station (H2), harpacticoid assemblages showed no significant temporal shifts, and neither did median grain size and mud fraction. Food availability (chla) did, however, change during the year. This contributes to the idea that the copepod assemblage at this station is primarily structured by hydrodynamics and its effects on sediment granulometry, and by predation pressure. Production of microphytobenthos in this type of sediment can be very similar to that in siltier sediments, but its turnover through hydrodynamics tends to be considerably higher (Middelburg et al. 2000, Herman et al. 2001, Stal 2003, Stal & de Brouwer 2003), resulting in lower MPB biomass. MPB of sandy habitats is diverse, with epipelic and epipsammic species, the latter lacking temporal shifts (de Jonge 1985, Ribeiro et al. 2013). At the same time, deposition and retention of organic matter from the water column are very limited, resulting in sediments with low organic matter content but high OM quality as indicated by chla/TOM and PRT/TOM ratios. In addition to their better adaptation to hydrodynamic disturbance, copepods like *P. spinicauda* and *A. intermedia* may be more efficient in feeding on epipsammic MPB resources. For *Paraleptastacus*, the latter scenario is supported by fatty acid and stable isotope data, which suggest a consistent (throughout the year) indirect dependence on MPB carbon through grazing on bacteria (Cnudde et al., chapter 3), while *Asellopsis* is a direct diatom grazer (Cnudde et al., chapter 3).

In all other stations harpacticoid assemblages exhibited pronounced temporal variability, both in species diversity (N_0, N_1) and, primarily, species composition, but not in total copepod abundances. This may indicate that overall, food availability does not limit copepod abundances in this intertidal area, but that shifts in resource composition and/or other environmental variables drive assemblage composition.

Temporal changes in the copepod assemblage were station-specific. Firstly, highest temporal variability was found in the low-intertidal mud flat station H1, with two clear temporal shifts (between June-August and between November-February) in copepod dominance i.e. from (1) a Tachidiidae-Miraciidae dominated assemblage to a Laophontidae-Ectinosomatidae dominated assemblage, and from (2) a Laophontidae-Ectinosomatidae-Harpacticidae assemblage to a Laophontidae assemblage (*Asellopsis intermedia*) with some Leptastacidae (*Paraleptastacus spinicauda*), and with a general species impoverishment. The latter assemblage resembled the sand flat assemblage. Indeed, this strong shift in

copepod assemblage composition coincided with a significant change in granulometry from silty in June to fine to medium sandy in February. Such pronounced changes in granulometry in the upper sediment layers are typical for many tidal flat stations in this part of the Westerschelde Estuary, which is characterized by substantial seasonal transport-deposition cycles of fine particulate matter (Herman et al. 2001). Concomitantly, these sediments can rapidly shift between two stable states as a result of a complex interplay between sedimentation-erosion, microphytobenthos development and biodeposition/bio-erosion from deposit-feeding macrofauna (the Molenplaat, Herman et al. 2001, van de Koppel et al. 2001, Weerman et al. 2011, Weerman et al. 2012). Compared to station MP2, however, our station H1 may be even more temporally variable, because its low intertidal location hampers development of stable microphytobenthos biofilms due to the shorter exposure time and to hydrodynamics. The two alternative stable states are easily noticeable in this tidal flat station, where a basically sandy sediment in winter, very comparable to mid-tidal sandy station H2, becomes covered with a layer of silt of variable thickness in summer (June). Accompanying this shift in sediment granulometry are increases in food availability (chla, LIP, fuc:chla) and in nutrient concentrations (NH_4^+, PO_4^+) towards summer.

The increased abundance of Laophontidae (*Asellopsis intermedia*) and Leptastacidae (*Paraleptastacus spinicauda*) when sediments turn more sandy relates to niche specialization. *Asellopsis intermedia* is typically a sand-dweller while the latter only occurs in the sediment interstices (see above). For these species, the presence of fine particulate matter may be unfavorable due to clogging of interstitial spaces (for *P. spinicauda*) or even of the feeding apparatus. By contrast, *Amphiascus* sp. 1 (Miraciidae) and *Tachidius discipes* (Tachiidae) attained high abundances when sediment was silty (June). Peak abundances in spring are typical for *T. discipes* (Heip 1979, Herman et al. 1984). In addition, *T. discipes* shows characteristics of a niche generalist: its temporal variability correlated to almost all environmental variables while its spatial distribution correlated to nearly none of the variables (Table 5), including food quantity and quality related factors which were highly different among stations. *Amphiascus* sp. 1 feeds on diatoms (Mine et al. 2005; see also intermediate levels of diatom-specific 16:1w7, chapter 3, De Troch et al. 2006) with a potentially high selectivity towards larger cell sizes (De Troch et al., 2005). With an increase in granulometry, MPB species and functional group composition may change (Hamels et al. 1998), and this may affect *Amphiascus* abundances. For Ectinosomatidae, the limited number of correlations with environmental factors (Table 5) suggests Ectinosomatidae to be largely unaffected by overall changes in sedimentary variables. We hypothesize that they are highly flexible, and this fits well with the fact that the epibenthic Ectinosomatidae are highly motile and occur in a range of habitats (mud, sand, phytal) (Hicks and Coull, 1983). Salt marsh stations (H3, H4 and H5) shared many harpacticoid species but their contributions to the copepod assemblages were highly variable over space and time. Their spatio-temporal patterns cannot be easily explained based on a few environmental variables. In the following discussion, we focus on the most striking patterns and on species with high variability in abundance.

For some species, temporal changes in abundances were similar among the different salt marsh stations: (1) presence of Cletodidae specific in June-August (in H3, H4, H5), (2) maximum *Platychelipus littoralis* abundances in June (in H3, H4, H5), (3) decreasing abundances of *Microarthridion littorale* from June to August (in H4, H5) followed by an increase in November (in H3, H4, H5), and (4) a *Paronychocamptus nanus* increase in November (in H3, H4, H5). Cletodidae were strongly restricted to the warmer June-August period. Considering their specialized trophic ecology with a high dependence on sulphur-oxidizing bacteria (Cnudde et al., chapter 3), we suggest that spatio-temporal shifts relate to the seasonality in physical sediment characteristics which in turn affects the sediment sulfur cycle.

We have no clear explanation for the peaking abundances of *Platychelipus littoralis* in the salt marsh stations in June, but this peak coincided with deviant values for several nutrient concentrations (increased NH_4^+ and PO_4^+, decreased NO_x). This species is unique in its non-swimming behavior and is therefore directly linked to the local conditions on a microspatial scale. Similarly, the causes of the spatio-temporal distribution for the species *Paronychocamptus nanus* en *Amphiascus* sp. 1, in H3 en in H3-H4 respectively,

remained unclear, though the contrasting median grain size in H3-H4 excludes predominant influences of sediment granulometry on the spatial distribution pattern for these two species. Furthermore, also tidal height and food availability differed among stations H3 and H4. Distribution patterns of *Microarthridion littorale* related to food availability, with high abundances in H5 relating to TOM and temporal variability within station H4 correlating to absolute quantities of autotrophic resources (chla, diadinoxanthin) and trophic interaction with diatoms supported by elevated proportions of diatom FA 16:1ω7 during November-February (Cnudde et al., chapter 3). The lack of clear explanations for the distribution of these species in the field should be searched in our limited knowledge on their individual (feeding) ecology. The correlations observed in the present study could be underpinned or rejected based on future data from lab experiments.

The distribution *of Robertsonia diademata* (Miraciidae) was highly specific, restricted to H3 and with a peak in August. Iwasaki (1999) similarly observed their specific occurrence in a sandy sediment compared to a vegetated salt marsh. Stringer et al (2012) found a negative correlation between *Robertsonia propinqua* and *Enhydrosoma* sp. (Cletodidae), and a negative correlation of the former to median grain size. This fits relatively well with our results, where a shift from a Cletodidae dominance in June to an *R. diademata* dominance in August at H3 coincided with a small decrease in median grain size (ca. 15 µm) and with changes in other sediment characteristics: in August the mud fraction, sorting sediment sorting and sediment skewness reached maximum values and especially skewness was twice as high compared to June.

The strong spatial dissimilarities among harpacticoid copepod assemblages correspond to strong differences among environmental conditions between habitats. Apart from a large difference in median grain size and mud content, habitat types differed substantially in resource quantity, quality and diversity. For instance, while diatoms are probably the dominant photoautotrophs at all stations (Sabbe & Vyverman 1991), abundances and proportions of Cyanobacteria and chlorophytes were higher in salt marsh stations than in tidal flat stations, thereby diversifying resource availability. In addition, the salt marsh stations are accretory stations which potentially receive and retain substantial inputs of organic matter from the water column. Together with the higher stability of these sediments, which allow a higher build-up of MPB biomass despite MPB productivity does not necessarily exceed that on tidal flat stations, this implies that food availability in the marsh stations is much higher than on the hydrodynamically more disturbed bare tidal flat stations. This is clearly translated in higher abundances and diversity of harpacticoid assemblages. At the same time, however, a considerable fraction of the deposited material consists of more refractory, low-quality detritus; as a consequence, the salt marsh stations are characterized by higher OM stocks but of lesser overall quality. Moreover, the availability of high-quality resources to consumers may be partly hampered by their mixing with silt and refractory detrital particles (Herman et al. 2001). BEST-BVSTEP analysis indicated that spatial heterogeneity in harpacticoid assemblage composition was primarily linked to total resource availability (TOM), OM turnover (pheo:chla ratio) and proportion of diatoms to total photoautotrophs (diato:chla). Temporal variation was linked to microphytobenthos (diato:chla, diadino:chla). While most of the factors listed above are intricately related to sediment granulometry, sediment grain size and silt content did not appear among the factors best explaining variation in copepod assemblage composition. Owing to the general variability of biotic factors over space and time, partly correlating to physical sediment characteristics but additionally affected by other non-sediment physical variables (temperature, pH), these may have had more weight in the statistical tests, overshadowing the potential primary role of sediment granulometry. The apparently high structuring role of food-related environmental variables was also illustrated at the species level. Significant positive correlations between the distribution of harpacticoid species in salt marsh stations and a large number of biotic variables, including proxies for food availability, food type (different photoautotropic taxa) and food quality were found. For harpacticoid species from the bare tidal flat stations, the lack of positive relationships with food-related variables is likely due to the direct and comparatively high physical impact in these more hydrodynamically controlled systems. But note that it is within the range of expectations that all food-related environmental variables are strongly linked to

sediment characteristics and correlations between harpacticoids distribution and certain food-related environmental variables do not necessarily imply causation. Actually, two studies from the same Paulina intertidal area, Gallucci et al. (2005) and Van Colen et al. (2010), have well demonstrated the structuring role of physical variables e.g. sediment grain size and hydrodynamic regime, on benthic assemblages, of predacious nematodes and deposit-feeding macrofauna, respectively. These studies stress the consequences of changing environmental conditions to biological traits and hence, ecosystem functioning.
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Chapter 3

Trophodynamics of intertidal harpacticoid copepods based on stable isotopes and fatty acid profiling

In preparation:

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ABSTRACT

Lower food-web interactions between the meiobenthos and basal food sources are key drivers of benthic energy fluxes. Yet, they are most challenging to reveal due to the complexity of benthic resources and small sizes of interacting organisms. By means of stable isotopes ($\delta^{13}C$, $\delta^{15}N$) and fatty acids, we examined the variability of *in situ* diets of harpacticoid species and families from a heterogeneous tidal flat – salt marsh area (5 stations sampled). This was done to describe trophic heterogeneity among harpacticoid species and spatio-temporal dietary shifts of individual species. At all stations, microphytobenthos played a central role in harpacticoid feeding although the pathway (direct/indirect) was uncertain. For a limited number of species, dietary contributions of suspended particulate matter and bacterial-derived energy were found. In salt marsh stations, consumption of *Spartina alterniflora* detrital matter was low, and in the sand flat station with poor harpacticoid diversity, co-occuring species showed dietary differentiation. Copepod taxa with complete trophic independence of microphytobenthos were *Paraleptastacus spinicauda*, Cletodidae and potentially also Ectinosomatidae. Moreover, Cletodidae were highly specialist feeders of chemoautotropic matter.

Key words: harpacticoid copepods, intertidal, fatty acids, stable isotope analysis

INTRODUCTION

Harpacticoid copepods often comprise an important fraction of the meiofauna in marine sediments, usually only surpassed in abundance by nematodes (Hicks & Coull 1983). Harpacticoid assemblages are structured by a range of abiotic and biotic habitat characteristics, such as temperature, salinity, food availability, biogenic structures, predation, etc. (e.g. Chandler & Fleeger 1987, Azovsky et al. 2004, Giere 2009). In spite of their abundance, their roles in energy transfer in marine sediments remain unclear. Harpacticoids transfer primary production to higher trophic levels, mainly to juvenile fish (Gee 1989). Their main food sources, in turn, probably consist of microalgae, mainly diatoms (Montagna et al. 1995, Buffan-Dubau & Carman 2000), but cyanobacteria, cilates, phytoflagellates, heterotrophic bacteria, detritus, exopolymeric mucus and fungi have also been reported as food for harpacticoids (for overview see Hicks & Coull 1983). Despite this broad dietary spectrum, there is little evidence to suggest that harpacticoids would be generalist feeders, and rather little is known about species-specific differences in their nutritional requirements, and hence on resource partitioning (Lee et al. 1976, Carman & Thistle

1985, Pace & Carman 1996, Buffan-Dubau & Carman 2000). When feeding conditions are unfavorable, harpacticoids survive on their lipid reserves (Weiss et al. 1996), adjust their feeding rate (Montagna et al. 1995), or shift to alternative food sources as observed for planktonic copepods (Ger et al. 2011). Furthermore, the flexibility of their diet in response to spatial and temporal environmental variation has important consequences for the copepod's value as food for higher trophic levels (John et al. 2001). Much of the available information on harpacticoid feeding selectivity and flexibility is derived from lab experiments, whereas studies revealing the *in situ* contribution of different food sources to the diet of harpacticoids are few (Carman & Fry 2002, Rzeznik-Orignac et al. 2008).

Trophic biomarkers, such as stable isotopes and fatty acids (FAs), are highly suitable to investigate trophic interactions *in situ* (Carman & Fry 2002, Kelly & Scheibling 2012). Complementary use of stable isotopes and FAs to disentangle meiofaunal trophic interactions, is highly recommended (El-Sabaawi et al. 2009, Leduc et al. 2009). Consumer fatty acid composition may clarify ambiguities on food source consumption that remain as a consequence of overlap of carbon isotopic signatures between resources (e.g. benthic microalgae and *Spartina anglica* detritus). For instance, a strong reliance on microphytobenthos (MPB) can be deduced from a high polyunsaturated fatty acid (PUFA) content of the consumers (indicator FA), with eicosapentaenoic acid (EPA, 20:5ω3) and decosahexaenoic acid (DHA, 22:6ω3) characteristic for the diatom and flagellate component of the MPB, respectively. Highly specific FA or 'marker FA' of food sources are, for instance, FA 16:1ω7 for diatoms and low-chained odd-numbered FAs 15:0 and 17:0 for bacteria (Kelly & Scheibling 2012, and references therein).

In highly dynamic ecosystems like temperate intertidal zones, small-scale habitat heterogeneity and temporal variability in environmental parameters result in high spatio-temporal variability of harpacticoid community structure (Azovsky et al. 2004, Cnudde et al. chapter 2). Whether and how these structural shifts are accompanied by shifts in resource utilization and partitioning has not been properly investigated yet while this is crucial for understanding the role of harpacticoids in benthic energy fluxes. Using both natural isotopic signatures and FA composition of harpacticoid copepods, this paper focuses on spatial and temporal dietary variability of harpacticoid species in a temperate intertidal zone with high habitat heterogeneity. Harpacticoids from five stations on a tidal flat and salt marsh area, differing in tidal height, granulometry and vegetation, were sampled on 4 occasions with a 3-month interval. The first aim of this study was to investigate the relative importance of different food sources such as microphytobenthos (MPB), suspended particulate organic matter (SPOM), bacteria, vascular-plant and macroalgal detritus and epiphytes for intertidal harpacticoids. This would give an impression of the trophic diversity of Harpacticoida in a heterogeneous ecosystem. The second aim was to examine dietary variability of species in space and time, which is an indication of their trophic plasticity.

MATERIAL AND METHODS

Study area

Harpacticoid copepods were collected from five stations in the intertidal zone of the Paulina tidal flat and salt marsh, located along the southern shore of the polyhaline zone of the Westerschelde Estuary (SW of The Netherlands, 51°20'55.4''N, 3°43'20.4''E). The five stations differed in terms of intertidal position (tidal height), granulometry and vegetation and therefore we presumed food availability and diversity to differ among habitat types. The hydrodynamic disturbance of the sediment surface and light exposure time (both related to hydrodynamics) is expected to impact microbial biofilm formation and stability as well as deposition of detrital matter from different origins (Herman et al. 2001). The five stations were geographically oriented over an east-west distance range of approximately 670 m and a north-south distance range of approximately 550 m. The first two stations (H1 and H2) were situated in the tidal flat area. Station H1 was located in the lower intertidal and exhibited a temporally variable granulometry in

the upper cms of sediment, while station H2 was located in the mid-intertidal and was characterized by fine sandy sediment with a negligible silt fraction. The other three stations H3, H4 and H5 are situated in or at the edge of the marsh area, although samples were always collected in unvegetated sediment spots. Station H3 is a sediment patch positioned in the mid to high intertidal surrounded by *Spartina anglica*. Samples were collected within 10 cm of the *Spartina* vegetation, in sediments dominated by the fine sand fraction and with a variable mud fraction of 0 to 25 %. Station H4 is located in the high intertidal, near *Spartina* vegetation as well as a small area with stones covered by *Fucus vesiculosus*. Samples were collected at about 1 m from the *Fucus* vegetation. Station H5 is positioned in a marsh gully surrounded by dense vegetation, dominated by a combination of *Spartina anglica*, *Aster tripolium and Atriplex portulacoides*. Samples were collected at the bed of the gully. Since stations H3, H4, and H5 were in close proximity of salt marsh vegetation, we refer to these as 'salt marsh stations'.

Sampling procedure

Four sampling campaigns at the Paulina salt marsh were performed in the year 2010-2011, covering the four calendar seasons: 2-3 June 2010 (spring), 31 August - 1 September 2010 (summer), 29-30 November 2010 (autumn) and 7-8 February 2011 (winter). Intertidal sediments were sampled for analysis of harpacticoid and sediment fatty acids and stable isotopes. Additionally, samples were taken for the analysis of harpacticoid communities, and of biotic and abiotic sediment characteristics including sediment granulometry, dissolved nutrients, total organic matter, phytopigment concentrations, lipid and protein concentrations, and bacterial abundances and diversity (see Cnudde et al., in preparation, chapter 2)

Harpacticoid copepods for isotopic and fatty acid analyses were sampled qualitatively by collecting the top 1 cm of the sediment (approx. 1 m^2) during low tide. Copepods were extracted by rinsing the sediment with fresh water over a 250 µm sieve. The harvested copepods were divided in two samples: one from which copepods were collected alive for fatty acid analysis; the other was stored at -20°C until processing for later stable isotope analyses. We also collected triplicate sediment samples for isotope and fatty acid analysis of bulk sediment particulate organic matter (SOM) by means of 3.5-cm diam. plexiglass cores. These sediment cores were sliced into 0-0.5 and 0.5-1cm layers. Suspended particulate material (SPOM) was obtained through filtration on a precombusted GF/F Whatman glass fibre filter of surface water collected near the low water level. Fresh and decaying leaves or thalli of cordgrass, *Spartina anglica,* and of the macroalga *Fucus vesiculosus* were collected, rinsed with MQ water to remove adhering sediment particles, and their epigrowth scraped off using a glass slide cover slip. This 'biofilm' material was collected in MQ water and then concentrated through centrifugation. Epiphytic biofilm samples and (biofilm-free) cordgrass/macrophyte material were stored at -20°C prior to isotopic analysis, and so were SOM and SPOM samples for isotope analysis. Copepod and sediment samples for fatty acid analysis were stored at -80°C.

Fatty acid analysis

Fatty acid samples were prepared (personal protocol) from living copepod specimens within max. 2 days after field sampling to minimize FA losses. On the first day, copepods were sorted from the sediment under a Leica dissecting microscope (magnification 180 x) using a Pasteur pipette. Batches of different copepod taxa were washed three times in 0.2 µm filter-sterilized and autoclaved artificial seawater (Instant Ocean, salinity of 28) (ASW) to remove external, cuticle-attached particles, and were stored overnight in a climate room (15 °C, 12h:12h light:dark) to allow defecation. The following day, copepods were given a final wash by transferring them through sterile ASW, and collected on a precombusted GF/F Whatman filter (diameter 25 mm). Filters were stored in Eppendorf tubes at -80°C until FA extraction. Target sample size was usually 100 specimens per filter, but actual sample size and number of replicates

depended on the abundance and biomass of the copepod taxa: down to 60 specimens per sample for the largest taxa (e.g. *Platychelipus littoralis* and *Harpacticidae*), and up to 500 specimens for *Paraleptastacus spinicaudus*. Ca. ten specimens of each copepod taxon that was sampled for FA analysis were preserved on ethanol for later species identification. For FAME (fatty acid methyl ester) analysis of sediments, 1 – 1.5 g of lyophilized and homogenized sediment was used.

Lipid extraction, fatty acid methylation and analysis of fatty acid methyl esters (FAMEs) were executed according to De Troch et al. (2012a). Lipid hydrolysis and fatty acid methylation were achieved by a modified 1-step MeOH-H_2S0_4 derivatisation method after Abdulkadir and Tsuchiya (2008). Aside from dilution (FAMEs in 300 µl and 750 µl hexane for copepods and sediment, respectively), FAME extraction and analysis was similar for copepod and sediments. FAMEs were separated using a gas chromatograph (HP 6890N) with a mass spectrometer (HP 5973) based on a splitless injection (i.e. 1 µl and 5µl of extract for sediment and copepods, respectively) at a temperature of 250°C on a HP88 column (Agilent J&W; Agilent). FAMEs were identified based on comparison of relative retention time and on mass spectral libraries (FAMES, WILEY) by means of the software MSD ChemStation (Agilent Technologies). Calculation of FAME concentrations (µg FA per g sediment dry weight) was based on the internal standard 19:0. The FA short hand notation A:BωX was used, where A represents the number of carbon atoms, B gives the number of double bounds and X gives the position of the double bound closest to the terminal methyl group (Guckert et al. 1985).

Since sampled copepods were collected and combined over the top one cm of the sediment, sediment FAs of the two depth layers (0-0.5 cm and 0.5-1 cm, originating from the same sediment core) were combined by summing the raw FA data of the depth layers (i.e. surface areas of chromatogram peaks) and converting these to FA amounts (in µg FA) per g sediment dry weight based on the internal standard (19:0). Absolute FA concentrations of sediment and copepods were converted to proportions of total sample FA content (in %).

Several potential resources for harpacticoid copepods have unique FAs (marker FA) or are characterized by a specific combination of FA (indicator FA) (Table 1a). The presence of marker FAs in the copepods and certain FA ratios (Table 1b) can specify the type(s) of food that were consumed. The long-chained poly-unsaturated FA (PUFAs), EPA (20:5ω3) and DHA (22:6ω3) are essential FA for consumers. Ratios EPA/DHA and 16:1ω7/16:0 ratios in excess of 1 are indicative of diatom feeding (herbivory), while low EPA/DHA and high PUFA/SFA (saturated fatty acids) ratios are characteristic of carnivory, although a low EPA/DHA ratio may also point at the relative importance of dinoflagellates. A dietary contribution of bacteria can be deduced from the sum of odd-chained FA and from 18:1ω7c (see Table 1).

Stable isotope analysis

Harpacticoids for stable isotope analysis were obtained by handpicking and washing specimens thoroughly in ASW using an eyed needle. The copepod samples were processed within maximum 2-3 days; they were maintained at 4°C for most of this time and kept cool during handling using pre-cooled ASW. Triplicate samples of each harpacticoid taxon were prepared for carbon isotope analysis. Generally each sample was composed of at least 20 specimens in a precombusted (450°C, 3h) aluminium capsule (2.5 x 6 mm, Elemental Microanalysis). For smaller species (e.g. *Paraleptastacus spinicauda*), considerably more individuals (typically 100) were collected in order to obtain sufficient biomass for reproducible measurements. For the most abundant harpacticoid taxa, we prepared one or more sample(s) for dual (i.e. carbon and nitrogen) isotope analysis (personal protocol). Such samples usually contained 60 to 150 specimens, but up to 500 for *Paraleptastacus spinicauda*. Samples of plant material, epiphytes, SPOM and SOM were dried at 60°C and ground with mortar and pestle for homogenisation. Samples of 5-6 mg of plant material were prepared in tin capsules. Epiphytes (4-6 mg), SPOM (4-6 mg) and sediment samples (40-80 mg) were prepared in silver capsules and acidified *in situ* with dilute HCl (1% v/v) to remove

carbonates (Nieuwenhuize et al. 1994). Capsules were dried overnight at 60°C, closed and stored in a dessicator until analysis. Stable carbon and nitrogen isotope ratios were analysed using a C-N-S elemental analyser coupled to an isotope ratio mass spectrometer (V.G. Optima, Micromass, UK and Sercon Ltd., Cheshire, UK). Isotopic ratios were expressed as δ values (‰) with respect to the Vienna PeeDee Belemnite carbon and atmospheric N_2 standards: $\delta X = [(R_{sample}/R_{standard})-1] \times 10^3$, where X is ^{13}C or ^{15}N and R is the isotope ratio (Post 2002). Similar as for sediment FA, sediment isotopic data shown here represent the top 1 cm, and have been obtained by averaging the $\delta^{13}C$ and $\delta^{15}N$ signatures of the 0-0.5 and 0.5-1 cm layers originating from the same sediment core.

Data analysis

Spatio-temporal differences in sediment resource availability and composition were analysed based on the univariate data including sediment $\delta^{13}C$, sediment $\delta^{15}N$ and total (absolute) FA content, as well as on the multivariate relative FA composition data. After log-transformation, total FA content matched the assumptions of normality and homogeneity of variances (tested with the Shapiro-Wilk test and the Levene test, respectively) and two-way ANOVA was performed using stations (stat) and months (mo) as fixed factors. Tukey's HSD-test was used for *a posteriori* pairwise comparisons. Isotopic data did not match the requirements for parametric ANOVA, even after log-transformation and in some cases also suffered from low replication. Therefore, these data were analysed with two-way Permutational ANOVA (PERMANOVA, main test and pair wise test) with stations (stat) and months (mo) as fixed factors. Variability in the fatty acid composition as well as variability in the proportions of individual FAs or in FA ratios were also inspected using multivariate or univariate PERMANOVAs. PERMANOVAs were performed with 9999 permutations and were based on a Euclidian distance or Bray-Curtis resemblance matrix, for univariate or multivariate tests, respectively. Homogeneity of dispersions was checked via the PERMDISP routine. When this homogeneity is not met, interpretation of significant factor effects should be done with due caution. For pair wise tests with less than 10 unique permutations, Monte Carlo p-values were interpreted (Anderson & Robinson 2003).

As for sediment isotopes, spatio-temporal variation in copepod $\delta^{13}C$ signatures was tested with two-way PERMANOVA because assumptions for parametric tests were not met. Since PERMDISP often indicated heterogeneity of dispersions, any significant differences between these copepod isotope data need to be interpreted with caution. No estimation of the contributions of food sources to copepod diet was performed using an isotope mixing model such as SIAR (Parnell et al. 2010, Fry 2013). The accuracy of model-fitting is expected to be low for spatio-temporal studies where sampling of potential food sources was incomplete (not at all times and all places) (Dethier et al. 2013), given the likelihood of substantial spatio-temporal variation in natural isotopic signatures of potential food sources (e.g. marine macrophytes, Dethier et al. 2013).

Spatio-temporal variability in relative FA composition of sediment and copepods were visualized by non-metric Multidimensional Scaling (nMDS) based on a Bray-Curtis resemblance matrix of untransformed relative FA profiles. Spatial and temporal differences in most abundant FA in sediments as well as FA contributing to the unique character of stations (% contribution to group similarity) or to differences among stations or sampling dates (% contribution to dissimilarity) were determined using a two-way Similarity Percentages (SIMPER) analysis. Additionally, a one-way SIMPER (factor month) was executed for each station to denote more specifically which FAs changed over time within that station. Variability in the proportions of individual FA was inspected using univariate PERMANOVAs. Copepod FA compositions were further compared non-statistically, by describing those marker FA or FA ratios with striking values.

Parametric analyses (assumption testing, ANOVA and post-hoc test) were performed in R. All other analyses were conducted in Primer V6 (Clarke & Gorley 2006), using the PERMANOVA + add-on package (Anderson et al. 2008).

Table 1. Literature-based overview of (a) indicator fatty acids of marine resources and of (b) some FA markers or ratios regularly applied to indicate consumers' diet. Selected literature primarily based on benthic resources and consumers. Strongly modified from Leduc et al. (2009). LC-SFA = long-chained saturated FA; PUFA = poly-unsaturated FA

a	**Resource**	**Reference**
16:1ω7c 20:5ω3 (EPA) limited in C_{18}-PUFA	Diatoms	Graeve et al. (1997), Ackman et al. (1968), Volkman et al. (1980), Kharlamenko et al. (1995)
18:2w6, 18:3w3	Cyanobacteria, ciliates, vascular plant/terrestrial detritus, green algae (Chlorophyceae), macrophyta, (non-diatom sources)	Boschker et al. (2005), Kharlamenko et al. (2001), Graeve et al. (2002), Nelson et al. (2002), Cook et al. (2004)
15:0, 17:0, 15:1ω1, 17:1ω1 18:1ω7c	Bacteria	Volkman et al. (1980); Findlay et al. (1990)
16:1ω7c, 18:1ω7c	Chemoautotrophe bacteria	Van Gaever et al. (2009)
18:1ω9c	Bacteria, Phaeocystis (marine phytoplankton), green algae	Nichols et al. (1982), Volkman et al. (1980), Sargent and Falk-Petersen (1981)
20:4ω6	Protists, macroalgae, cyanobacteria	Graeve et al. (2002), Zhukova and Kharlamenko, (1999), Howell et al. (2003)
22:6ω3 (DHA) C_{18}-PUFA	Dinoflagellates	Sargent et al. (1987)
14:0	Prokaryotes (also diatoms)	Volkman et al. (1980); Findlay et al. (1990)
LC-SFA (with > C_{20})	Terrestrial plant debris	Douglas et al. (1970)

b	**Diet**	**Reference**
C16:1ω7/16:0	Diatoms	Ackman et al. (1968) (planktonic)
EPA/DHA	Diatoms/dinoflagellates	Kelly and Scheibling (2012)
C_{18} PUFA	Non-diatom feeding	Kayama et al. (1989)
Σ C15:0-C17:0	Bacteria	Kelly and Scheibling (2012)
20:1ω9	Carnivorous feeding (or de novo biosynthesis)	Graeve et al. (1997)
C18:1ω9c (oleic acid)	Carnivorous/detritivorous copepod	Sargent and Falk-Petersen (1981)
PUFA/SFA DHA/EPA	Carnivorous	Cripps and Atkinson (2000)

RESULTS

Characterization of sedimentary organic matter

Isotopic signature

Carbon isotopic signatures of sediment particulate organic matter (POM) differed between stations but not between sampling dates (main test: stat: $p < 0.001$, mo and stat x mo: ns) (Fig. 1). The sandy station H2 was ^{13}C-enriched compared to other stations (pairwise, all $p < 0.001$) with a $\delta^{13}C$ value of -18.3 ± 1.0 ‰ (mean ± SD), while stations H1 and H5 were ^{13}C-depleted (pairwise, all $p < 0.05$ except H1-H5: ns) with $\delta^{13}C$ value of -22.6 ± 0.5 ‰. Nitrogen isotopic signatures also differed mainly between stations but the interaction effect station x month was also significant (main test, stat: $p < 0.001$, mo: ns, stat x mo: $p < 0.01$; PERMDISP for stat x mo impossible – due to less than three replicates per group), with a clear difference between the isotopically heavier muddy salt marsh stations H4 and H5 and the other stations (pairwise, all $p < 0.01$, but H4-H5: ns, H1-H2-H3: ns). $\delta^{15}N$ values in H4 and H5 averaged 8.2 ± 0.6 ‰ and in other stations 6.4 ± 1.1 ‰. The significant interaction term mainly reflects the following differences in temporal behavior of sediment organic matter $\delta^{15}N$ between stations: no significant temporal variation at all for station H2, while stations H4, H5 and also H1 increased in $\delta^{15}N$ during the warmer period (pairwise, $p < 0.05$ for Aug-Febr in H4 and H5, and $p < 0.05$ for June-Febr in H1), and H3 decreased in $\delta^{15}N$ in late spring compared to late winter (pairwise, $p < 0.05$ for June-Febr in H3).

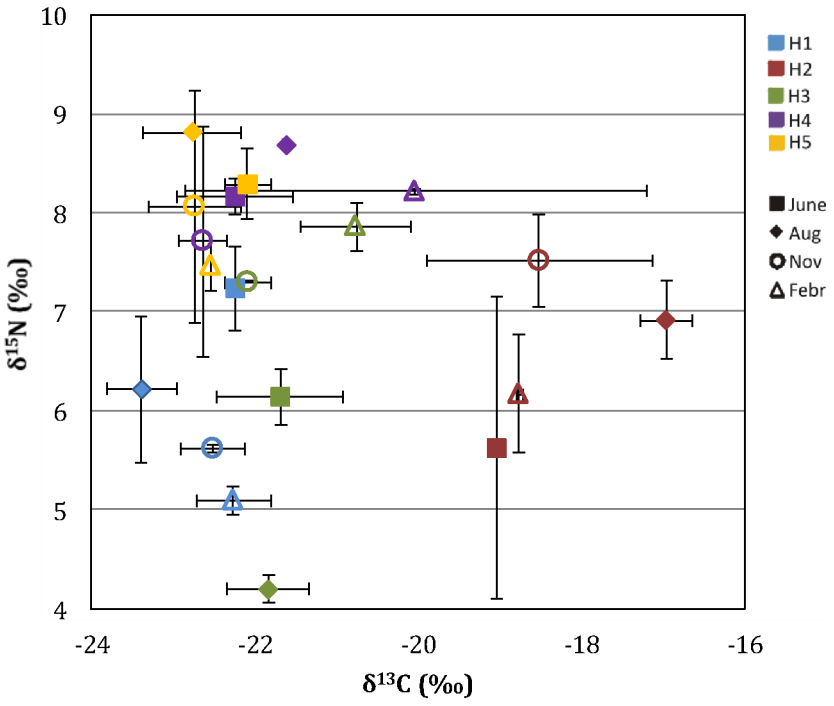

Fig. 1. $\delta^{13}C$ and $\delta^{15}N$ signatures of the sediment top 1 cm (mean ± SD, n = 2): stations are indicated by colors, sampling dates are indicated by symbols.

Fatty acid content

Considerable variability in sediment FA among replicates (small-scale patchiness) was present in both total FA content (Fig. 2; error bars) and FA composition (Fig. 3; sample spread), but variability was low for station H2. Total FA content showed complex spatio-temporal variation (main test: stat: $p < 0.01$, mo: $p < 0.05$, stat x mo: $p < 0.001$). There was a tendency of higher total FA amounts in stations H4, H5 and H1 (921 ± 297 µg/g and 793 ± 350 µg/g, 620 ± 420 µg/g, respectively) and lowest FA amounts, together with lowest temporal variability, in sandy station H2 (613 ± 196 µg/g) (Fig. 2). In addition, temporal changes within stations were only significant for H1 ($p < 0.05$ for June-Aug, June, Nov and Nov-Febr), and the exact timing of maximum and minimum FA content was station-specific (Fig. 2).

Similarly, H1 and H2 had the highest and lowest temporal variability in FA composition, respectively (Fig. 3). Overall, FA composition exhibited significant spatio-temporal differences (main test: stat, mo and stat x mo: all $p < 0.001$; PERMDISP of stat x mo: $p = 0.009$), but visually observed trends were not always strongly confirmed by the significance levels from pair wise PERMANOVA tests. nMDS (Fig. 3) did not clearly aggregate sediments according to station or month, but there were some trends: on the spatial scale, differences in FA composition were most explicit between the sand flat (H2) and salt marsh stations H3, H4 and H5 (pairwise within each month, most $p < 0.05$), positioned at the left and right side of the nMDS, respectively. Again, station differentiation was time-dependent. For instance, the sediment of H1 had a unique FA pattern in June only (pairwise within June, H1 versus all other stations, all $p < 0.05$), and the often quite similar sediments from the salt marsh area did differ from each other in November (H3, H4 and H5, all pair wise combinations, $p < 0.05$). Temporal fluctuations in FA composition were station-specific (main test: stat x mo: $p < 0.001$), but a general trend was noticeable with a shift in FA composition between warmer and colder periods: for stations H1, H2 and H3, February samples aggregated, and for stations H4 and H5 November-February samples were separated from June-August samples. EPA was not a characteristic FA in June and August (see further, < 10% contribution to group similarity) as a result of low EPA relative contributions (< 10% abundance) compared to November-February sediments.

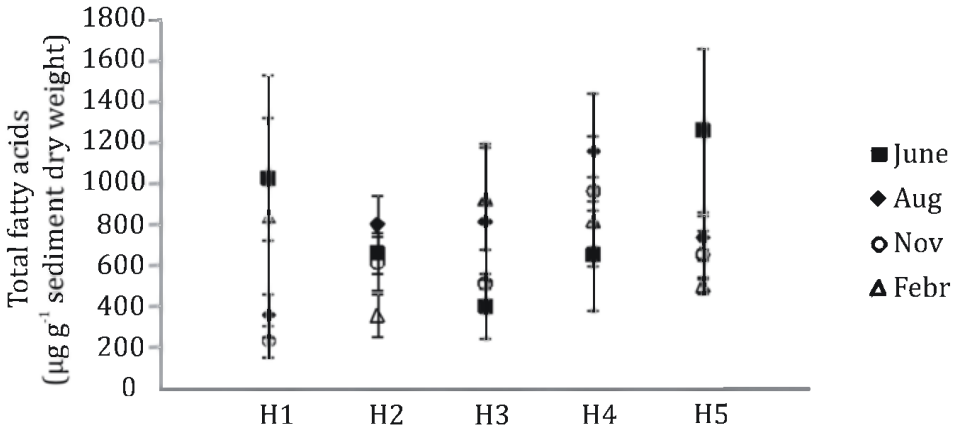

Fig. 2. Spatio-temporal variation in total fatty acid content of sediments (mean ± SD, n = 3)

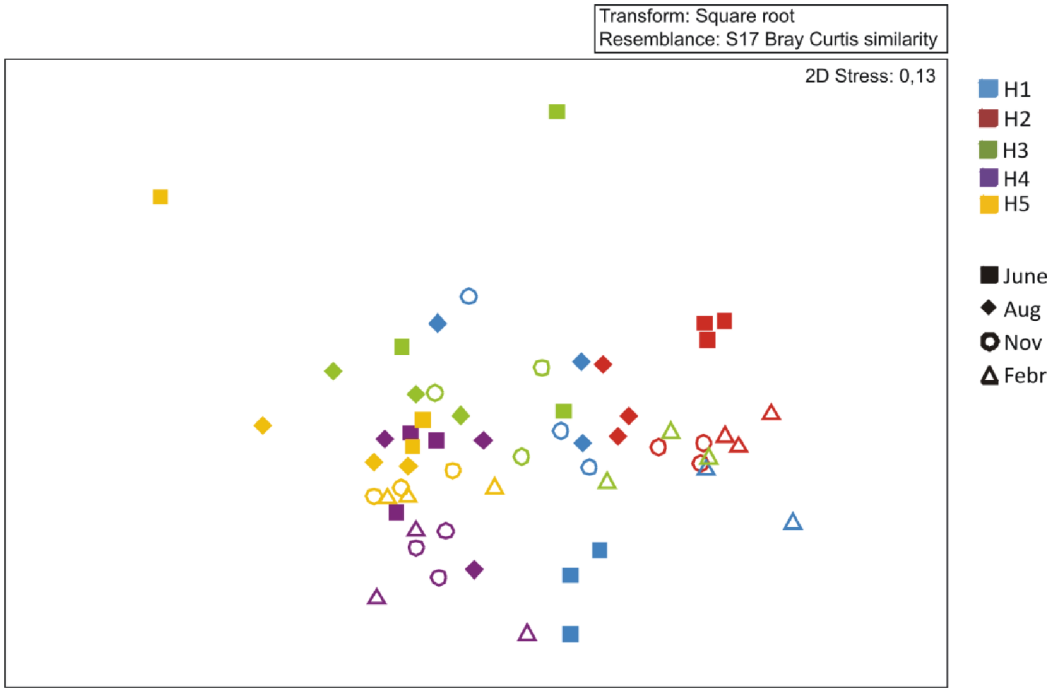

Fig.3. nMDS of sediment fatty acid composition, based on untransformed data: stations are indicated by colors, sampling dates by symbols.

Overall, the most characteristic sediment fatty acids were 16:1ω7 (diatom-specific), 16:0 and EPA (20:5ω3) (≥ 10% contribution to similarity within stations and to similarity within months, based on 2-way and 1-way SIMPER, respectively) and generally, their % contributions were a reflection of their relative abundances. These three FA constituted up to 78.0 % (cumulative abundance) of the FAs in H2, considerably more than in salt marsh stations H4 and H5, where they constituted ca. 57.0 %. The latter two stations further differentiated from tidal flat sediments H1 and H2 by the bacteria-specific FA 15:0 (≥ 10% contribution and ≥ 10% abundance). Highest proportions (and absolute concentrations) of bacteria-specific FA 15:0 and also 15:1ω5 were found in H4, containing 6 and 10 times higher levels, respectively, than at station H2. Station H5 is further characterized by FA 18:1ω9, which could originate from bacteria or phytoplankton (Table 1a). When present, C24:0 from vascular plant litter/detritus was only a minor component of sediment FA content, with relative abundances of < 1.3 %.

Spatial and temporal variability in sediment FA were often accounted for by the same characteristic FA. FA differences between station H2 and stations H3, H4 and H5 were attributed to higher levels of diatom-related FA 16:1ω7 and EPA in H2, and to higher levels of 15:0, 15:1ω5 and 18:1ω9 in the other stations. FA % contributions to dissimilarity among months revealed the following patterns: EPA generally increased in relative abundance towards winter (February), but contributed only little to temporal variation at station H4. Similarly, the other main PUFA, DHA, showed an increased relative abundance in colder months. Bacteria-specific FA (15:0 and also 15:1ω7) showed a reverse trend (e.g. station H3, H5), i.e. decreasing in February. In station H5, the temporal changes in EPA and 18:1ω9 were opposite.

Isotopic signatures of candidate resources

Potential food sources were characterized by specific $\delta^{13}C$ and $\delta^{15}N$ signatures, irrespective of spatial or temporal variability (Fig. 4; and data shown in addendum III - Table S1). Fresh *Spartina* and *Spartina* detritus in an early stage of decomposition were isotopically the heaviest carbon sources; while strongly decomposed, fibrous *Spartina* detritus was slightly more depleted in ^{13}C and its $\delta^{13}C$ overlapped with MPB

(ca. -17 to -14 ‰) (Fig. 4). MPB signatures of the Paulina tidal flat – salt marsh area were obtained from the study of Moens et al (2005a). Epiphytic biofilms had intermediate $\delta^{13}C$ (ca. -20 ‰), whereas *Fucus* detritus and SPOM were more depleted in ^{13}C (ca. -23 ‰). Nitrogen isotopic signatures increased from SPOM and MPB (ca. 6 ‰) to epiphytes (ca. 10 ‰), *Spartina* (ca. 13 ‰) and *Fucus* (ca. 15 ‰). $\delta^{15}N$ of *Fucus* even exceeded that of copepods (Fig. 4).

The spatial variability in POM carbon isotope signatures mentioned before, spanned a range of 4 ‰ in $\delta^{13}C$ values, from -18.3 ± 1.0 ‰ (H2, n = 8) to -22.5 ± 0.4 ‰ (H5, n = 8), the latter revealing a predominant contribution of settled SPOM to bulk sediment OM.

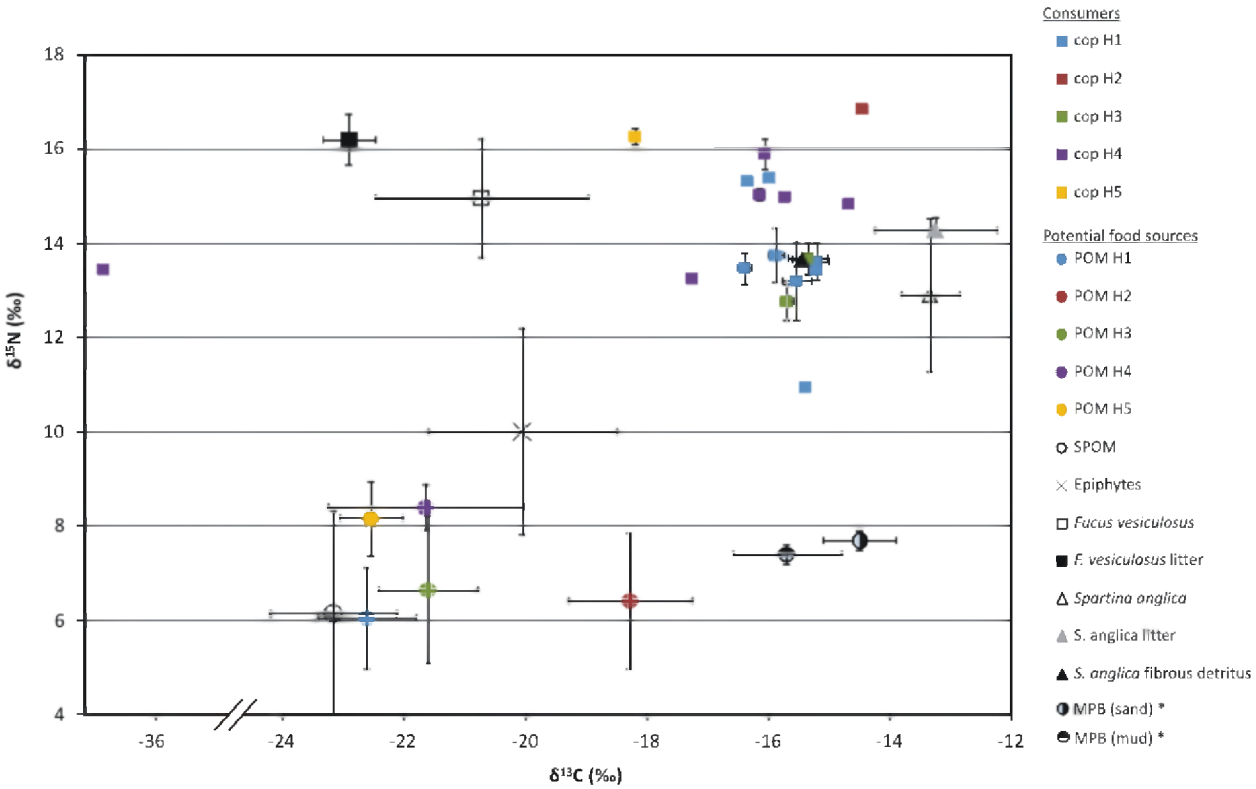

Fig. 4. $\delta^{13}C$ and $\delta^{15}N$ signatures (mean ± SD) of copepod as consumers (cop) and its candidate food sources in the Paulina study area. POM = sediment particulate organic matter, SPOM = suspended particulate organic matter, Epiphytes = epiphytes from *Fucus vesiculosus* and *Spartina anglica*, MPB = microphytobenthos. Asterics (*) indicates data that originated from the study of Moens et al. (2005). MPB data of Moens et al (2005) were collected in 2004, from a sandy and muddy flat, having similar granulometry and tidal position as stations H2 and H4 in current study. Copepod data point represent individual species from a certain month and station. If replicates were present, these were averaged (and indicated by error flags).

Isotopic signatures of harpacticoid copepods

Harpacticoid $\delta^{13}C$ values in the Paulina tidal area ranged from -40.3 to -12.1 ‰, with Cletodidae having extremely depleted values (mean $\delta^{13}C$ = -36.0 ± 2.7 ‰). Even though Cletodidae $\delta^{13}C$ values spanned a range of 6 ‰ (Table S2), compared to all other copepod taxa, their ^{13}C depletion was clearly consistent over all stations (H3, H4 and H5) and times. When considering all copepod taxa, copepod carbon isotopic data differed among stations and months (stat, mo: $p < 0.001$, stat x mo: ns, PERMDISP for stat and mo: $p = 0.018$, $p = 0.0001$). Highest $\delta^{13}C$ values were found in H2 (all $p < 0.001$) and lowest values in stations H3, H4 and especially H5 (compared to other stations, all $p < 0.05$, in between H3-H4-H5, all $p \geq 0.05$) (Fig.

5a). This pattern was still present, though less outspoken, when excluding the values of Cletodidae (Fig 5b). The absence of a correlation between the standard deviations on the $\delta^{13}C$ per station and time, and the number of species analysed (S) (Spearman rank correlation for S), strongly indicates that despite dissimilarity among data sets of the stations, in terms of species richness, spatial and temporal variability in $\delta^{13}C$ values is primarily caused by interspecific differences.

The majority of harpacticoid copepod species had average $\delta^{13}C$ signatures between -14 and -18 ‰ (Fig. 6). Aside from Cletodidae, the copepod taxa with lowest $\delta^{13}C$ values were *Paronychocamptus nanus* (mean $\delta^{13}C$ = -16.5 ± 2.8 ‰, with lowest value of -23.5 ± 1.2 ‰ at H5 in November), *Amphiascus* sp. 1 (mean $\delta^{13}C$ = -17.0 ± 1.8 ‰ with lowest value of -20.3 ± 0.7 ‰ at H5 in November) and *Microarthridion littorale* (mean $\delta^{13}C$ = -17.5 ± 1.7 ‰ with lowest value of -20.5 ± 1.7 ‰ at H4 in August). However, the $\delta^{13}C$ of these species was not consistent over time and stations. *P. nanus* $\delta^{13}C$ varied significantly (stat, mo, stat x mo: all $p < 0.01$), but this variation was largely limited to deviant values in H5 in November (spatial dissimilarity among H5 and the other stations: all $p < 0.01$). *Amphiascus sp. 1* $\delta^{13}C$ did not significantly change over time but did consistently exhibit spatial differences (stat: $p < 0.05$, mo and stat x mo: ns) between station H5 and stations H1 and H4 (H5-H1 and H5-H4, $p < 0.05$). Finally, significant temporal variability was found for *M. littorale* (stat: ns, mo: $p < 0.01$ and stat x mo: $p < 0.05$), a species which we found in generally high abundances in most stations and at most times. Temporal differences for *M. littorale* were, however, restricted to stations H4 (between June-Aug and Aug-Nov, both $p_{MC} < 0.05$) and H5 (between Aug-Nov and Febr-Nov, both $p_{MC} < 0.05$).

Copepod taxa with highest $\delta^{13}C$ values were *Asellopsis intermedia* (mean $\delta^{13}C$ = -14.3 ± 1.2 ‰, with highest value of -12.6 ± 0.1 ‰) and *Paraleptastacus spinicauda* (mean $\delta^{13}C$ = -14.1 ± 1.2 ‰ with highest value of -12.7 ± 0.6 ‰), for both at station H2 in February. The latter species did not exhibit significant spatial or temporal variation in $\delta^{13}C$ (stat, mo: ns, stat x mo: not tested due to limited dataset), while the former did (stat, mo: both $p < 0.01$, stat x mo: ns). In fact, a majority of copepod taxa showed significant spatial and/or temporal variation in $\delta^{13}C$. From the taxa with clear shifts mentioned earlier, the maximal temporal range of $\delta^{13}C$ was up to 7.8 ‰ for *P. nanus* in station H5 (between Nov-Febr) and its maximal spatial range of $\delta^{13}C$ was also 7.8 ‰, between H1 and H5 (in November). Copepod taxa with no spatio-temporal variability were restricted to *P. spinicauda* (statistical significance see above, range in $\delta^{13}C$ = 4‰) and Harpacticidae (range in $\delta^{13}C$ = 1.9‰; station, month, station x month, all $p > 0.05$). An overview of $\delta^{13}C$ values per copepod taxon over all stations and sampling months is given in Table S2 (addendum III).

The carbon isotopic ratios of copepods most closely resembled those of microphytobenthos (MPB) and the fibrous *Spartina anglica* detritus (Fig. 4). Comparatively ^{13}C-depleted signatures, primarily observed for copepod taxa from H5, may result from the consumption of epiphytic biofilms, but a mixture of MPB/*Spartina* detritus and SPOM may equally yield such an intermediate consumer $\delta^{13}C$. $\delta^{15}N$ of copepods covered a range of ca 5.5 ‰ (from 11.0 ‰ for *Tachidius discipes* at H1 to 16.9 ‰ for *P. spinicauda* at H2) (Fig. 4), which is equivalent to two or three trophic levels. Among H1 and H4 copepods, variability in $\delta^{15}N$ was relatively small (2-3 ‰). The $\delta^{15}N$ of many copepod samples was at least 5 ‰ higher than that of MPB. The restricted dataset of copepod $\delta^{15}N$ data does not allow to draw conclusions about species-specific and spatio-temporal variability in nitrogen isotope signature.

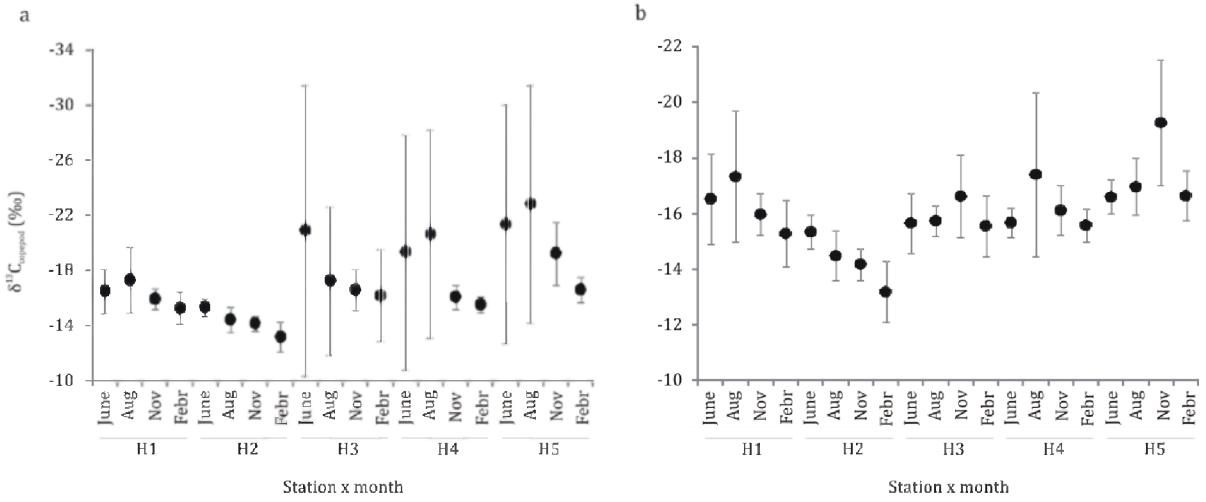

Fig. 5. Spatio-temporal $\delta^{13}C$ signatures of copepod communities as the average of $\delta^{13}C$ values of the participating copepod species (mean ± SD, n = variable), (a) with and (b) without inclusion of Cletodidae (present in H3, H4 and H5 only)

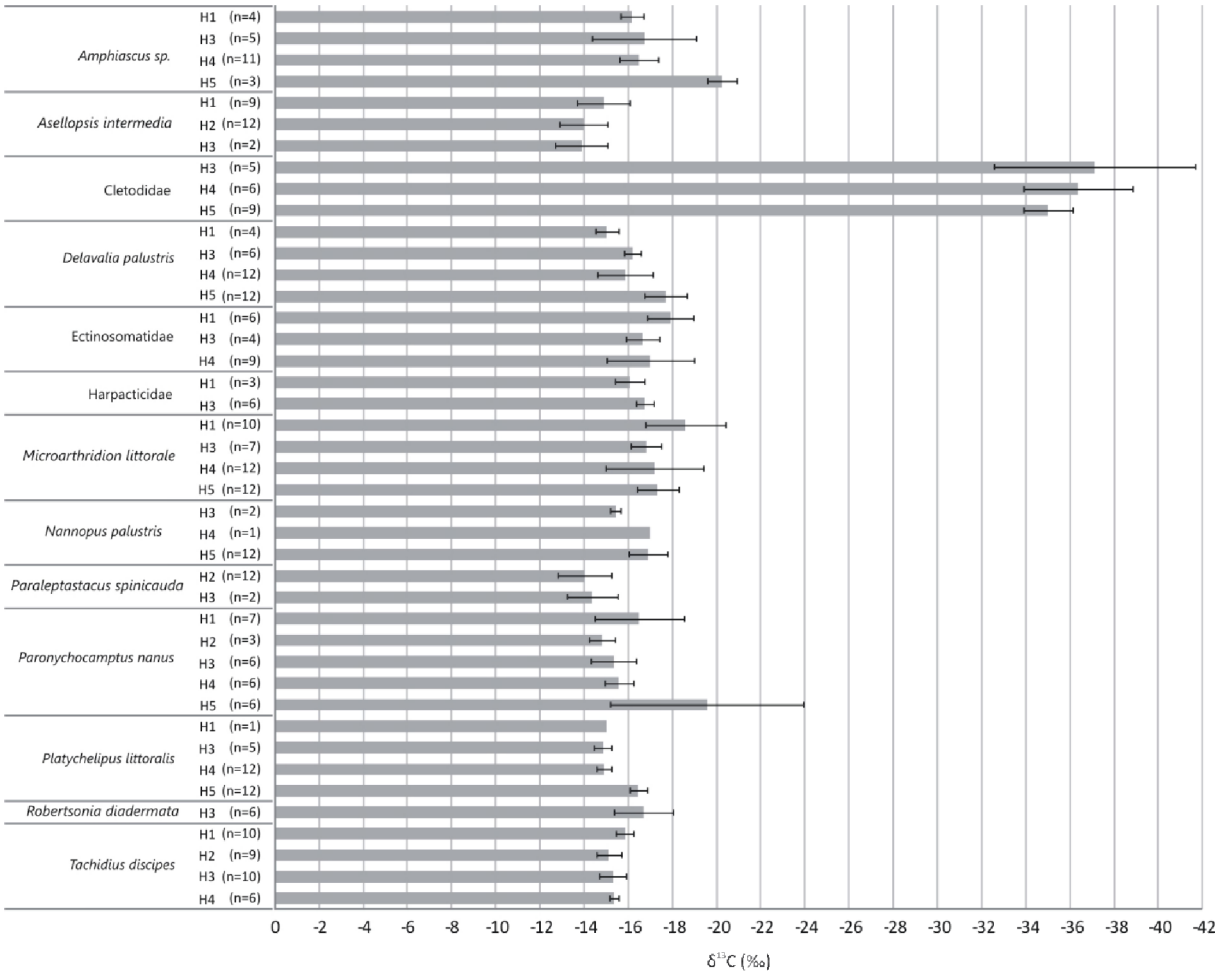

Fig. 6. Stable carbon isotope signatures of harpacticoid taxa from different stations (mean . Presented data are averaged values (± SD) of samples from the four sampled months.

Harpacticoid FA profiles

An nMDS showed no clear grouping of copepod samples by station or by sampling date (Fig. 7), although there was a tendency for copepods sampled in June-August (positioned at the left side) to be separated from copepods sampled in the colder period November-February (at the right side of the nMDS) (Fig.7), in line with the results of a two-way PERMANOVA (stat: ns, mo: $p < 0.001$, stat x mo: $p < 0.01$, PERMDISP: $p = 0.0003$). Total FA content of copepods varied over species and months (PERMANOVA; spec, mo: both $p < 0.001$, species x month: $p < 0.01$), with species being 2 to 10 times more FA-depleted during spring (air temperature of 30°C) compared to winter (pairwise, for each species, $p < 0.05$ for June-Febr). The highly abundant PUFAs, DHA (stat: ns, mo: $p < 0.001$, stat x mo: $p < 0.05$) and EPA (stat, stat x mo: ns, mo: $p < 0.001$) strongly differed over time. Copepods sampled in November and February showed higher amounts of DHA (pairwise, multiple $p < 0.05$, Nov-Febr: $p > 0.05$). We need to note here that due to this general temporal change in copepod FA composition, especially the PUFAs, dietary ratios FA/PUFA (e.g. PUFA/SFA used as indicator for carnivory) must be interpreted with caution.

Furthermore, copepods did not group by copepod taxon (taxa not shown in fig. 7). Exceptions to this rule were *Paraleptastacus spinicauda* and *Nannopus pallustris* (Fig. 7), the former also lacking variation in $\delta^{13}C$ values which are, in addition, positioned vary opposed from each other in the nMDS (fig. 7., encircled samples).

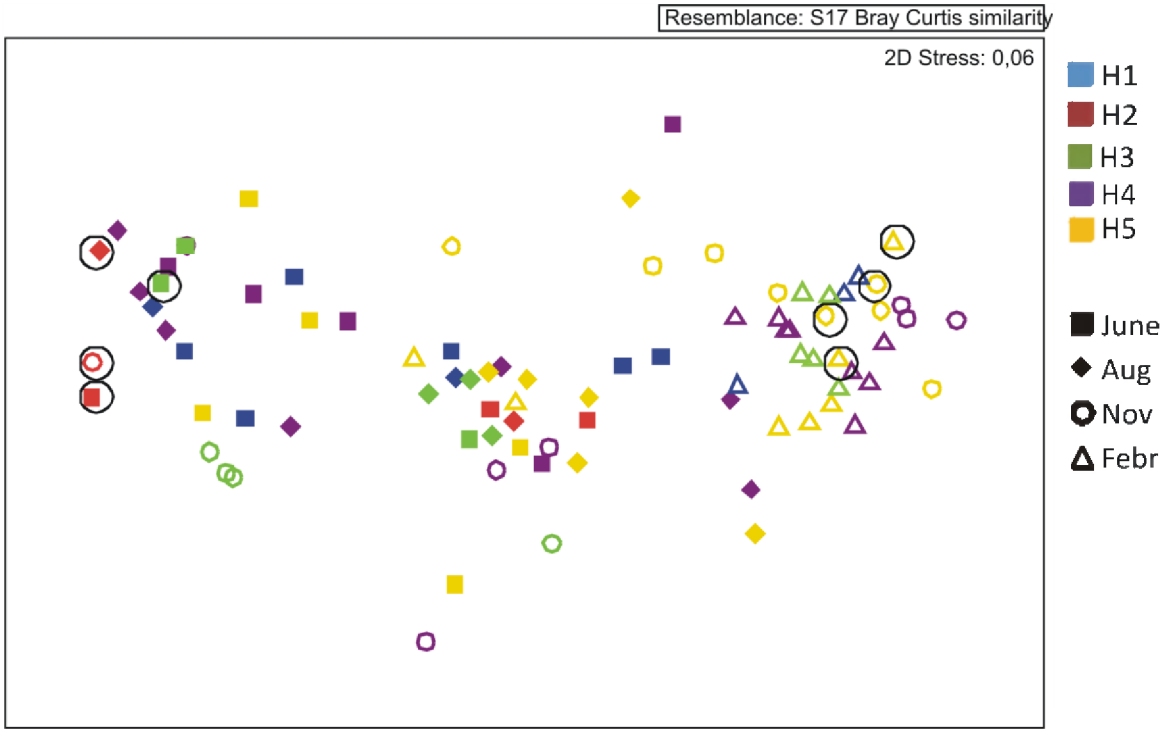

Fig. 7. nMDS based on relative FA profiles from copepods originating from different stations (indicated by colors) during 4 months (indicated by symbol). Encircled symbols at left and right side are *Paraleptastacus spinicauda* and *Nannopus palustris* samples.

The FA profile of *P. spinicauda* was characterized by (1) the (nearly) complete absence of conventional diatom FAs, i.e. PUFAs (EPA, DHA) and 16:1ω7, (2) the high abundance of total bacterial FA Σ 15:0-17:0, and (3) considerable proportions of 14:0 (Table 2; Table 3). FA profiles of *N. palustris* were dominated by PUFAs (> 50 % of total FA), with a predominance of EPA, intermediate levels of 16:1ω7 (9-12%) and the

presence of C_{18}-PUFA (Table 2; Table 3). More generally, C_{18}-PUFA were present in low proportion (≤ 2.3 %, constituting. 18:2ω6 and 18:3ω3) and in nearly all species, but were absent in *P. spinicauda, Cletodidae and Ectinosomatidae*. Highest values were measured in *Amphiascus sp. 1* and *Nannopus palustris*. Spatially, C_{18}-PUFA were absent or low in copepods from stations H1 and H2. Copepods can only obtain C_{18}-PUFA through uptake of non-diatom food sources (Table 1a).

16:1ω7 (characteristic for diatoms) was present in nearly all copepod species, except for Cletodidae, *P. spinicauda* and Ectinosomatidae, and at considerably higher levels (on average 11.7 %) than the C_{18}-PUFA, albeit with high spatio-temporal variability for most species. Copepods generally attained high EPA/DHA ratios (characteristic for herbivory), with values larger than 1 and were highest for *Platychelipus littoralis* and *D. palustris* (> 2.1, with few exceptions). In addition, the latter contained remarkably high proportions of bacterial FA (Σ15:0-17:0). Values of ratio 16:1ω7/16:0 were lower than 1. PUFA were nearly absent in Cletodidae, *P. spinicauda* and Ectinosomatidae. I these species, other trophic biomarker FA (ratio), e.g. PUFA/SFA and FA 20:1ω9, were present in low levels. However, *P. spinicauda* contained up to 15 % bacterial FA (Σ 15:0-17:0, Table 3), which in all other copepods except *D. palustris* contributed < 4 %. The one sample of Cletididae did not contain substantial levels of bacterial FA. FA 18:1ω7 is a potential bacterial marker (Table 1a). Because of its similar retention time as FA 18:1ω9c, this FA could not be separately identified. FA 18:1ω7 was part of the reported 18:1ω9c levels, which constituted less than 2 % of Cletodidae FA. Next to EPA-rich diatoms, DHA-rich dinoflagellates are an important component of the MPB and preferential feeding on dinoflagellates would result in EPA/DHA < 1. Low values of 18:1ω9c occurred for *Tachidius discipes*, *Microarthridion littorale* and *P. spinicauda*.

Variability in copepod FA profiles cannot easily be associated with observed spatio-temporal shifts in copepod $\delta^{13}C$ since not only those species with high $\delta^{13}C$ variability, but most copepod species showed a certain level of variation in the marker/indicator FA (proportions of 16:1ω7, EPA, DHA C_{18}-PUFA and bacterial FA and ratio EPA/DHA). The strongest $\delta^{13}C$-depleted *Amphiascus sp. 1*, observed in November at H5, showed lowest EPA/DHA and lowest proportion of bacterial FA (Table 2 and 3). For the highly $\delta^{13}C$-depleted *P. nanus* and $\delta^{13}C$-enriched *A. intermedia*, in November at H5 and in February respectively, no complementary FA samples are present. However, for *P. nanus* in February, we noticed a lower 16:1ω7 proportion and remarkably low PUFA content (EPA + DHA = 17%) considering the pattern of PUFA-enrichment of most copepods during colder months, which could be indicative for copepods' independence of microphytobenthos during that period.

			Relative fatty acid profile																
			C14:0	C15:0	C15:1ω5	C16:0	C16:1ω7	C17:0	C17:1ω7	C18:0	C18:1ω9t	C18:1ω9c*	C18:2ω6	C18:3ω3	C20:1ω9	20:4ω6	20:5ω3	22:6ω3	
Amphiascus sp.	H1	June	2.14	0.44		28.87	11.39	1.52		11.16	0.84	5.73			0.64	0.59	24.35	12.35	n = 2
	H3	Febr	1.13	0.63		16.86	12.34	1.10		4.14	1.62	9.99	1.49	0.68	0.64	0.52	32.5	16.33	
	H4	Nov	1.08	1.83	0.27	9.99	7.06	1.49	4.52	2.19	2.00	5.79	1.18	0.53	0.30	0.54	35.66	25.58	
		Febr	0.93	0.69		15.30	8.06	1.28	1.63	4.07	1.4	7.29	1.45	0.40	0.41	0.40	34.95	21.73	
	H5	Nov	1.00	0.58		14.64	7.3	0.83		4.94	2.44	5.88	1.72	0.62	0.47	0.65	33.01	25.93	
Asellopsis intermedia	H1	Febr	1.36	0.86		23.10	19.84	0.85	0.50	4.11	1.24	2.45	0.56		0.36	1.87	24.83	18.09	
	H2	June	2.49	0.76		35.71	19,00	0.88		10.34	0.78	2.33				1.09	13.21	13.41	n = 2
	H5	Nov	2.03	0.39		21.97	9.32	0.39		6.31	1.24	1.11	0.50			0.86	30.89	24.98	
Cletodidae	H4	Aug	1.81	0.49		54.72	0.53	0.58		40.21		1.67							
Delavalia palustris	H1	June	1.89	1.01		28.83	9.57	1.51		11.95	1.21	4.77	0.61		1.69	1.96	23.80	11.19	
	H4	Aug	2.74	4.94	0.71	35.93	8.65	4.89	4.22	16.61	0.87	4.09	0.84	1.30	1.02	3.36	25.04	11.04	n = 2
		Nov	4.58	9.82	0.39	33.59	17.07	5.08	10.44	7.87	0.77	0.84	1.04	0.82	0.74	0.33	5.84	0.77	
		Febr	1.23	1.95	0.48	15.74	9.24	2.42	3.07	4.47	1.12	7.60	1.09	0.39	2.30	1.37	32.13	15.42	
	H5	June	3.44	9.67	0.96	32.98	15.4	5.37	6.14	10.31	0.36	3.61			0.78	0.70	7.74	2.52	
		Aug	1.24	4.21	0.82	21.45	7,00	5.33	3.95	10.46	0.72	4.76	0.63	0.93	1.34	4.20	21.68	12.36	n = 2
		Nov	1.29	1.25	0.33	11.15	9.41	1.07	3.20	2.58	0.60	5.13	1.20	0.39	1.57	1.55	40.96	18.32	
		Febr	2.41	1.40	0.29	19.20	12.08	0.75	1.32	5.00	0.90	7.13	1.84	0.38	2.15	1.30	33.19	11.30	n = 2
Ectinosomatidae	H1	June	2.33	1.02		51.72	2.46	2.14		24.16							10.52	5.65	
	H4	June	2.65	1.37		55.96	1.76	7,00		32.31		2.86					2.22		
Microarthridion littorale	H1	Aug	4.16	1.31		57.81	4.35	1.63		29.82	0.92								
		June	5.46			58.60	15.49			16.21							4.24		
	H3	Febr	1.41	0.39		18.98	14.5	0.43		3.60	0.74	3.07	0.37	0.20		0.86	25.46	29.98	
	H4	June	0.58	0.38		54.65	8.09	0.56		26.23		1.34					4.68	3.49	
		Aug	3.51	1.10		58.28	11.1	0.81		21.81	0.56	1.36					3.30	1.54	n = 2
		Nov	3.33	1.07		43.13	16.00	0.72		15.87	1.01	2.30	0.49			0.36	9.21	6.68	n = 3
		Febr	1.51	0.58		19.18	14.87	0.80	1.38	3.34	1.07	4.79	1.11	0.22	0.49	0.51	28.81	22.59	n = 3
	H5	June	3.10	0.75		44.21	18.86	0.53	0.15	10.8	0.66	2.02	0.17			0.35	9.69	8.98	n = 3
		Aug	3.07	0.96		39.13	14.37	0.83		9.11	1.35	1.67	0.71			0.90	13.85	14.58	n = 3
		Nov	1.55	0.39		30.59	6.79	0.37		15.25	0.81	1.19	0.57	0.20		0.59	18.26	23.37	n = 3
		Febr	3.48	0.75		39.70	16.04	0.76		9.71	1.68	3.03					14.37	10.49	
Nannopus palustris	H5	Nov	1.31	0.50	0.07	16.75	8.80	0.43		4.51	1.37	4.17	1.25	0.17	0.58	0.65	30.89	28.53	n = 2
		Febr	1.76	0.46		17.11	12.92	0.45		3.24	1.60	5.96	1.86	0.23	0.84	0.47	33.37	19.71	n = 2
Paraleptastacus spinicauda	H2	June	6.90	6.30		58.59		8.72		19.49									
		Aug	0.93	0.42		57.76	0.28	1.09		37.93	0.18	0.12					0.37	0.44	
		Nov	4.49	4.41		58.40		10.05		22.64									
	H3	June	2.77	1.68		55.67	2.84	2.99		31.05	0.60	2.40							
Paronychocamptus nanus	H3	Aug	3.13	1.03		37.53	20.11	1.31		11.46	0.88	2.52	0.39	0.63		0.93	11.13	8.94	
		Nov	5.89	2.00		63.00	15.68	1.63		11.60	0.60								n = 3
		Febr	1.75	0.42		19.62	14.81	0.47		3.52	1.34	2.51	0.36	0.22	0.52	0.76	34.12	19.83	n = 2
	H5	Febr	4.00	0.61		45.62	11.8	0.54		15.77	2.60	1.96					13.51	3.59	
Platychelipus littoralis	H4	June	2.34	0.68		41.83	15.85	0.54		15.28	1.25	4.47	0.41		0.53	0.89	11.16	4.98	n = 2
		Aug	1.33	0.86		23.69	16.68	0.88	0.37	5.47	1.15	5.90	0.48	0.22	0.58	1.24	28.35	12.8	
		Nov	1.33	0.70	0.12	13.38	9.39	0.62		2.86	1.06	3.60	0.50	0.13	0.71	1.26	38.97	25.43	n = 2
		Febr	1.48	0.42		16.02	12.04	0.59		2.70	0.99	6.21	0.92	0.22	0.81	0.73	40.37	16.5	
	H5	June	4.96	1.41		43.06	6.68	0.96		29.78		13.14							
		Febr	1.63	0.36		18.12	14.13	0.39		3.28	0.91	6.16	1.03	0.21	0.80	0.65	38.68	13.65	
Tachidius discipes	H1	June	5.23	1.54		58.33	9.07	1.2		24.63									
		Febr	2.30	0.47		14.92	13.75	0.23		2.62	1.21	3.98	0.65	0.23	0.25	0.56	27.52	31.33	n = 2
	H2	June	2.11	1.42		38.74	8.05	1.70		17.33	0.68	1.36				0.95	9.4	19.09	n = 2
	H3	June	1.71	1.18		45.49	18.33	1.70		12.08	0.35	2.83				0.90	7.62	7.81	
		Aug	3.63	1.37		43.57	14.73	1.33		14.54	1.46	1.61					10,00	7.76	
		Nov	5.32	1.63		31.99	28.63	0.69		5.34	2.99	3.74				0.42	10.86	8.39	
		Febr	1.74	0.05		16.64	12.51	0.29		4.32	2.09	3.94	0.44	0.14	0.20	0.41	27.23	30,00	
	H4	Febr	2.43	0.51		21.65	12.40	0.45	0.47	5.62	1.85	4.27	0.35	0.21	0.28	0.23	23.63	25.90	n = 2

* or C18:1ω7c

Table 2. Relative fatty acid profiles of harpacticoid species. If more than one replicate, the number of replicates is indicated (right).

			Diatoms	Diat./Flagel.	non-diatom	Bacteria	Bacteria (partially)	Carnivorous	Carnivorous	
			16:1ω7/16:0	EPA/DHA	C_{18} PUFA	Σ15:0-17:0	C18:1ω9c*	C20:1ω9	PUFA/SFA	
Amphiascus sp.	H1	June	0.4	2,00		1.96	5.73	0.64	0.85	n = 2
	H3	Febr	0.731	1.99	2.17	1.73	9.99	0.64	2.16	
	H4	Nov	0.71	1.39	1.71	3.31	5.79	0.3	3.83	
		Febr	0.53	1.61	1.85	1.98	7.29	0.41	2.65	
	H5	Nov	0.5	1.27	2.33	1.41	5.87	0.47	2.82	
Asellopsis intermedia	H1	Febr	0.86	1.37	0.56	1.71	2.45	0.36	1.5	
	H2	June	0.54	1,00		1.64	2.33		0.57	n = 2
	H5	Nov	0.42	1.24	0.5	0.79	1.11		1.84	
Cletodidae	H4	Aug	0.01			1.06	1.67			
Delavalia palustris	H1	June	0.33	2.13	0.61	2.52	4.77	1.69	0.83	
	H4	Aug	0.33	2.27	2.14	9.83	4.09	1.02	1.13	n = 2
		Nov	0.51	7.59	1.86	14.9	0.84	0.74	0.14	
		Febr	0.59	2.08	1.48	4.37	7.6	2.3	1.95	
	H5	June	0.47	3.07		15.04	3.61	0.78	0.18	
		Aug	0.34	1.81	1.24	9.54	4.76	1.34	0.94	n = 2
		Nov	0.84	2.24	1.59	2.32	5.13	1.57	3.6	
		Febr	0.63	2.94	2.23	2.15	7.13	2.15	1.68	n = 2
Ectinosomatidae	H1	June	0.05	1.86		3.16			0.2	
	H4	June	0.03			2.24	2.86		0.02	
Microarthridion littorale	H1	Aug	0.08			2.94				
		June	0.26						0.05	
	H3	Febr	0.76	0.85	0.58	0.82	3.07		2.29	
	H4	June	0.15	1.34		0.94	1.34		0.1	
		Aug	0.2	2.14		1.92	0.68		0.06	n = 2
		Nov	0.41	1.6	0.49	1.79	2.3		0.32	n = 3
		Febr	0.77	1.31	1.33	1.38	4.79	0.49	2.1	n = 3
	H5	June	0.44	1.13	0.17	1.28	2.02		0.36	n = 3
		Aug	0.37	0.95	0.48	1.79	1.67		0.57	n = 3
		Nov	0.24	0.78	0.77	0.75	1.19		1,00	n = 3
		Febr	0.4	1.37		1.5	3.03		0.46	
Nannopus palustris	H5	Nov	0.53	1.1	1.42	0.93	4.17	0.58	2.66	n = 2
		Febr	0.76	1.69	2.1	0.91	5.96	0.84	2.42	n = 2
Paraleptastacus spinicauda	H2	June				15.02				
		Aug		0.84		1.51	0.12		0.01	
		Nov				14.46				
	H3	June	0.05			4.67	2.4			
Paronychocamptus nanus	H3	Aug	0.54	1.24	1.02	2.34	2.52		0.4	
		Nov	0.25			3.63				n = 3
		Febr	0.76	1.72	0.59	0.89	2.51	0.52	2.15	n = 2
	H5	Febr	0.26	3.77		1.15	1.96		0.26	
Platychelipus littoralis	H4	June	0.41	2.17	0.41	1.22	4.47	0.53	0.32	n = 2
		Aug	0.7	2.21	0.7	1.74	5.9	0.58	1.34	
		Nov	0.7	1.53	0.63	1.33	3.6	0.71	3.51	n = 2
		Febr	0.75	2.45	1.14	1.01	6.21	0.81	2.77	
	H5	June	0.16			2.38	13.14			
		Febr	0.78	2.83	1.24	0.75	6.16	0.8	2.28	
Tachidius discipes	H1	June	0.16			2.74				
		Febr	0.92	0.88	0.87	0.7	3.98	0.25	2.94	n = 2
	H2	June	0.21	0.51		3.12	1.36		0.48	n = 2
	H3	June	0.4	0.98		2.88	2.83		0.26	
		Aug	0.34	1.29		2.71	1.61		0.28	
		Nov	0.89	1.29		2.32	3.74		0.44	
		Febr	0.75	0.91	0.58	0.34	3.94	0.2	2.53	
	H4	Febr	0.58	0.91	0.56	0.96	4.27	0.28	1.65	n = 2

* or C18:1ω7c

Table 3. Marker fatty acids (in %) or fatty acid ratios in harpacticoids, indicative for copepods' diet.

DISCUSSION

Spatio-temporal variability in resource availability

The biggest contrasts in sediment isotopic signatures and FA profiles were found between the sandy station (H2) and the muddy salt marsh stations (H4, H5). Bulk organic matter at H2 had an MPB-dominated isotopic signature. Comparatively higher hydrodynamic disturbance of the sandy sediment minimizes accumulation of silt and retention of ^{13}C-depleted detrital organic matter. Hence, the $\delta^{13}C$ signature of the sediment surface is mainly a reflection of the autochtonous primary production by MPB. However, this MPB does not accumulate as stable biofilms as it does on nearby siltier and less hydrodynamically disturbed sediments, leading to lower MPB biomass in sandy sediment, even though the overall rates of primary productivity may be very comparable in both sediment types (Herman et al. 2001). MPB isotopic data used in the present study were coined from previous work in the Paulina intertidal area (Moens et al. 2002, Moens et al. 2005a). They are in the range of typical saltmarsh and tidal flat MPB (Currin et al. 1995, Riera et al. 1996, Deegan & Garritt 1997) and overlap with isotopic values of decomposed *Spartina* (Middelburg et al. 1997). Fresh *Spartina anglica* tissue, SPOM and bulk sediment $\delta^{13}C$ data (POM) from the current study matched well with earlier measurements from the polyhaline part of the Schelde Estuary (Middelburg et al. 1997, Middelburg & Nieuwenhuize 1998, Moens et al. 2002). At most stations, with the exception of H2, sediment organic matter $\delta^{13}C$ closely resembled that of SPOM (see Fig. 4), illustrating the strong retention of deposited phytoplankton and other detritus. SPOM deposition appeared most pronounced in June and could be clearly observed in the field: the upper few cms were siltier and richer in detritus than deeper sediment layers (Cnudde et al., 2013, in preparation, chapter 2).

Especially stations H4 and H5 (^{13}C-depleted and ^{15}N-enriched as a result of oxic organic matter degradation (Lehmann et al. 2002) can be considered mainly detritus-based systems with high and more diverse resource availability, as also shown by their higher FA content and FA diversity. In such environments, there is a positive feedback between MPB biofilms and silt deposition, but the MPB has lower turnover rates and is often less available to grazers than in sandier sediments (Herman et al. 2001). In terms of sediment granulometry, station H3 resembled more closely H2, indicating a higher hydrodynamic activity. At the same time, it is situated amidst pioneer *Spartina* vegetation, and its POM $\delta^{13}C$ was not significantly different from that of the siltier sediments. Concentrations of bacterial FA at this station were, however, comparatively limited. *Spartina anglica* was the dominant vegetation in the immediate vicinity of H3, H4 and H5, but our $\delta^{13}C$ demonstrate that its detritus input at these stations is limited, in line with data from other salt marshes (Middelburg et al. 1997). The high $\delta^{15}N$ in the muddy salt marsh stations suggest intensive microbial nitrification-denitrification processes (Lehmann et al. 2002, and ref herein). These stations were also characterized by high harpacticoid abundances and biomass (Cnudde et al., in preparation, chapter 2), suggesting high food availability and/or quality (Ahlgren et al. 1997, de Skowronski & Corbisier 2002, Sevastou et al. 2011).

Copepod resource utilization

Considering the substantial habitat and temporal coverage of the present sampling campaign, the variation in natural stable carbon isotope signatures of harpacticoid copepods was relatively small. With a $\delta^{13}C$ range of -18 to -12.5 ‰ and a high prominence of diatom-specific FA (e.g. 16:1ω7), we can conclude that the majority of copepod species rely predominantly on MPB. Significant contributions of ^{13}C-depleted sources, i.e. SPOM or detrital vascular plant material (C_{18}-PUFAs), were more rare. These sources were, however, manifest in *Paronychocamptus nanus* (particularly at H5 in February, with a $\delta^{13}C$ value of -23.52 ± 1.18 ‰), *Amphiascus* sp. 1 (at H5 in November, with $\delta^{13}C$ value of -20.3 ± 0.7 ‰) and, to a lesser degree, *Microarthridion littorale* (at H4 in August, with $\delta^{13}C$ value of -20.5 ± 1.7 ‰). *P. nanus* and *Amphiascus* sp. 1 were previously considered detritus and diatom feeders, respectively (Hicks 1971, Heip 1979), but no

study has specifically tackled their *in situ* feeding habits. *M. littorale* has been well studied because of its near omnipresence in tidal flats worldwide. At the Paulina tidal flat, it was present at all stations (except H2) and at all times. It can feed on benthic and planktonic microalgae (Decho 1986, Decho & Fleeger 1988, Santos et al. 1995) but also on bacteria. Their ability to bioconvert essential PUFA such as DHA (De Troch et al. 2012a) may be an important aspect behind this 'generalist' feeding behaviour. For other harpacticoid species, the smaller significant variability in $\delta^{13}C$ signatures (narrow range of 2‰) could result from a more selective feeding behaviour on different microbenthic algal species, depending also on spatial-temporal changes in MPB composition. Lab experiments by De Troch et al. (2006, 2012b) and Wyckmans et al. (2007) have documented that harpacticoid species can select among diatoms by diatom size, age or species. A high dependence of harpacticoids on MPB carbon is in line with previous researches (Santos et al. 1995, Riera et al. 2004, Galvan et al. 2008). Importantly, however, our stable nitrogen isotope data showed a spread of 5.5 ‰ among different harpacticoid species, and a mean nitrogen isotopic fractionation of 6 ‰ between harpacticoid copepods and diatoms, which is nearly twice the expected value for a single trophic step (Post 2002, McCutchan et al. 2003). The latter result suggests harpacticoid copepods can obtain MPB carbon indirectly, through one or even two trophic intermediates, rather than by direct grazing on MPB. Caution is due when interpreting this fractionation between MPB and copepods, because the MPB isotopic data used here have not been obtained at the same time as the copepod data. $\delta^{15}N$ signatures of marine sources can exhibit substantial spatio-temporal variation (Riera et al. 2000, De Brabandere et al. 2002, Dethier et al. 2013). Nevertheless, the explicit variation in $\delta^{15}N$ among copepod species strongly suggests that they span more than one trophic level. For the two copepod samples with $\delta^{15}N > 16$ ‰ (Fig. 4), there is no obvious indication of a higher trophic level (e.g. carnivorous feeding) in their FA profiles, although their FA profiles indicate a lower dependence on MPB: (1) *P. spinicauda* (station H2, red data point) showed no affinity at all with the FA characteristics of primary producers, and (2) for *M. littorale* (station H5 – November, yellow data point), FA composition had a predominance of DHA instead of EPA, which indicated dinoflagellate feeding or carnivory; 16:1ω7 proportions were intermediate compared to other species. For nematodes from the Paulina area, $\delta^{13}C$ also generally point at MPB as the main basal carbon source. $\delta^{15}N$ data, however, demonstrate that several species obtain this carbon indirectly, and not always in accordance with expectations from morphology-based feeding types (Moens et al. 2005a, subm.). For harpacticoid copepods, however, mouth parts provide only little information on their feeding strategy (De Troch et al. 2006). There are several potential scenarios which may help to explain the spread in $\delta^{15}N$ in harpacticoid copepods in our study. First, some harpacticoid copepods may feed partly or predominantly on bacteria (Rieper 1982), which in turn may derive a considerable portion of their carbon from MPB, for instance from their exopolymer secretions (EPS) (Decho & Moriarty 1990). In our study, the FA patterns of *D. palustris* and *P. spinicauda*, for instance, clearly indicate at least a partial dependence on bacterial food sources. The carbon isotopic signature of heterotrophic bacteria is often very similar to that of their carbon sources (Boschker & Middelburg 2002), but can also deviate by up to 11 ‰ (Macko & Estep 1984). In contrast, nitrogen fractionation by sedimentary bacteria is poorly predictable and depends on the molecular nature of the organic nitrogen source (including C:N ratio, biosynthetic and metabolic pathways e.g. degree of transamination). Even among similar nitrogen sources, ^{15}N fractionation can vary from strongly negative (Macko and Epstein 1984) to strongly positive (McCarthy et al. 2007). Second, some harpacticoid copepods may feed on heterotrophic ciliates and/or flagellates, which in turn consume MPB, be it through grazing on cells or consumption of EPS (Rieper 1985). With DHA levels dominating over EPA, consumption of dinoflagellates by *M. littorale* and *Tachidius discipes* is, for instance, plausible. Third, some harpacticoids may be predators of other, MPB-grazing metazoans, such as nematodes, ostracods or harpacticoid nauplii (Lazzaretto & Salvato 1992, Lehman & Reid 1992, Kennedy 1994, Seifried & Dürbaum 2000, Dahms & Qian 2006). However, copepod FA profiles did not reveal carnivorous feeding (PUFA/SFA and 20:1ω9 low). Fourth, harpacticoids may re-utilize their own fecal pellets and associated microbes (e.g. De Troch et al. 2009).

When comparing stable isotope data from different stations in our study, the dependence of sandy-sediment copepods on MPB was more pronounced than that of copepods from other, more accretory

stations. However, FA data did not fully support this conclusion, in that the abundant interstitial copepod *P. spinicauda* had very low contributions of MPB-characteristic FA. The other two dominant species from station H2 were *Asellopsis intermedia* and *Tachidius discipes.* FA data support the view that the former is primarily a diatom-feeder, at least in station H2 (and H1) where 16:1ω7 contributed 19 % to copepod FA and contrasting to only 9% in H5 (based on one sample only), while the latter species is not (low EPA/DHA ratio, low 16:1ω7).

No *in situ* data of these harpacticoid species are available from other biomarker studies, but our results line up with trophic knowledge obtained from more classical approaches. Interstitial copepods are considered as non-diatom feeders (Joint et al. 1982), browsing on sediment grains and scraping the epiflora which is largely composed of epipsammic bacteria (Noodt 1971, Feller 1980). For species from the genus *Paraleptastacus* specifically, previous suggestions of bacterial feeding were based on the absence of diatoms in the gut of *P. klei* (Azovsky et al. 2005) and the tolerance of *P. espinulatus* to high organic inputs with high bacterial activity (Hockin 1983). In contrast, the sand dwelling *A. intermedia* showed tidal migration, moving to the sediment surface during low tide and hence to MPB biofilms (Joint et al. 1982). *T. discipes* is the only species where DHA levels exceeded EPA and partial trophic reliance on protists is indicated.

The few species from muddy sediments for which our stable isotope data indicated variable or no reliance on MPB were *Paronychocamptus nanus* and *Amphiascus* sp. 1, both from station H5 in November, and Cletodidae. To our knowledge, no empirical evidence about their trophic ecology has hitherto been published. The more depleted $\delta^{13}C$ of *P. nanus* and *Amphiascus* sp. 1 ($\delta^{13}C \leq$ -20 ‰) compared to that of co-occuring species ($\delta^{13}C \geq$ -18.5 ‰) suggests a stronger reliance on SPOM ($\delta^{13}C$ averages -22.8 ‰; Table S1). For *P. nanus*, however, the more depleted $\delta^{13}C$ was not consistent over time, the data in February ($\delta^{13}C$ = -15.7 ‰) resembling those of other species and suggesting a closer link with MPB. . For *Amphiascus* sp 1, a lower presence of diatoms indicators (16:1ω7, EPA/DHA) and a small increase in C_{18}-PUFAs in station H5, indeed support the idea of higher contribution to SPOM to their diet.

Cletodidae are clearly specialist feeders relying on a completely different carbon source than the other copepods that were analysed here. Their very light carbon isotopic signatures are characteristic for chemoautotrophic bacteria. Sulphide production in these largely anoxic marsh sediments is high, even though sulphide concentrations in the Paulina intertidal area are on average low because of rapid scavenging by the high Fe^{2+} and Mn^{2+} concentrations. Interestingly, our results on Cletodidae are confirmed by a report on Cletodidae from seagrass-vegetated stations in the Mira estuary, Portugal (Vafeiadou et al. in prep). To our knowledge, there have been only two reports of harpacticoid copepods from shallow-water environments with similar strong reliance on chemoautotrophic carbon, but the identity of the species were unknown in these cases: one at a mudflat station in the Eastern Scheldt estuary (Moens et al. 2011) and one from shallow subtidal sediments in the North Sea (Franco et al. 2008). Van Gaever et al. (2006) found ^{13}C-depleted values of -51 ‰ for a species morphologically similar to the harpacticoid *Tisbe wilsoni* at a cold methane-venting seep in the Barents Sea, demonstrating reliance on methanotrophic bacteria. These few published results on harpacticoid copepods are in accordance with similar results on particular nematode species from shallow waters (Ott et al. 2004), deep-sea (Van Gaever et al. 2006, 2009, Tchesunov et al. 2012), mangrove (Kito & Aryuthaka 2006, Moens et al. unpubl., Bouillon et al. 2008) and seagrass (Vafeiadou et al. in prep) sediments, confirming that chemoautotrophic carbon may be an important energy source for several meiofaunal taxa. Whether in Cletodidae this reflects some sort of symbiotic relationship, as reported in several marine nematode genera (e.g. Polz et al. 1992, Riemann et al. 2003), or rather a direct and selective grazing (as in Halomonhystera disjuncta from an active, methane-venting mud volcano,Van Gaever et al. 2006, 2009) remains to be established. Unfortunately, we could only obtain a single successful FA profile of Cletodidae, yielding somewhat equivocal results: the lack of essential PUFA such as EPA and DHA, confirms their independence of MPB. But at the same time, the abundances of bacterial-specific FA (Kharlamenko et al. 1995) and those

specifically for chemoautotrophic bacteria (Table 1a; Van Gaever et al., 2006) were low, with levels comparable to other species and lower than in bacterivorous *D. palustris* and *P. spinicauda*.

Most of the interspecific, spatial and temporal variation in $\delta^{13}C$ was in the range of 2 ‰. Whether this points at shifts in the copepod diets, for instance with increasing or decreasing contributions of MPB, or at different taxa of MPB being consumed, cannot be derived from our data. Small spatio-temporal variations in resource $\delta^{13}C$ (Dethier et al, 2012), and/or shifts in the composition of these resources (for instance the MPB community) may equally explain the spatio-temporal variation in consumer isotopic data. A second difficulty, related mostly to the use of FA as trophic biomarkers, is the fact that species consuming the same food source may differentially assimilate and metabolize trophic markers.

For some species, observed $\delta^{13}C$ variation in combination with changes in relative FA proportion did illustrate shifts in diet. For instance, the spatial variation in $\delta^{13}C$ values of *Asellopsis intermedia* related to a different reliance on benthic (epipsammic) diatoms among the stations: diatom reliance was clearly higher in stations H1 and H2 (tidal flats) compared to H5. For *Microarthridion littorale* at station H5, the isotopic difference between November and February was ascribed to a change from flagellate consumption (DHA>EPA) in combination with some assimilation of vascular plant detritus (C_{18}-PUFA), to a higher diatom grazing (EPA>DHA, 16:1ω7, no C_{18}-PUFA). For *Delavalia*, FA data suggest the use of two food sources, i.e. bacteria (15:0, 17:0) and diatoms (high EPA/DHA).

Copepods showed a high FA diversity in February, accompanied by the highest proportions and absolute concentrations of PUFAs EPA and DHA), in accordance with sediment organic matter PUFA content at this moment. Possible explanations include (1) an early MPB bloom at the end of February, and/or (2) an increased PUFA accumulation by copepods for overwintering during November (winter). With a seasonal change in temperature, copepods produce a FA reserve for overwintering and copepods overwinter in diapauses with a reduced metabolic rate (Kattner & Krause 1989, Lee et al. 2006, Falk-Petersen et al. 2009). However, exact hibernating strategy differences among copepods are unknown, some species will end up being lipid poor after winter while others were able to maintain enough lipid reserves to spawn in spring (Kattner & Krause 1989). Moreover, copepod lipid content before overwintering can be highly variable, depending on whether the copepod species still produced a late-summer generation (Kattner & Krause 1989).

General conclusion

MPB, mainly diatoms, was of high dietary importance for the majority of intertidal harpacticoid taxa over the entire tidal flat – salt marsh area. Copepods spanned at least two trophic levels, and whether MPB carbon is mainly channeled directly or indirectly to harpacticoids remains unclear. There was little evidence for a role of *Spartina* detritus as a resource for copepods. SPOM contributed significantly to the diets of a limited number of species. In spite of the general importance of MPB as a major carbon source for a majority of our copepod species, food source utilization patterns were diverse and species-specific. In addition to species-specific trophic differences, spatio-temporal patterns also occurred, and particular species had at least partly different diets depending on the station where they were found.

Cletodidae consistently used chemoautotrophic energy, a trophic link which for harpacticoid copepods had hitherto only been reported from a deep-sea vent system and a subtidal flat.

Chapter 4

Substrate-dependent bacterivory by intertidal benthic copepods

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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 ^{13}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 protistsns 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 (Desvilettes 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}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-occuring 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 Huntemanniidae) 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 µm 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 µm Millipore filter to remove flagellates and eukaryotes. From 100 µl 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^{-1}) and bacto peptone (DIFCO, 5 g L^{-1}). 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^{-1} ^{13}C glucose (D-glucose, U-$^{13}C6$, 99%, Cambridge Isotope Laboratories, Inc.) was added. As a control for the effectiveness of ^{13}C-labeling, parallel bacterial cultures were incubated in an identical liquid medium with ^{12}C glucose. After 24 h of growth, the growth medium was replaced by sterile seawater to remove any remaining ^{13}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 µg 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 ($\delta^{13}C$ > 8000‰) 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±3.1 µm in length. The diatom cultures were grown non-axenically in tissue culture flasks (175 cm^2 surface) with 20mL 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 µmol photons m^{-2} 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}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 $2.5x10^6$ diatom cells/Petri dish. All experimental units received $1x10^{11}$ ^{13}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}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°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°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}C from the copepod body (Moens et al. 1999d). Subsequently, the aluminum capsules were oven-dried overnight at 60°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

$\delta^{13}C$ 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}C label is expressed as specific uptake ($\Delta\delta^{13}C = \delta^{13}C_{sample} - \delta^{13}C_{control}$) and as ^{13}C uptake per unit copepod biomass. The control signal refers to the $\delta^{13}C$ 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}C (I, in µg ^{13}C), 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 of the control ($F_{control}$) and the sample (F_{sample}), 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 $R_{VPDB} = 0.0112372$. Subsequently, total ^{13}C

uptake was converted to total bacterial carbon assimilation per unit copepod carbon (in µg C), calculated by dividing uptake I by sample biomass (organic carbon content) and taking into account the atomic % ^{13}C in bacterial biomass (9.7 %). Individual carbon contents of the copepod species were, in decreasing order, 1.51 ± 0.10 µg C, 1.05 ± 0.06 µg C, 0.85 ± 0.04 µg C and 0.55 ± 0.02 µg C (mean ± 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 % H_2SO_4-methanol solution since 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 µL injected per run) and split mode (for bacteria, 0.1 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 150 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

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 (± 1 SD, n = 4) in the substrate experiment and in the time-series experiment

Mortality (%)		B	BD	BS	BDS
Substrate exp.					
Platychelipus	T_4	8 (±7)	25 (±16)	19 (±6)	24 (±3)
Microarthridion	T_4	13 (±9)	12 (±3)	100 (±0)	96 (±3)
Time-series exp.					
Platychelipus	T_4	3 (±3)	3 (±2)		
	T_9	18 (±7)	2 (±2)		
Nannopus	T_4	1 (±1)	3 (±2)		
	T_9	6 (±3)	2 (±2)		
Delavalia	T_4	8 (±9)	5 (±5)		
	T_9	34 (±12)	9 (±5)		

The specific ^{13}C uptake levels ($\Delta\delta^{13}C$) 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}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).

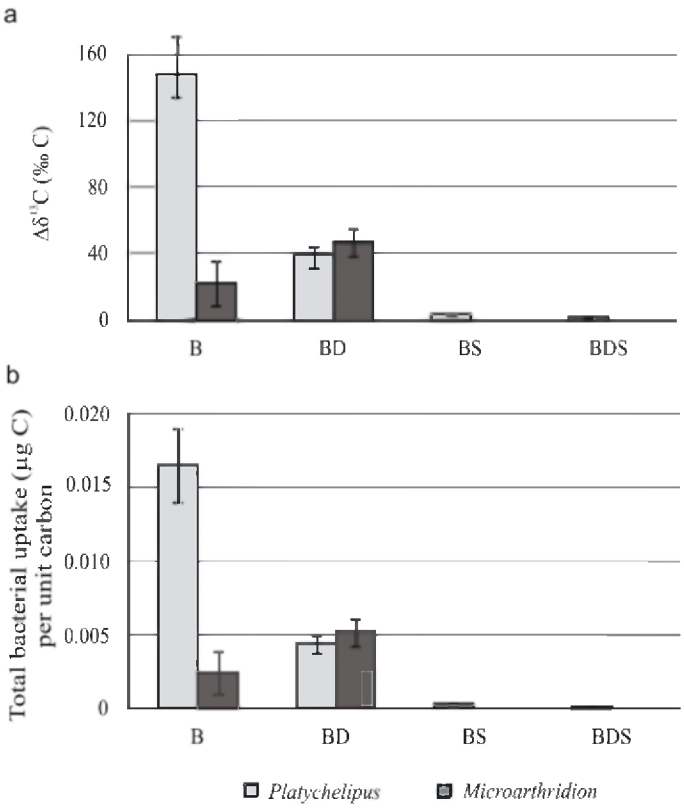

Fig. 1. Substrate-dependent assimilation of bacterial carbon (mean ± 1 SD, n = 4) by *Platychelipus* and *Microarthridion* after 4 days of grazing on a bacterial mixture without a primary substrate (treatment B) and in the presence of diatoms (treatment BD), sediment (treatment BS) and the combination diatoms+sediment (treatment BDS). Assimilation of bacterial carbon is expressed as (a) specific uptake $\Delta\delta^{13}C$ 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 ± 4 % (mean ± 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 ± 7 % and 34 ± 12 % (n = 4), respectively (*post hoc* test, $0.05 < p < 0.1$ and $p < 0.01$, respectively). Mortality of *Nannopus* remained low (6 ± 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.	***Platychelipus***	
	control	0
	B	-7
	BD	+122
	BS	-11
	BDS	+257
	Microarthridion	
	control	0
	B	-25
	BD	+79
Time series exp.	***Platychelipus***	
	control	0
	B-T4	-41
	B-T9	-56
	BD-T4	+71
	BD-T9	+117
	Nannopus	
	control	0
	B-T4	-59
	B-T9	-61
	BD-T4	-44
	BD-T9	-31
	Delavalia	
	control	0
	B-T4	-27
	B-T9	+21
	BD-T4	+67
	BD-T9	+240

^{13}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 $\delta^{13}C$ ($p < 0.001$, *post hoc* test $p < 0.05$): -14.9 ± 0.2‰, -16.1 ± 0.4‰ and -17.1 ±0.3‰ (n = 3) for *Platychelipus*, *Nannopus* and *Delavalia*, respectively. At the end of the experiment (at T_9), all three copepod species showed ^{13}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_4), *Delavalia* showed the lowest specific uptake (81 ± 44 ‰), which was nevertheless still higher than the specific uptake by *Microarthridion* (22 ± 13 ‰) in the corresponding treatment of the substrate experiment. The specific uptake by *Platychelipus* was the highest (196 ± 48 ‰) among the three species, and closely resembled that in the substrate experiment (148 ± 22 ‰) (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}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}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 µg C_{bact} per µg C_{cop}) and quintupled for *Delavalia* (from 0.01 to 0.06 µg C_{bact} per µg C_{cop}). For *Nannopus* the uptake of bacteria was twice as high (from 0.015 to almost 0.03 µg C_{bact} per µg C_{cop}) in the presence of diatoms but of no significance, not time-related or substrate-dependent.

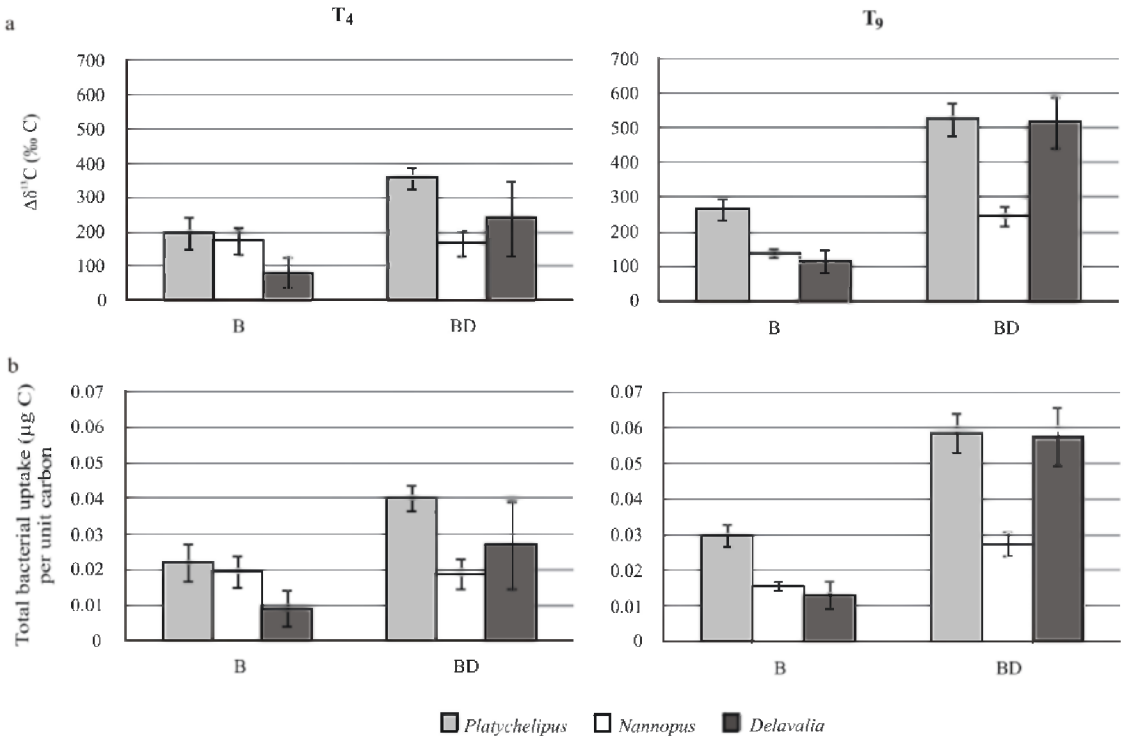

Fig. 2. Assimilation of bacterial carbon (mean ± 1 SD, N = 3) by *Platychelipus*, *Nannopus* and *Delavalia* after 4 days (T_4) and 9 days feeding (T_9), in the absence of a substrate (treatment B) and in the presence of a diatom substrate (treatment BD). Assimilation is expressed as (a) specific uptake $\Delta\delta^{13}C$ 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_0 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_{MC} = 0.001), with a dissimilarity of 50.5 % (SIMPER, data not shown). C16:1ω7 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 C18:1ω9 (relative abundance 37.6%, also found in diatoms but in very low amounts) and C17:1ω7 (relative abundance 10.7 %), whereas C20:5ω3 (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 C18:3ω6 and C22:6ω3 (docosahexaenoic acid, DHA) for diatoms.

The three copepod species, whilst originating from the same sampling station, had distinct FA compositions at T_0 (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, C17:1ω7 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, C15:1ω5, C17:0 and C17:1ω7) for *Delavalia*, *Nannopus* and *Platychelipus* was, respectively, 23.4 ± 2.5 %, 4.8 ± 0.6 % and 1.8 ± 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_0 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_{MC}). Significance codes: '*' P_{MC} <0.05, '**' P_{MC} < 0.01, '***' P_{MC} <0.001

Groups	t	P	Unique perms	P_{MC}
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

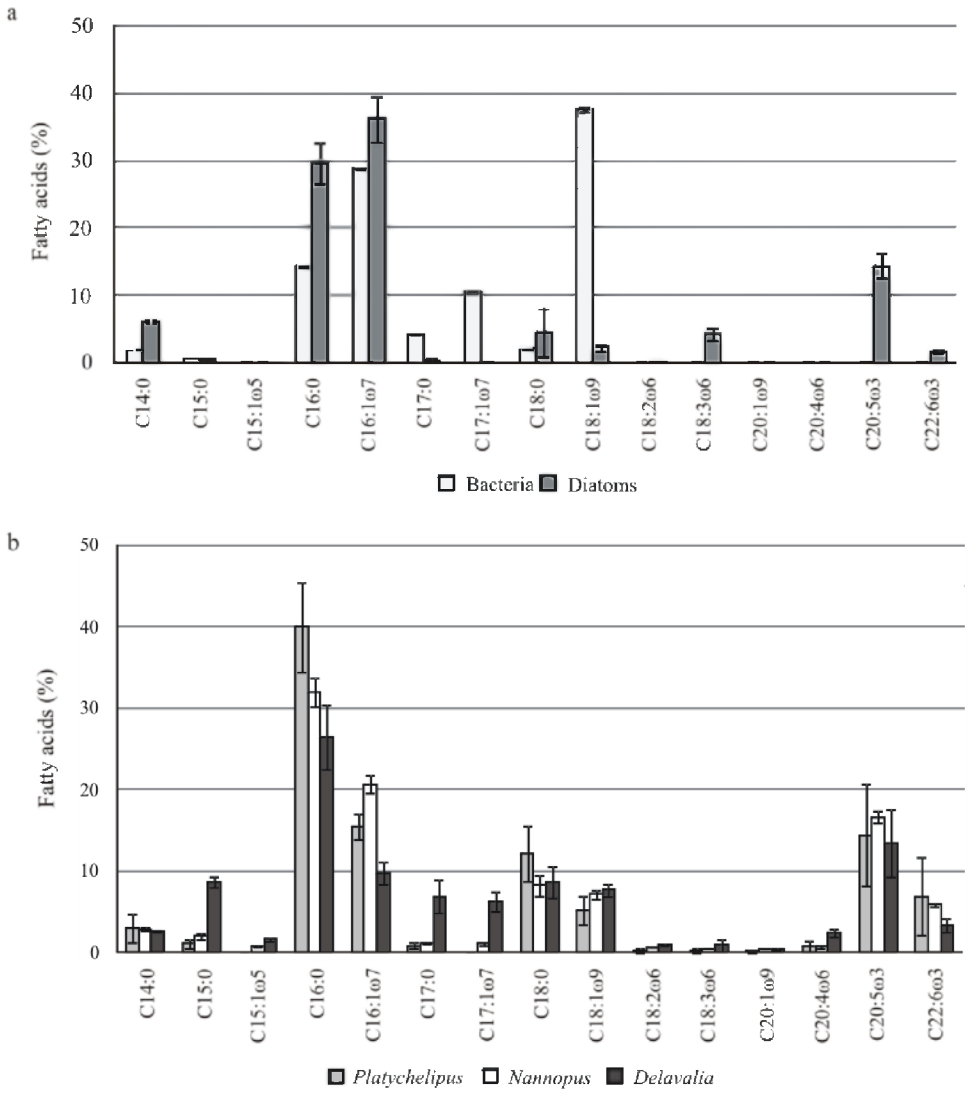

Fig. 3. Relative fatty acid composition (%) of (a) the food sources (bacteria and diatom monoculture) and of (b) the copepod species at time T_0

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 C16:1ω7 (found in both diatoms and bacteria) and three PUFA (C18:3ω6, C20:5ω3, 22:6ω3), 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: C16:1ω7, the bacterial biomarker C18:1ω9, 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 C16:1ω7, of diatom biomarker FA (C20:5ω3, C18:3ω6, C22:6ω3, C14:0) and of the bacterial biomarker C18:1ω9. No gain in C17:1ω7, 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_0, 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	C16:1ω7	23.26
	C15:0	14.49
	C17:0	12.3
	C16:0	12.08
	C17:1ω7	11.14
DEL-PLA	C16:0	22.13
	C15:0	12.61
	C17:1ω7	10.29
	C17:0	9.89
	C16:1ω7	9.29
	C20:5ω3	8.43
NAN-PLA	C16:0	24.51
	C16:1ω7	15.95
	C20:5ω3	13.02
	C18:0	12.8
	C22:6ω3	9.96

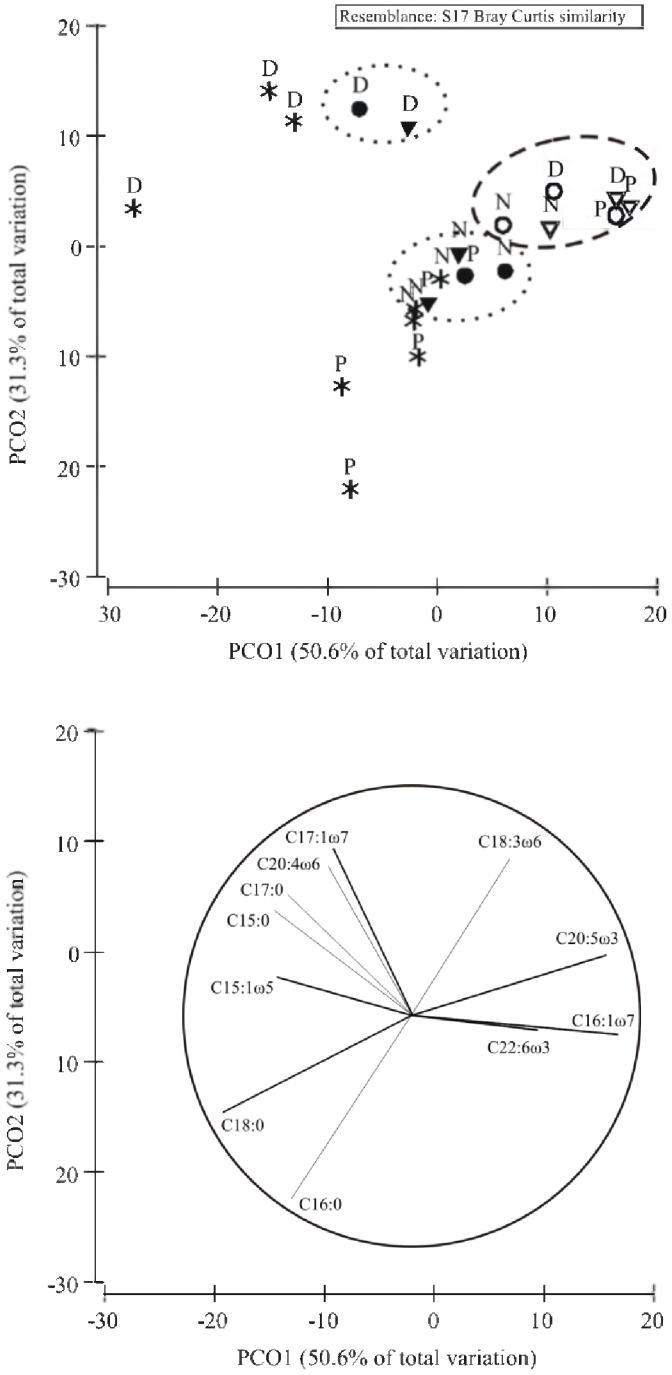

Fig. 4. Principal Coordinate (PCO) analysis of *Platychelipus* (P), *Nannopus* (N) and *Delavalia* (D) based on their natural relative fatty acid composition (*) and composition after experimental grazing during 4 days (○) and 9 days (▽ (■ – grazing on bacteria, □ – grazing on bacteria and diatoms). 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

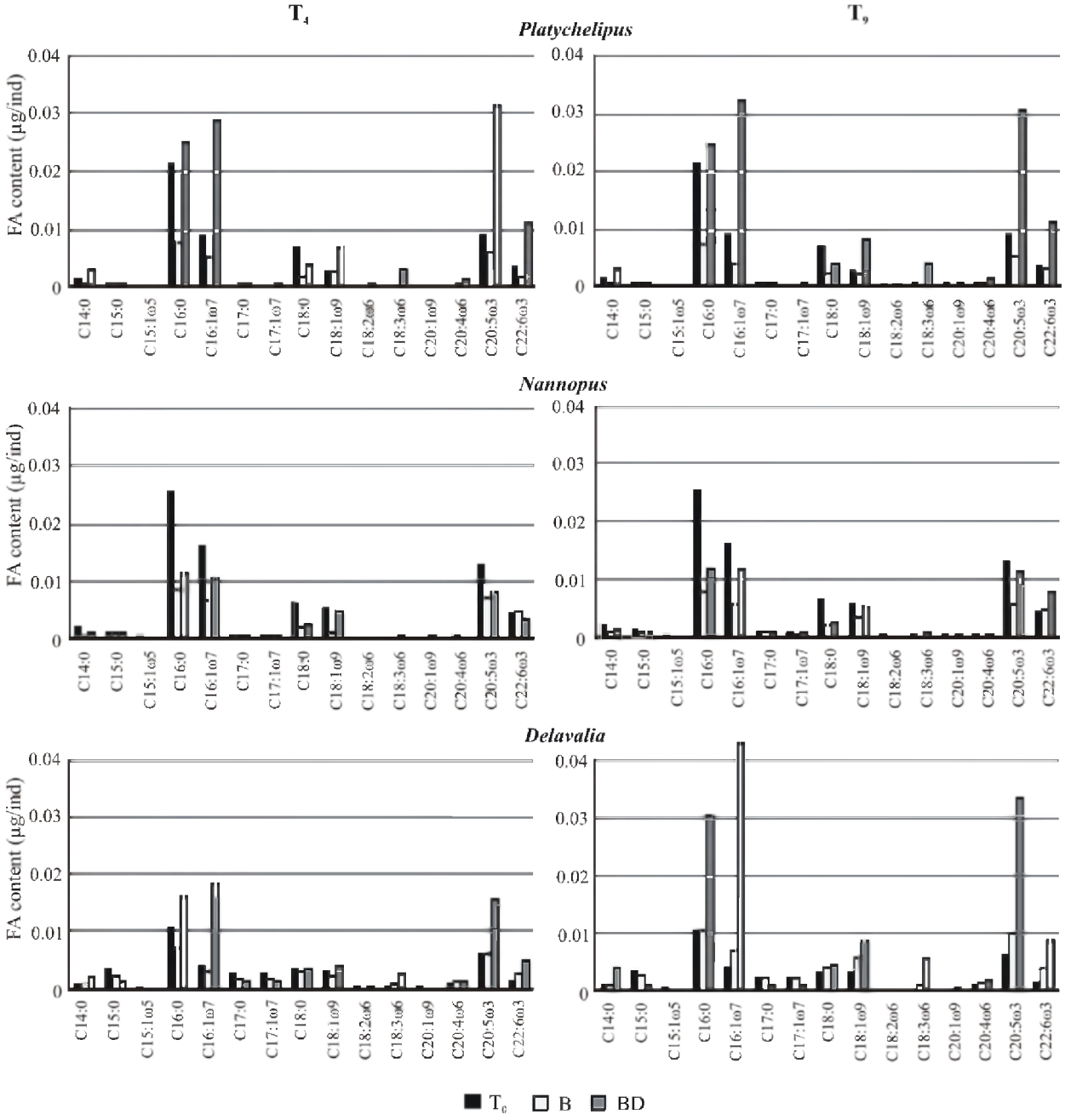

Fig. 5. Fatty acid composition (µg ind^{-1}) of the copepods *Platychelipus, Nannopus* and *Delavalia* after grazing during 4 days (T_4) and 9 days (T_9) on bacteria without an additional substrate present (treatment B) and with a diatom substrate (treatment BD), compared to fatty acid profiles of copepods 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 µm (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 (< 5µm), 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 µm and a 62 % mud fraction, compared to natural sediment from the copepod habitat with a median grain size 126 µm 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 interferred with grazing of copepods (*Paramphiascella fulvofasciata*, *Nitocra spinipes*) on diatoms (De Troch et al. 2006).

On the other hand, the increased FA content of *Platychelipus* in the BDS versus BD treatment suggests that the presence of sediment stimulated diatom assimilation by *Platychelipus*. Hence, unlike in *Microarthridion*, the lack of bacterial uptake by *Platychelipus* 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 *Platychelipus* 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, *Platychelipus* 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. *Platychelipus, 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 *Platychelipus* 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 $\delta^{13}C$ 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 C16:1ω7 and C20:5ω3, 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 *Platychelipus littoralis* and *Delavalia palustris* and their general trophic ecology is, however, lacking. The abundances of Bacillariophyceae FA in these species varied considerably. Their natural $\delta^{13}C$ signals (*Platychelipus* 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 $\delta^{13}C$ 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, C17:1ω7). *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), *Pseudostenhelia 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 demonstates 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: C16:1ω7 and C18:1ω9. Little or no incorporation of less abundant FA (C16:0, C17:1ω7 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 C18:3ω6 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 C18:1ω9 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.

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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).

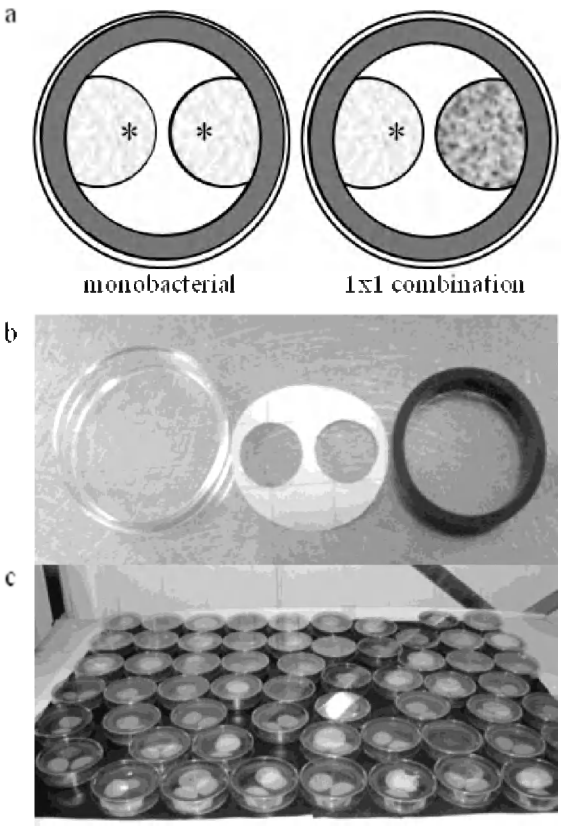

Fig. 1. Experimental setup: (a) two types of food microcosms, a monobacterial treatment (left) and a combination treatment (right), containing two biofilms of one bacterial species or of two different bacterial species, respectively [asterisk (*) indicating the ^{13}C-labelled biofilms]; (b) microcosms 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).

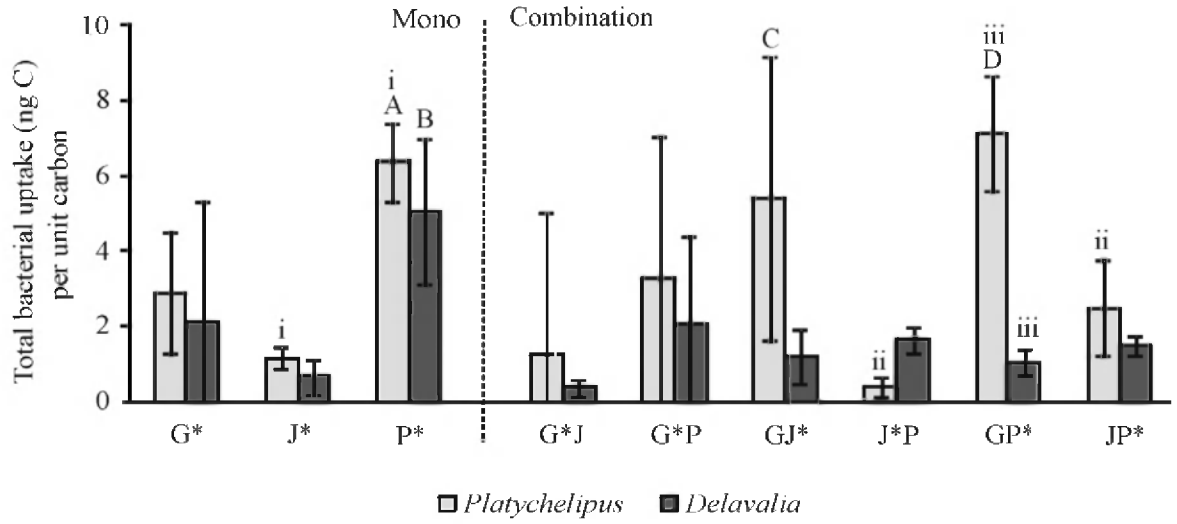

Fig. 2. Carbon uptake by *Platychelipus* and *Delavalia* in each bacterial treatment expressed as total carbon uptake per unit copepod carbon. Monotreatments included 2 monobacterial biofilms of one strain (G* = *Gramella*, J* = *Jannaschia*, P* = *Photobacterium*, with * indicating the ^{13}C labelling). 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.

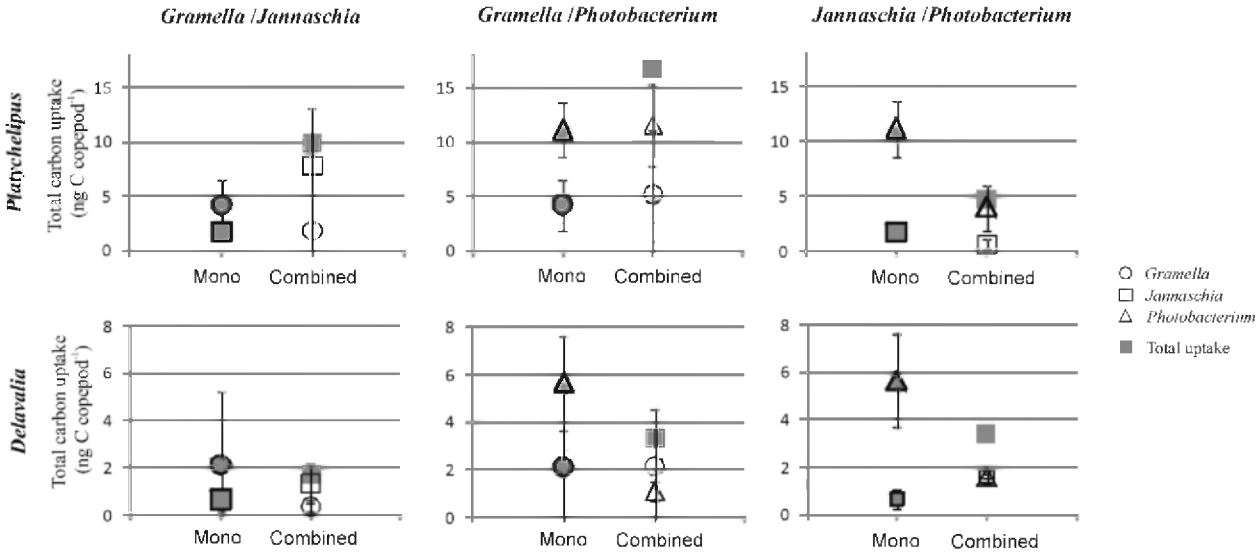

Fig. 3. Total carbon uptake values (per copepod) of each bacterial strain (symbols ○, □, Δ) by *Platychelipus* and *Delavalia* in mono versus combined treatments. Total bacterial uptake in each treatment is indicated by grey squares (■). For combination treatments, total bacterial uptake is calculated 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

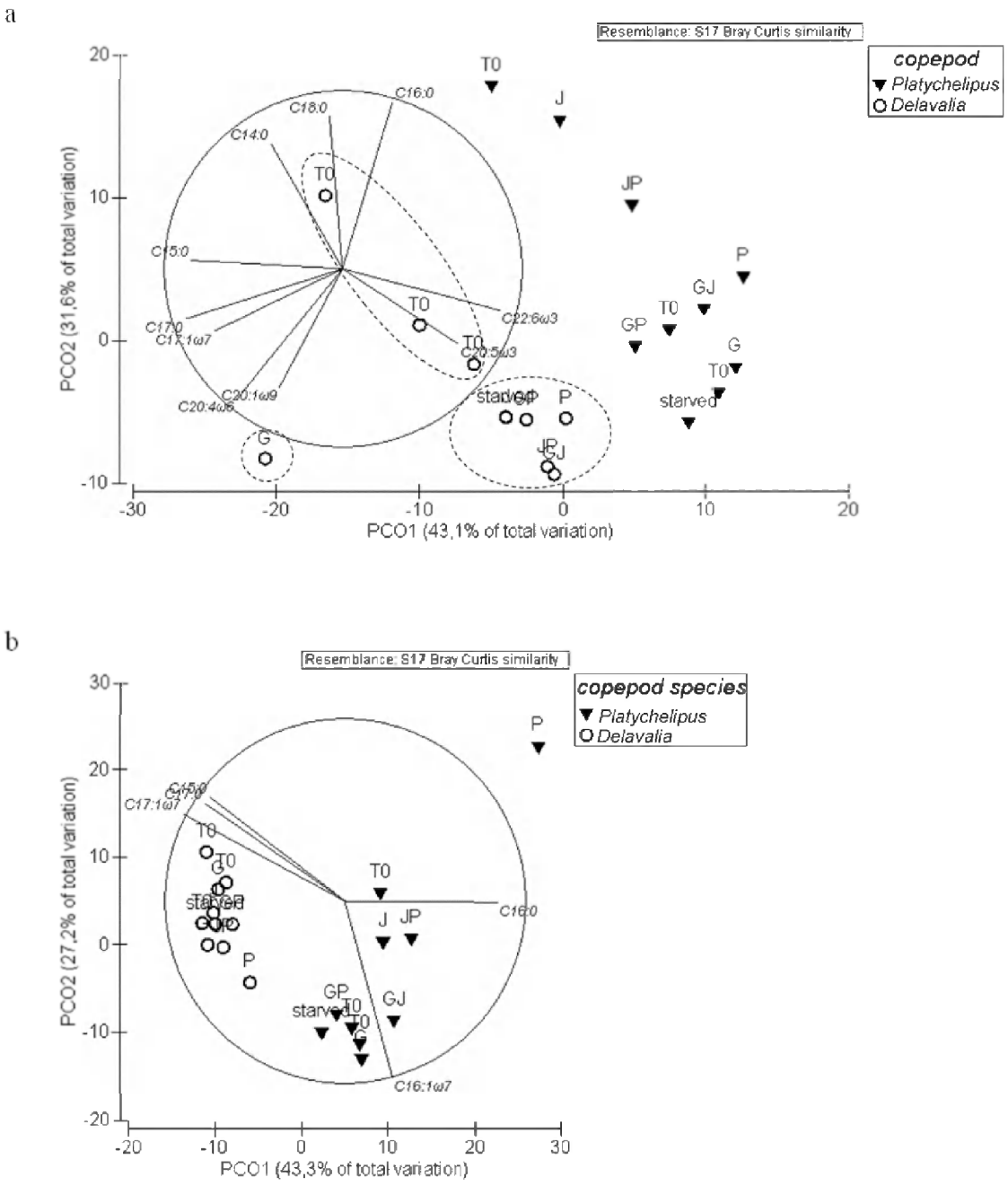

Fig. 4. Principal Coordinate (PCO) analysis of *Platychelipus* (▼) and *Delavalia* (○) FA profiles at the beginning of the experiment (T0) and after 9 days of incubation in the bacterial treatments, based on (a) relative total FA and on (b) relative copepod FA without PUFA. The vectors represent individual 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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Chapter 6

Structural and functional patterns of active bacterial communities during aging of harpacticoid copepod fecal pellets

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ABSTRACT

Copepod fecal pellet (fp) dissolved organic matter is consumed by free-living bacteria, while particulate matter is degraded by bacteria packed inside the fp ('internal') or attached to the fp surface after colonization from the environment (external). This study analyzed the contribution of 'internal' and external fp bacteria to the active bacterial community associated with the fp from two copepod species, *Paramphiascella fulvofasciata* and *Platychelipus littoralis*, during 60 h of fp aging in seawater. Despite early colonization (within 20-40 h), fp enrichment by seawater bacteria as deduced from RNA-based DGGE after 60 h was limited. In contrast, 'internal' bacteria showed high phylotype richness. The majority of 'internal' bacterial phylotypes persisted on aged fp and together represented half of the active bacterial community. Food source strongly impacted 'internal' bacterial diversity, though the exact origin of fp 'internal' bacteria, as either undigested food-associated bacteria or as copepod gut bacteria, could not be unambiguously determined. 'Internal' bacteria of fresh fp showed a high functional diversity (based on Biolog assays) to which *Vibrio* sp. contributed significantly. In terms of bacterial diversity and functional potential, degradation of copepod fp by 'internal' bacteria is equally important as by bacteria which colonize fp from the outside.

Keywords: copepod fecal pellet, fecal pellet degradation, active bacterial communities, harpacticoid copepods, 16S rRNA, DGGE, Biolog EcoPlate™

INTRODUCTION

Marine copepods assimilate approximately 30-60 % of the food they ingest (Thor et al. 2007). Hence a considerable fraction is voided as fecal pellets (fp) and dissolved organic carbon (DOC) (Smetacek 1980, Jumars et al. 1989, Turner 2002, Møller et al. 2003, Olesen et al. 2005). Copepod fp have a high C:N ratio compared to the original food (Morales 1987). In terms of quantity and quality, fp may contribute significantly to the pool of particulate and dissolved detrital carbon in the oceans. Since a substantial part can be recycled (Turner 2002), the carbon contribution of copepod fp to the detrital pool may be less than 10 % in neritic environments (Smetacek 1980). Nevertheless, fp are still a high-quality food source for bacteria which in turn upgrade the fp quality for metazoan consumers (Johannes & Satomi 1966). As a result, fp are rapidly degraded in the upper water layers and only a limited fraction reaches the sediment (Smetacek 1980, Urban-Rich et al. 1999, Wassmann et al. 1999, Wexels Riser et al. 2007). No reports on the general fate and bacterial degradation rates of benthic copepod fp exist, notwithstanding benthic fp

are also rich in bacteria (De Troch et al. 2010) and may even be grazed by harpacticoid copepods (Koski et al. 2005, De Troch et al. 2009, Møller et al. 2011), a process potentially enhancing fp degradation.
The majority of the fp organic matter eventually enters the microbial loop (Jacobsen & Azam 1984, Anderson & Tang 2010). In addition to protozooplankton, heterotrophic bacteria are important fp degraders (Poulsen & Iversen 2008). The DOC plume released from the fp immediately after egestion, nurtures the growth of free-living bacteria and protists (Cho & Azam 1988, Urban-Rich et al. 1999, Thor et al. 2003). Pellet-associated bacteria solubilize the fp content and degrade the surrounding membrane, producing more labile DOC and releasing smaller POC (Jacobsen & Azam 1984, Roy & Poulet 1990, Thor et al. 2003). They also lower the fp C:N ratio (Fukami et al. 1981). Zooplankton mediates the turnover of fp through fp fragmentation (coprorhexy/-chaly), whereby the increase in fp surface:volume ratio and reduction in fp sinking rates facilitate bacterial colonization (Noji et al. 1991, Poulsen & Kiørboe 2005, Reigstad et al. 2005, Iversen & Poulsen 2007, Wexels Riser et al. 2007).
The metabolic activity of the bacterial flora associated with copepod fp is multiple times higher than those of free-living seawater bacteria (Tang 2001, Thor et al. 2003). Bacteria may colonize fp from the environment (external activity) (Honjo 1976, Turner 1979, Jacobsen & Azam 1984, Delille & Razouls 1994), but may also be delivered by the copepod itself as gut bacteria or transient, digestion-resistant bacteria packed within the pellet (internal activity) (Lawrence et al. 1993, De Troch et al. 2010). Note that 'internal' refers to the origin of the bacteria but not necessarily to the actual location of their activity, which may extend to the fp surface. A low abundance of 'internal' bacteria compared to the strong colonization of fp immediately after egestion (Honjo 1976, Turner 1979), suggests a degradation driven from the 'outside-in'. In other cases, however, a high survival of bacteria in copepod guts and a resulting high bacterial abundance inside the fp (Lawrence et al. 1993) has been observed, along with a limited bacterial abundance on the fp exterior (Gowing & Silver 1983), supporting the idea of an 'inside-out' degradation.

To obtain more insight into the importance of the 'internal' and external fractions of active bacteria during the degradation process of fp of benthic copepods, we investigated the successive changes in the structure of bacterial communities during 60-h aging of fp in natural seawater. Immediately after egestion, fp exclusively contain copepod-associated bacteria. These 'internal' fp bacteria can comprise enteric bacteria ('resident') as well as undigested food bacteria that survived gut passage ('transient'). If shortly after egestion, fp become enriched in external bacteria, freshly produced and degrading fp are expected to show a clear divergence in their composition of the active bacterial community. In addition, a shift in bacterial community composition may be accompanied by a shift in the functioning of the bacterial community. Genetic and metabolic community profiling of the bacterial communities associated with fp of different age were achieved by means of RNA-based denaturing gradient gel electrophoresis (DGGE) and Biolog EcoPlate™ carbon substrate utilization assays, respectively.

We focused on the following questions:

(1) Is there an important change in the structure of the active bacterial community during fp aging? Since bacterial attachment to copepod fp occurs within the first few hours, followed by bacterial division on the fp surface (Jacobsen and Azam, 1984), we hypothesize that the 'internal' bacterial community typical of freshly egested fp will rapidly (within hours) be replaced by, or at least become strongly enriched with, external bacteria.
(2) Do the 'internal' bacteria originate from the consumed food source or from the copepod's intestinal flora?
(3) Is there a divergence in bacterial community functionality (metabolic capabilities) of freshly produced vs aged fp? Gowing and Silver (1983) suggested that 'internal' fp bacteria may be metabolically different from bacteria on the exterior of the fp.

MATERIAL AND METHODS

Extraction of harpacticoid copepods and gut clearance

The benthic copepod species *Platychelipus littoralis* (family Laophontidae) and *Paramphiascella fulvofasciata* (family Miraciidae) (henceforth referred to by their genus names) were used for fp production. *Platychelipus* was collected from an intertidal creek in the Paulina salt marsh in the polyhaline reach of the Westerschelde estuary (SW Netherlands, 51°20'55.4''N, 3°43'20.4''E). Specimens were extracted from the silty sediment by rinsing over a 250-µm sieve and subsequent handpicking under a stereomicroscope using a Pasteur pipet. *Paramphiascella* was handpicked from a laboratory batch culture, originating from an intertidal area in Helgoland (Germany), reared in 1-l glass beakers with artificial seawater (ASW, salinity c. 32, Instant Ocean® salt, Aquarium Systems, France), and fed a diet mainly consisting of the cultured benthic diatom *Seminavis robusta*. *Seminavis robusta* was obtained from the diatom culture collection of the Laboratory for Protistology and Aquatic Ecology (Ghent University). Diatom cells were grown non-axenically in cell tissue culture flasks with f/2 culture medium (Guillard 1975) based on autoclaved ASW (salinity 28) and under the same incubation conditions of copepods, i.e. at 15 °C under a 12:12 h light:dark regime. Diatom cultures were kept in exponential growth phase and the f/2 medium was refreshed regularly.

Batches of copepods (1500 to 2500 specimens) were washed multiple times by sequentially transferring specimens into sterilized ASW (salinity 28, 0.2-mm filter-sterilized and autoclaved) in order to remove loosely attached bacteria and particles adhering to the copepods. The batches comprised adult specimens only and consisted of a randomly sorted mixture of males and females representing the male-female ratio from the field or the copepod culture. Subsequently, copepods were placed in sterilized ASW-filled Petri dishes (52 mm diameter, 150 copepods per dish) in a climate room at 15 °C (near *in situ* temperature) and a 12:12 h light:dark regime for 24 h to allow gut clearance. The same temperature and light conditions were applied throughout this study for laboratory feeding of copepods, fp production and fp aging. The overall preparation of a batch of copepods (isolation, washing and placing in ASW-filled dishes) was completed within ca. 4 - 8 h. The released gut content of *Platychelipus* specimens, or 'natural fp' (sample notation: nat), composed of food ingested prior to copepod capture, was sampled for bacterial analysis. After 24h, the natural fp of *Platychelipus* were collected from all Petri dishes. All these freshly egested fp were pooled and the batch of fp was processed and aged as described further for laboratory fp.

Grazing, fecal pellet production and fecal pellet degradation

Laboratory fp (sample notation: lab) of the two benthic copepod species were obtained by feeding copepods cultured *S. robusta* diatom cells (strain 85A, about 35 µm in length), followed by gut clearance in sterilized ASW. For this, field-caught and cultured copepods with emptied guts were allowed to graze for one day in sterilized ASW-filled Petri dishes containing *Seminavis* cells *ad libitum* (> 3 x 10^3 cells). Copepods retrieved from the diatom Petri dishes were rapidly and thoroughly washed with ASW and placed in clean ASW-filled dishes for a 24-h defecation period as described above. Freshly egested fp were harvested within 24 h after egestion using an eyed needle, and transferred to sterilized ASW a few times to remove loosely attached bacteria. The freshly egested fp were not exposed to natural seawater and the associated active bacteria thus originate exclusively from the copepod, either as transient (undigested) food-associated bacteria or as resident bacteria from the copepod gut or exoskeleton. The batch of fresh fp was split into 4 smaller batches: one batch was used immediately for preparing a sample of fresh fp, representing the 'internal' fp bacteria, and the other three were independently aged in Petri dishes filled with natural seawater (NSW) under the same conditions (12h:12 h light:dark and 15 °C) for 20 h, 40 h and 60 h, respectively. The natural seawater was first filtered over a 2.0-µm pore-size filter to remove suspended organic particles and protists, but not the free-living bacteria. From one batch of fresh fp, four samples were obtained (fresh fp, 20-h aged fp, 40-h aged fp and 60-h aged fp) (unless fp yield was too

low) which were further analysed with DGGE. Replicated fp samples originated from subsequent fp collection actions, using 1500 to 2500 newly harvested copepods (Table S1, biological replicates), namely three *Platychelipus* copepod batches and two *Paramphiascella* copepod batches. However, due to a low fp production by *Platychelipus*, less than three replicates of fp samples were included (Table S1). From each harvest, *Seminavis* and NSW were also sampled (see 'Sample preparation for bacterial analysis').

Similarly, a second series of setups with *Platychelipus* and *Paramphiascella* was used to sample fp for metabolic profiling by means of Biolog EcoPlate™ assays. In contrast to the series for DGGE-analysis, no successive fp degradation samples were prepared. Priority was given to obtaining replicate samples of the fresh fp and 60-h aged fp originating from the same fp batch (technical replicates, table S1) since the strongly diluted bacterial inoculates (1.8 ml, see further) for Biolog EcoPlate™ analysis may cause variability in the carbon source utilization patterns (Garland & Lehman 1999). Here as well, *Seminavis*, NSW and copepods were screened on EcoPlates alongside fp.

Sample preparation for bacterial analyses

Each fp sample was composed of 100 fp, collected in a 2-ml eppendorf tube containing 200 µl of sterilized ASW. Due to the low bacterial abundances of fresh fp on the fp exterior (Gowing & Silver 1983) and the potential lower efficiency of extracting the 'internal' fp bacteria, extracted RNA concentrations were low (in the range of 0 to 5 ng μl^{-1}, i.e. around the lower detection limit of Nanodrop 2000) and cDNA transcription results from preliminary tests were negative. Hence samples of fresh fp were incubated another 24 h to allow 'internal' bacteria to proliferate while avoiding the risk of contamination with new bacterial strains.

To assess the origin of fp bacteria based on DGGE profiling, aliquots of the *Seminavis* culture and the filtered NSW were sampled during setups. Copepod samples, consisting of 3 pooled *Platychelipus* or *Paramphiascella* specimens, were collected after laboratory feeding on *S. robusta.* Some additional copepods (5 to 10 adults) were collected after laboratory feeding followed by gut clearance. Bacterial analysis of the latter will thus represent the resident copepod bacterial flora and/or bacteria associated with the exoskeleton. Samples for bacterial RNA-extraction were centrifuged at high speed (13000 rpm, 15 min); the supernatant was removed and the 'dry' sample was 'flash'-frozen in liquid nitrogen and stored at -80 °C until further analysis.

Samples of fp, *Seminavis*, NSW and copepods (only with emptied gut) for the EcoPlates™ assays were all diluted with sterilized ASW to a final volume of 1.8 ml ASW, the volume needed to fill 32 EcoPlate wells with 55 µl, and homogenized for 1 h at 200 rpm using a mechanical shaker to detach bacteria. Samples were centrifuged at low speed (500 rpm for 1 min) to spin down all organic particles except the bacteria. The supernatant with suspended living bacteria was immediately inoculated into the EcoPlate (see further). An overview of all samples analysed is given in table S1.

RNA-based DGGE fingerprinting

The diversity of active bacteria was profiled by PCR-DGGE of the 16S rRNA of the active bacterial community (Anderson & Parkin 2007). Total RNA extraction from fp, *Seminavis* and copepod samples was performed using the NucleoSpin® RNA XS Kit (Macherey-Nagel) developed for small RNA amounts. In addition to chemical cell lysis following the manufacturer's protocol, 3 x 30 s mechanical disruption with a bead beater at 30 Hz was included (silicon beads, 1.0 mm). For this purpose, the recommended volumes of the kit reagents were consistently doubled, respecting manufacturer's reagent ratios. After on-column DNase treatment, RNA was eluted twice in 10 µl nuclease-free water to increase RNA yield. An additional DNA digestion in the RNA eluate was done using TURBO™ DNase (Ambion) with incubation at 37 °C for 1 h. TURBO™ DNase was deactivated afterwards by adding EDTA (15 mM final concentration) followed by a

10-min incubation at 75 °C. Reverse transcription of RNA to cDNA was performed with the Sensiscript® Reverse Transcription Kit (Qiagen) using the two-tube method, i.e. cDNA synthesis and cDNA amplification in separate tubes. Prior to cDNA synthesis, RNA extracts were checked to assure they were DNA-free. All RNA extracts were subjected to PCR (identical to PCR for cDNA amplification, see below). No DNA bands were observed on a 1 % agarose gel (20 min, 100 V). For cDNA synthesis by reverse transcription (RT), preparation of the master mix and performance of the RT reaction were executed following the manufacturer's recommendations, using 10 µM random hexamer primer (Fermentas,Thermo Scientific), 10 units RNase inhibitor (Qiagen) per reaction, and 1 µl of RNA template. From the cDNA, the variable V_3 region of the 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR), using the universal bacterial primer set 357f and 518r (Yu & Morrison 2004) (Sigma Aldrich) with a GC-clamp coupled to the forward primer (Temmerman et al. 2003). The 50 µl PCR mixture was prepared with 2 µl cDNA template as in Temmerman et al. (2003), but instead of $MgCl_2$ and Taq polymerase, 0.25 µl Top Taq (5 U μl^{-1}) was used. In order to increase amplification specificity and reduce the formation of spurious byproducts, a touchdown PCR (Don et al. 1991) was applied using an Eppendorf Thermal Cycler. After 5 min denaturation at 94 °C, the touchdown PCR was performed during 10 cycles including 30 s denaturing at 94 °C, 30 s annealing starting at 61 °C with a 0.5 °C $cycle^{-1}$ decrement (until 56 °C), and an extension at 72 °C for 1 min. In the next 25 cycles of regular PCR, annealing was done at 56 °C, ending with a final extension for 30 min at 72 °C. A negative control, i.e. PCR mix without addition of template DNA, was included in each PCR. Because of low cDNA yields, all samples were subjected to a second PCR using an identical touchdown PCR program but only 10 cycles of regular PCR. PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega), and cDNA concentration was measured using a NanoDrop® 2000 (Thermo Scientific). 600 ng cDNA were analyzed by DGGE using a 8 % (w/v) polyacrylamide gel with a 35 - 70 % urea-formamide gradient (Temmerman et al. 2003) and using the Bio-Rad DCode System (Nazareth, Belgium). DGGE was performed in 1x TAE buffer for 16 h at 75 V and at 60 °C. Gels were stained for 30 min with 1x SYBR Gold nucleic acid gel stain (Invitrogen, Merelbeke, Belgium) in 1x TAE buffer and digitally visualized using a charge-coupled device camera and the Bio-Rad Quantity One software. On each DGGE gel, 3 lanes were loaded with a reference (on the outer lanes and in the middle of the gel). The reference was composed of V_3 region amplicons of 11 bacterial strains originating from the sandy and silty sediments from the Paulina salt marsh, and allowed normalization of the fingerprint profiles within and among DGGE gels using the BioNumerics software version 5.10 (Applied Maths, St.-Martens-Latem, Belgium).

Carbon substrate utilization

The capacity of fp bacterial communities to metabolize different substrates (functional potential) was assessed by means of Biolog EcoPlates™ (Biolog Corporation, USA) with a community level physiological profile (CLPP) of ecological communities as output (Insam 1997). Biolog EcoPlates™ contain 31 ecologically relevant carbon sources and 1 blank well (no substrate) in triplicate. A colorless tetrazolium redox dye attached to the substrates was reduced to a violet formazan as a consequence of substrate oxidation by the inoculated bacteria. Color formation was quantified spectrophotometrically, generating a carbon substrate utilization pattern (CSUP) composed of 31 substrate absorbance values (OD, 'optical density').

Under sterile atmosphere, each 1.8-ml homogeneous bacterial suspension (see 'Sample preparation for bacterial analyses') was distributed over the substrate wells (55 µl $well^{-1}$). The control well was inoculated with sterilized ASW. Absorbance measurements at 595 nm used a VICTOR™ Multi-label Microplate Reader (Perkin Elmer); 25 measurements of 0.5 s each were performed in each well on a daily basis. The first reading was executed immediately after inoculation of the plates (day 0). Plates were incubated at 15 ^{0}C and were measured for 10 - 15 days.

At final reading, 30 µl of colored wells were subsampled for screening of bacterial diversity on DGGE, based on DNA as all bacteria were expected to be active. After centrifugation at 13,000 rpm for 20 min, the

supernatant was removed from the tubes and the remaining pellets were stored at -20 °C. Total DNA was extracted by alkaline lysis (Baele et al. 2000) using 10 µl alkaline lysis buffer and 90 µl milliQ water. DNA amplification by PCR was executed as described above and loaded on DGGE. To obtain an impression of the diversity of substrate oxidizing bacteria, DGGE bands were excised for sequencing. DNA was eluted from the gel by 10-min incubation at 65 °C in TE 1x buffer. DNA was purified by re-amplification and separation on DGGE 2 to 5 times to achieve optimal purification. Purified DGGE bands were sequenced by Macrogen Corporation (The Netherlands). Sequences were aligned to sequences from the NCBI Genbank database using the BLAST program and analysed by the DECIPHER chimera check program (Wright et al. 2012). Partial 16S rRNA gene sequences were identified using the Ribosomal Project Database (RPD). Sequences have been deposited in EMBL under accession numbers HF955287 to HF 955396.

Data Analysis of DGGE fingerprints

DGGE gels were normalized and analyzed using the BioNumerics software (version 4.61, Applied Maths, Sint-Martens-Latem, Belgium). Each band within a DGGE pattern represents a bacterial phylotype or OTU (operational taxonomic unit). Variations in band intensities within a pattern suggest differential contributions of phylotypes to the active community. Band intensity reflects the total RNA amount of a phylotype within the fp sample. In contrast to DNA-based DGGE, it is not necessarily an indicator of phylotype cell abundance because it may also in part represent the average cell activity of a phylotype since the activity level is partly reflected by the cellular rRNA content (Kerkhof & Ward 1993, Milner et al. 2001). OTUs among samples were classified as the same OTU when they were positioned within a 1 % range (of total pattern length) from each other. Variability in the community structure of fresh fp was determined by cluster analysis, using the Pearson's correlation coefficient and the Unweighted Pair Group Method with the Arithmetic Mean (UPGMA) algorithm. Bacterial diversity of differently aged fp was assessed by phylotype richness (number of DGGE bands, S), Shannon-Wiener diversity index (H') and Simpson's evenness index (1-λ'). The effect of fp aging on community composition was investigated by multivariate principle coordinates analysis (PCO). The PCO was constructed using square-root transformed relative band intensity data and a Bray-Curtis resemblance matrix. DGGE bands correlated to PCO axes were assigned by Spearman correlation (70 % threshold).

To quantify the step-wise change in community structure over each 20-h time period (in %), moving window analysis of DGGE profiles was applied (Marzorati et al. 2008). Herein, the difference between DGGE profiles of consecutive time points was calculated as 100 – similarity % using Pearson correlation similarity values and data were plotted on a time axis. Finally, as a possible indication of the functional organization of ecological communities (Marzorati et al. 2008), species distribution curves or Pareto-Lorenz curves (Lorenz 1905) were constructed. Cumulative band numbers of OTUs ranked from high to low band intensity were plotted on the x-axis and their respective cumulative relative band intensities were represented on the y-axis. Changes in community evenness are deduced from the position of the curves in accordance with the theoretical perfect-evenness-line (45° diagonal). The cumulative relative abundance (y-value) of 20 % of the OTUs (x-axis, 0.2 value) can be a measure for high, medium or low functionally organized communities (Wittebolle et al. 2008).

By comparing *Seminavis* and copepod DGGE profiles, the origin of fresh fp bacteria ('internal' bacteria) was deduced. Main phylotypes of fresh fp, i.e. with a medium (5 - 10 % relative band intensity) to high contribution (> 10 % relative band intensity) to the active bacterial community, were considered to persist when their contribution to the community remained more than 5 or 10 % on aged fp.

Data analysis of Biolog EcoPlatesTM

Bacterial metabolic activity rate of fp, *Seminavis*, copepods and NSW was assessed by the average well color development (AWCD). AWCD is the average of the 31 substrate wells, after correcting OD values for background absorbance by subtracting the OD value of the blank well (Garland & Mills 1991). Differences

in AWCD at final measurement were determined by one-way ANOVA using SS type II for unbalanced datasets and respecting the assumption of normality and homogeneity of variances, tested with the Shapiro-Wilk test and Levene test, respectively. To compensate for differences in AWCD among samples of different origin (see 'Results'), owing to unstandardized inoculum densities, samples were compared at similar AWCD level, generally referred to as the single-time-point approach (Garland 1996). A standard AWCD value of 0.2 was chosen, equal to the lowest observed AWCD among all samples. This AWCD was observed for fp samples in the present study. This standard value was reached after 10-15 days for fp samples but at different time points for other sample types (*Seminavis*, copepods, NSW). Analyses were executed on net absorbance values, i.e. OD values corrected for blank well OD and for substrate-specific background noise by subtraction of the substrate OD value at time T_0 (Nair & Ngouajio 2012). A positive substrate response was defined by a visually observed well coloration with a net absorbance value ≥ 0.2. All OD values lower than 0.2 were set to zero.

To depict differences in bacterial functional diversity between fresh and aged fp, principle components analysis (PCA) was executed based on normalized OD values. For normalization, OD values were divided by the average well color development (AWCD) (Garland 1996). Significant difference in CSUP between the *a priori* defined groups of fresh and aged fp was tested with a 1-way ANOSIM for each copepod species separately.

The potential of fp bacterial assemblages to utilize substrates was further assessed by recording substrate richness S, i.e. the number of positive responses per sample. Based on chemical composition, the substrates of EcoPlates belong to 6 substrate guilds (carbohydrates, carboxylic acids, polymers, amino acids, amines and miscellaneous compounds) (Zak et al. 1994). Utilization of each guild was calculated as the number of positive responses within the guild over all replicate samples. The utilized substrate richness per guild was standardized for the number of sample replicates and for substrate guild size since different guilds are composed of a different number of substrates (7 carbohydrates, 9 carboxylic acids, 4 polymers, 6 amino acids, 2 amines and 3 miscellaneous compounds). Substrate guilds were equally weighted by using a guild-specific correction factor calculated as the number of substrates within the guild divided by number of substrates of the largest guild (i.e. carboxylic acids, composed of 9 substrates) (Preston-Mafham et al. 2002).

ANOVA analysis was performed using the software package R, version 2.14.1 (R Development Core Team 2012) and all other analyses in PRIMER v6 with PERMANOVA add-on software (Clarke & Gorley 2006, Anderson et al. 2008).

RESULTS

Active bacterial communities from fresh fp

Multiple active phylotypes were found on all freshly produced fp (Fig. 1). 'Internal' bacterial communities of fp differed between the two copepod species and their food sources (UPGMA, lowest similarity level 12%). The two fp samples of cultured *Paramphiascella* showed a high similarity of 91%, in contrast to the variability observed between fp samples of the field-caught *Platychelipus*. *Paramphiascella* fp also contained a lower phylotype richness (S = 7 - 9) than *Platychelipus* fp (S = 7 - 16). Among the *Platychelipus* fp, there was no separate grouping according to fp origin (Fig. 1). Firstly, similarity between the natural fp was very low, the three samples clustered at the 12% similarity level. Secondly, laboratory fp showed a similar variability in associated bacteria and clustered closely with a natural fp sample (at the 87 %, 60 % and 93 % similarity level, respectively) (Fig. 1). Additionally, *Platychelipus* laboratory fp were not closely clustered with laboratory fp produced by *Paramphiascella* fed the same *Seminavis* diet.

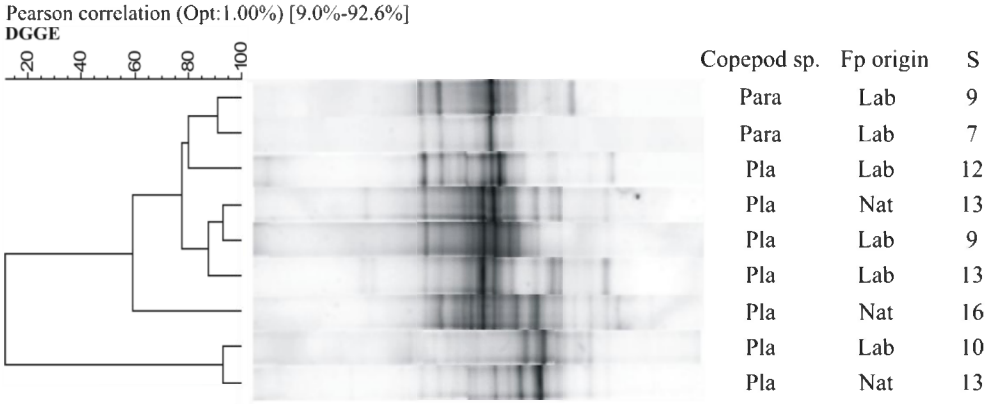


Fig. 1 Similarity between RNA-based DGGE profiles of fresh fecal pellets (fp), including natural (nat) and laboratory (lab) fp of copepods *Platychelipus* (Pla) and *Paramphiascella* (Para). S: number of DGGE bands.

We noted an increased similarity between copepod bacteria and *Seminavis* bacteria after feeding on *Seminavis*, from 3.2 % to 48.2 % for *Paramphiascella* (Fig. S1a). After releasing gut content, similarity of copepod-associated bacteria to those associated with the *Seminavis* food source dropped to 15.6 % (Fig. S1a) due to loss of 5 OTUs. Only two prominent OTUs remained associated with the copepods, of which the OTU with highest band intensity was *Seminavis*-related and the other an original copepod-related OTU. Also for *Platychelipus* (Fig. S1b), after removal of the gut content, the copepod bacterial flora showed reduced similarity with *Seminavis*, from 49.6 % to 31.3 %. Due to gut clearance, the copepod bacteria changed drastically for both copepod species. Additionally, for *Paramphiascella*, comparison of bacteria from copepods obtained from culture and copepods obtained after one time feeding on *Seminavis* showed a pronounced change in copepod flora (Fig. S1a). The origin of fp bacteria can be traced and the specific contribution of food bacteria and copepod bacteria to the active bacterial community of fresh fp was deduced by comparison of the DGGE profiles of laboratory fp with those of *Seminavis* and copepods. Of the main fp bacterial phylotypes (contributing > 5 % to the active community), half appeared to originate directly from the food (Table 1, grey) since they were shared with *Seminavis* samples and, if present in the copepod, they were lost after gut clearance, e.g. the DGGE band at position 60.9% (alias phylotype 18, Fig. 2). For *Platychelipus* (Table 1a), 4 out of 7 phylotypes were related to the food source, corresponding to 36 % of the total band pattern intensity. For *Paramphiascella* (Table 1b), 3 out of 6 phylotypes related to the food source, corresponding to a cumulative OTU abundance of 71.5 %. Other phylotypes were found in common for *Seminavis* and copepods after gut clearance (Table 1, hatched cells), and their exact origin therefore remains uncertain. None of the main fp phylotypes were found uniquely related to the copepod itself.

a

		Platychelipus			
Band	OTU	Fp (n = 4)	Food (n = 7)	Cop - fed (n = 2)	Cop - empty (n = 2)
39.3 %	7	5.0 (4.8)	5.9 (5.1)	0.9 (0.2)	
48.2 %	11	5.4 (5.0)	24.9 (9.8)	4.8 (1.5)	15.1 (3.1)
52.4 %	13	20.0 (15.7)	2.8 (6.3)	4.7 (1.3)	14.0 (3.6)
54.0 %	14	4.9 (9.8)	0.7 (1.9)		
55.0 %	15	19.4 (13.6)	1.3 (3.5)		6.0 (2.6)
60.9 %	18	7.1 (11.5)	4.8 (4.1)	19.2 (3.3)	
64.4 %	19	10.2 (18.7)	7.3 (6.7)	19.1 (5.1)	4.1 (0.9)

b

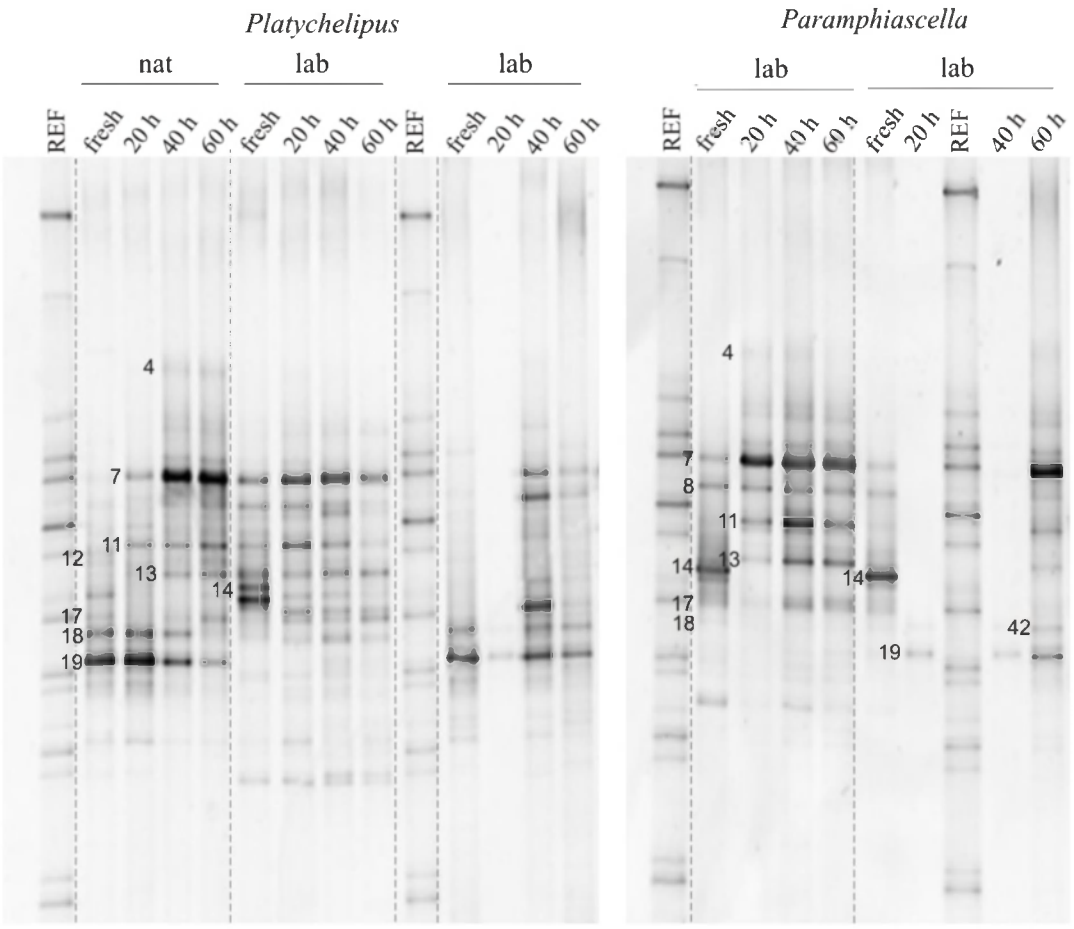
		Paramphiascella			
Band	OTU	Fp (n = 4)	Food (n = 4)	Cop - fed (n = 2)	Cop - empty (n = 2)
39.3 %	7	7.7 (2.3)	26.9 (17.1)	11.0 (6.7)	6.0 (0.4)
43.0 %	8	10.2 (2.1)	12.1 (5.3)		
51.0 %	12	8.0 (10.7)	0.8 (1.5)	5.2 (4.1)	1.0 (0.5)
54.0 %	14	51.4 (13.4)	11.7 (7.9)	33.0 (13.5)	
57.8 %	17	9.9 (4.2)	10.6 (4.0)	13.0 (9.4)	
59.3 %	42	5.1 (5.9)			

Table 1. Origin of 'internal' fp bacteria associated with (a) fresh *Platychelipus* fecal pellets (fp) and (b) fresh *Paramphiascella* fp. Overview of main fp DGGE bands (relative band intensity > 5 %) and their presence in the *Seminavis* used during the same experimental setup (Food) and in copepods before (Cop – fed) and after gut clearance (Cop - empty). Fp bacteria originating from *Seminavis* are marked grey, fp bacteria found on both *Seminavis* and copepods are hatched. Presented data are averaged relative band intensities (corresponding standard deviation between brackets)

Shifts in bacterial community structure during aging of fp

The DGGE gels (Fig. 2) visualize the genetic community structure of the active bacteria of both freshly egested and degraded fp (20, 40 and 60 h). At first glance, numbers of OTUs on degraded fp did not appear much higher than those on fresh fp, though overall OTU richness S was a little higher on all aged fp: fp of *Platychelipus* increased with 2 - 4 OTUs and fp of *Paramphiascella* with 4 - 7 OTUs, as they aged. Three samples with only two bands and an overall weak profile were considered PCR artefacts.

During degradation, the structure of the active bacterial community from fresh fp changed immediately. The PCO (Fig. 3a), explaining 70.3 % of total variation (PCO 1: 48.9 %; PCO 2: 21.4 %), plotted 3 of the fresh fp separately from aged fp, while 2 fresh fp samples were still positioned close to the 20-h aged fp. Fresh fp are located on the negative side of both axes. Seven OTUs were correlated for more than 70% with PCO 1 (OTU 4, 11, 13, 18, 19) or PCO 2 (OTU 7, 14) (OTUs indicated in Fig. 2). Only one OTU (OTU 4) was unique for aged fp. The other OTUs were at least once part of the fresh fp community. Three of these phylotypes were previously denoted as possibly originating from *Seminavis* (OTU 7, 14, 18) and three were undefined (OTU 11, 13, 19). Furthermore, from these six 'internal' phylotypes, band intensity of three OTUs (OTU 7, 11, 13) increased or remained fairly constant over time, while two OTUs (OTU 18, 19) tended to diminish and one (OTU 14) was completely lost within 20 h of aging in NSW.



	Platychelipus												*Paramphiascella*									
	natural fp				laboratory fp								laboratory fp									
	f	20 h	40 h	60 h	f	20 h	40 h	60 h	f	20 h	40 h	60 h	f	20 h	40 h	60 h	f	20 h	40 h	60 h		
S	13	12	13	15	12	16	15	14	11		13	14	8	9	14	15	7			11		
H'	1.61	**1.59**	**1.90**	1.92	**1.80**	**2.20**	2.19	2.11	1.39		1.93	1.78	1.64	**1.62**	**2.04**	1.96	1.35			1.55		
1-λ'	0.71	**0.70**	**0.79**	0.77	**0.80**	**0.86**	0.83	0.83	0.64		0.84	0.78	0.75	**0.73**	**0.84**	0.83	0.63			0.67		

Fig. 2 Bacterial community associated with degrading fecal pellets (fp), from fresh fp to 60-h aged fp with a 20-h time interval, visualized by DGGE profiles (unprocessed gels, REF: reference lane) and corresponding indices for diversity (phylotype richness S and Shannon-Wiener index H') and evenness (Simpson's index 1-λ'). Natural (nat) and laboratory (lab) fp originated from *Platychelipus* and *Paramphiascella.* Numbers on the gels indicate relevant OTUs.

Since the majority of aged fp grouped together in the PCO (Fig. 3a), differences in DGGE profiles between differently aged fp are relatively small, indicating that during aging the bacterial communities did not change systematically over time. Moving window analysis (Fig. 3b) illustrates a pronounced change (42 %) in the community composition of *Platychelipus* natural fp between 20 - 40 h incubation in NSW. At the same time, Shannon-Wiener diversity (H') increased from 1.59 to 1.90 and community evenness (1-λ') from 0.70 to 0.79 (Fig. 2), but there was no clear increase in phylotype richness (S). For *Platychelipus* laboratory fp, a major change (62 %) was equally observed in the 20 -40 h period or within the first 20 h. The fp of the cultured *Paramphiascella* showed a prominent change (62 %) within the first 20 h (Fig. 3b), visible in Fig. 2 by a change in dominance (from OTU 14 to OTU 7), a complete loss of OTU 14 and the emergence of new OTUs (11, 13). Elevated values of diversity indices S, H' and 1-λ' after 40 h (Fig. 2) rather than 20 h indicate a change in the 20 – 40 h period. In the fp degradation series of *Paramphiascella* fp which missed the 20-h and 40-h aged samples (see Fig. 2), the 60-h aged fp differed strongly from the

fresh fp, but no precise timing of an abrupt change could be determined (Fig. 3a, b). Overall, for all fp the community changes beyond 40 h incubation were relatively minor.

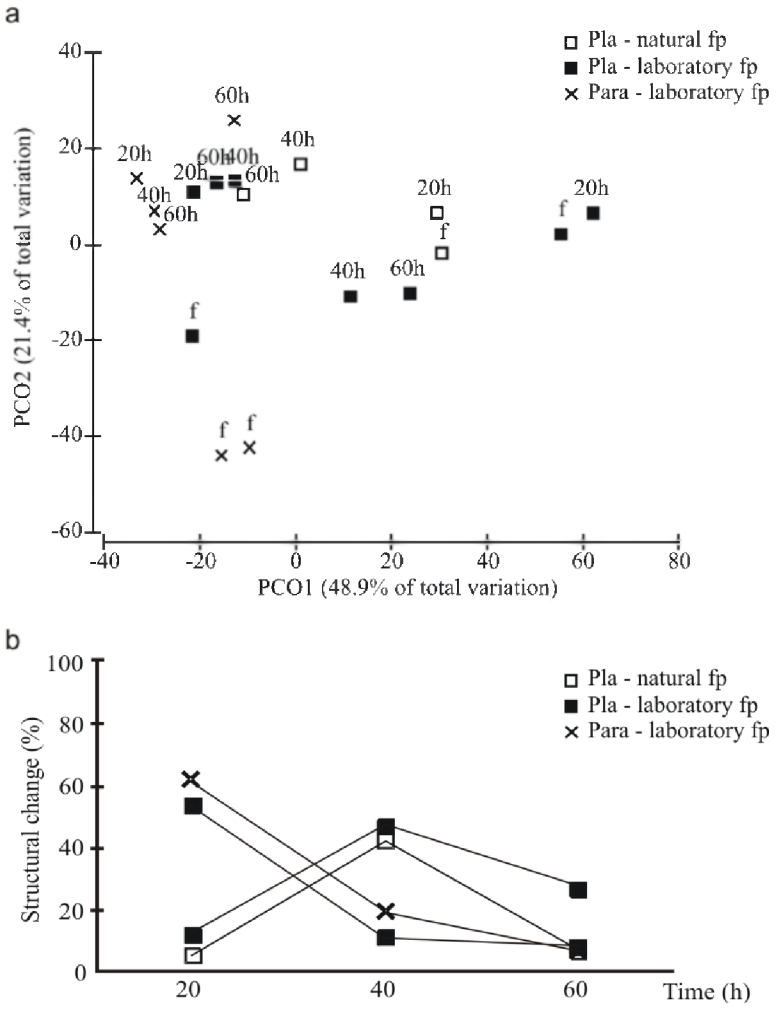


Fig. 3 Successive change in the DGGE profiles of active bacteria during fecal pellet (fp) degradation, from fresh fp to 20-h, 40-h and 60-h aged fp assessed by (a) multivariate PCO analysis and (b) by univariate moving window analysis. In the latter, data points indicate the % change of the bacterial community that occurred during a 20-h time period. Used DGGE profiles are presented in fig. 1a.

The Pareto-Lorenz evenness curves of fresh and aging fp for two selected degradation sample series (Fig. 4a, b) are highly similar and strongly convex curves. The change in evenness during fp aging showed no clear trend, except for lowered curves of *Platychelipus* (Fig. 4a). Over all fp, differing in age and from both copepod species, the y axis values ranged from 0.55 to 0.80, representative of a medium (y-value ~ 0.45) to highly (y-value ~ 0.8) functional organized community (Marzorati et al. 2008). Marzorati and co-workers (2008) defined functional organization as the ability of a community to organize in an adequate distribution of dominant and resilient microorganisms and to counteract the effect of a sudden stress exposure, e.g. environmental change.

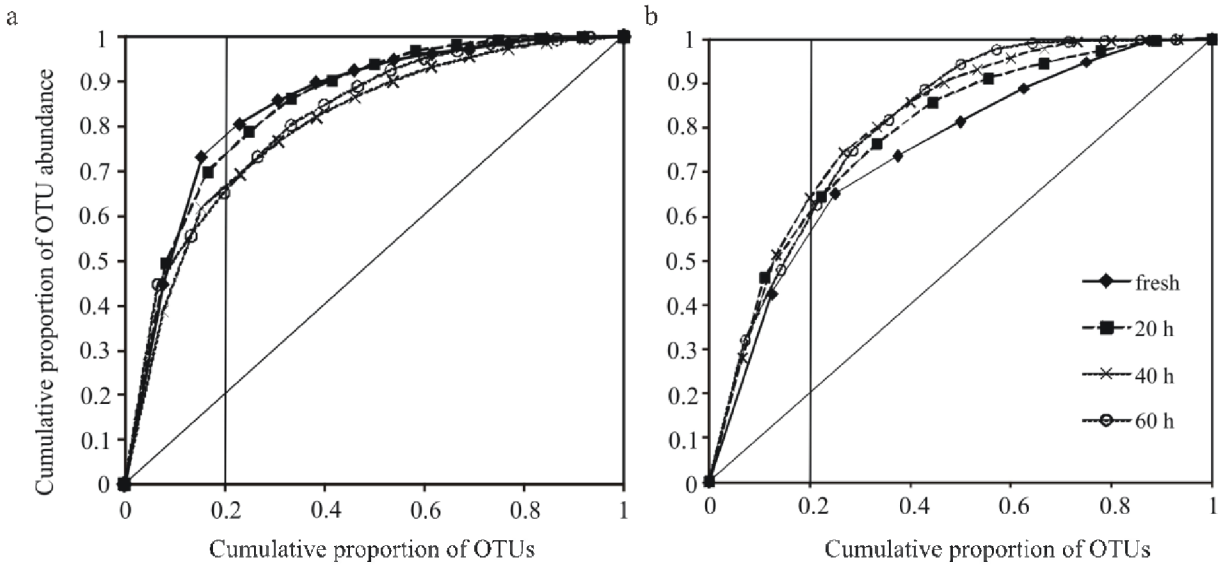


Fig. 4 Pareto-Lorenz curves of active bacterial communities associated with aging (a) natural *Platychelipus* fecal pellets and (b) laboratory *Paramphiascella* fecal pellets. The diagonal line represents perfect evenness. Curves are compared at the 0.2 x-axis value.

For both *Platychelipus* and *Paramphiascella* fp, a number of 'internal' phylotypes of fresh fp still contributed to the active community after 60 h aging of the pellet in NSW and thus persisted in presence of NSW bacteria. For *Platychelipus* fp (natural and laboratory fp), 66.7 ± 28.9 % (i.e. 4 OTUs) of the phylotypes with an originally high contribution to the fresh fp community (band intensity > 10 % of DGGE profile) still contributed > 10 % to the aged community (Table 2a) and thus persisted. From the phylotypes with a relative band intensity of 5 - 10 % or medium contributing phylotypes, another 16.7 ± 28.9 % persisted (i.e. 2 OTUs) (Table 2a). Overall, 'internal' bacteria represented almost half of the 60-h aged bacterial community, namely 46 % in *Platychelipus* fp (sum of 40.5 ± 24.5 % and 5.5 ± 5.1 %, for the > 10 % and the 5 - 10% contributing OTUs, respectively) and 56 % in *Paramphiascella* fp (sum of 4.3 ± 2.1 % and 51.9 ± 23.6 %, for the > 10 % and the 5 – 10 % contributing OTUs, respectively) (Table 2b). For the latter, the importance of medium-contributing phylotypes increased during degradation, from 30.7 ± 5.6 % to 51.9 ± 23.6 %, but the importance of highly-contributing 'internal' phylotypes reduced strongly, from 67.2 ± 2.9 % to 4.3 ± 2.1 % (Table 2b). These results showed that during aging, on all fp there was an almost even contribution (fifty-fifty) of the original, 'internal' fp phylotypes and other fp phylotypes, supposedly originating from the NSW.

Table 2 The presence of 'internal' bacteria during fecal pellet (fp) aging: (a) their persistence in aging fp, expressed as the % of OTUs from fresh fp, of which DGGE band intensity in aged communities at least equals the original band intensity in fresh fp bacterial communities; (b) their cumulative contribution to the aged active community, expressed as the summed band intensities of all persisting 'internal' OTUs compared to total DGGE profile intensity (in %).
'Internal' bacterial phylotypes are grouped as highly contributing or medium contributing phylotypes according to their initial contribution to the fresh fp bacterial community (band intensity > 10 % or 5-10 %, respectively).

a

		Persistence over time (%)								
		Highly contributing phylotypes*					Medium contributing phylotypes**			
Fp sample		# OTUs	fresh	20 h	40 h	60 h	# OTUs	fresh	20 h	40 h	60 h
Platychelipus	nat	2	100	100	50	50	2	100	50	50	50
	lab	4	100	25	50	50	2	100	100	50	0
	lab	2	100	50	100	100	1	100	0	0	0
	average		100	58.3 (38.2)	66.7 (28.9)	66.7 (28.9)		100	50 (50)	33.3 (28.9)	16.7 (28.9)
Paramphiascella	lab	2	100	0	0	0	5	100	60	80	80
	lab	2	100			0	3	100			33
	average		100			0		100			56.5 (33.2)

b

		Contribution of total aged active community over time (%)							
		Highly contributing phylotypes*				Medium contributing phylotypes**			
Fp sample		fresh	20 h	40 h	60 h	fresh	20 h	40 h	60 h
Platychelipus	nat	73	69.7	30.4	12.5	12.9	8.9	7.5	10.1
	lab	83.1	38	51.1	50.6	11.2	29.9	11.7	6.3
	lab	79.3	100	32.1	58.3	6	0	1.4	0
	average	78.5 (5.10)	69.2 (31.0)	37.9 (11.5)	40.5 (24.5)	10.0 (3.6)	12.9 (15.4)	6.9 (5.2)	5.5 (5.1)
Paramphiascella	lab	65.1	0	3	5.8	34.6	79.6	69.7	68.6
	lab	69.2			2.8	26.7			35.2
	average	67.2 (2.9)			4.3 (2.1)	30.7 (5.6)			51.9 (23.6)

* with > 10 % contribution to the active community ** with 5-10 % contribution to the active community

Metabolic potential of fp bacteria

AWCD (violet coloration) in EcoPlate assays differed among sample types based on inoculum density differences ($p > 0.001$). NSW showed a rapid colour development, i.e. at final measurement (after 10 days) being 5 - 10 times higher than fp, *Seminavis* and copepod samples ($AWCD_{NSW} = 1.03$, $AWCD_{fp} = 0.22$, $AWCD_{Seminavis} = 0.14$, $AWCD_{copepod} = 0.14$; N = 3, 27, 3, 9 resp.; SD all ± 0.10) (post hoc, all $p < 0.001$), as well as the most complex CSUP (27 out of 31 substrates used). Analyses of the CSUPs were executed on an AWCD set-point of approximately 0.20 OD (see Material and methods). Note that for NSW, where AWCD 0.2 OD is already reached at day 3, the analysed CSUPs may not represent the complete metabolic potential of NSW bacteria but included only the fastest responding substrate reactions. Functional diversity measured by CSUP differed between fresh fp and 60-h aged. In a PCA (Fig. S2), fresh and aged fp grouped almost separately (PC 1 and PC 2 explaining 41.5 % and 23.4 % of the total variation, respectively). The difference in CSUP of fresh and aged fp was small and insignificant for both *Platychelipus* (ANOSIM, R = 0.158; p = 0.012) and *Paramphiascella* (ANOSIM, R = -0.125, p = 0.61), respectively, although aged fp samples grouped closely together (except for one outlier) while spreading of fresh fp samples was high. This represents a relatively high variability in CSUPs of fresh fp which diminished during fp aging. In case of *Platychelipus*, fresh fp bacteria used 7 ± 2 substrates (N = 10) while aged fp bacteria consistently used a lower number of substrates (S = 2 ± 2, N = 7) (Fig. 5a, b). Besides substrate richness, substrate OD values - being indicative of bacterial metabolic activity (cell activity or cell abundance) - of aged fp samples were generally low while these fp were exposed for 60 h to free-living seawater bacteria with a higher metabolic potential (S = 7 ± 2, N = 3) (Fig. 5a, b). On the other hand, fresh fp bacteria utilized more substrates than *Seminavis* and copepod bacteria where substrate richness was only 2 ± 0 (N = 3) and 5 ± 3 (N = 8), respectively (Fig 6a, b, c). A reduced substrate utilization pattern

of aged fp was not found for *Paramphiascella* fp (Fig. 5c), where substrate richness S was 7 ± 3 (N = 6) for fresh fp and 8 ± 6 (N = 2) for aged fp.

The most frequently utilized substrates by fp bacteria were (in decreasing order): tween 80 (19 responses), N-acetyl-D-glucosamine (18 responses), D-mannitol and D-cellobiose (each 15 responses), glycogen (10 responses), α-D-lactose (8 responses), tween 40 and L-threonine (each 7 responses) and L-asparagine and putrescine (each 6 responses). Fp bacteria were able to metabolize all substrate guilds in contrast to bacteria from *Seminavis*, copepods and NSW (Table S2). For all sample types carbohydrates and polymers were the main utilized guilds. Laboratory fp bacteria utilized tween 40 and miscellaneous compounds (pyruvic-acid-methyl-ester, D,L-α-glycerol phosphate), which were unique for *Seminavis* and copepod CSUPs, respectively, and were not used by seawater bacteria. For NSW, substrate richness increased strongly at higher AWCD readings, but in this study only the fast responding substrates have been included (Fig 6d).
A total of 127 DGGE bands originating from positive wells and covering the entire pattern spread (data not shown), were successfully sequenced. The observed substrate oxidations were realized by fp bacteria belonging predominantly to Gammaproteobacteria and Alphaproteobacteria. Further sequencing revealed the following genera: *Pseudomonas*, *Pseudoalteromonas*, *Marinomonas*, *Alcanivorax* and *Thallassospira* for fp of both copepod species. From *Platychelipus* fp, additional genera were *Halomonas*, *Rhodovulum*, *Photobacterium* and a dominant presence of *Vibrio*. A few Bacteroidetes (*Myroides*, *Flavobacterium*) were retrieved only from *Platychelipus* fp.

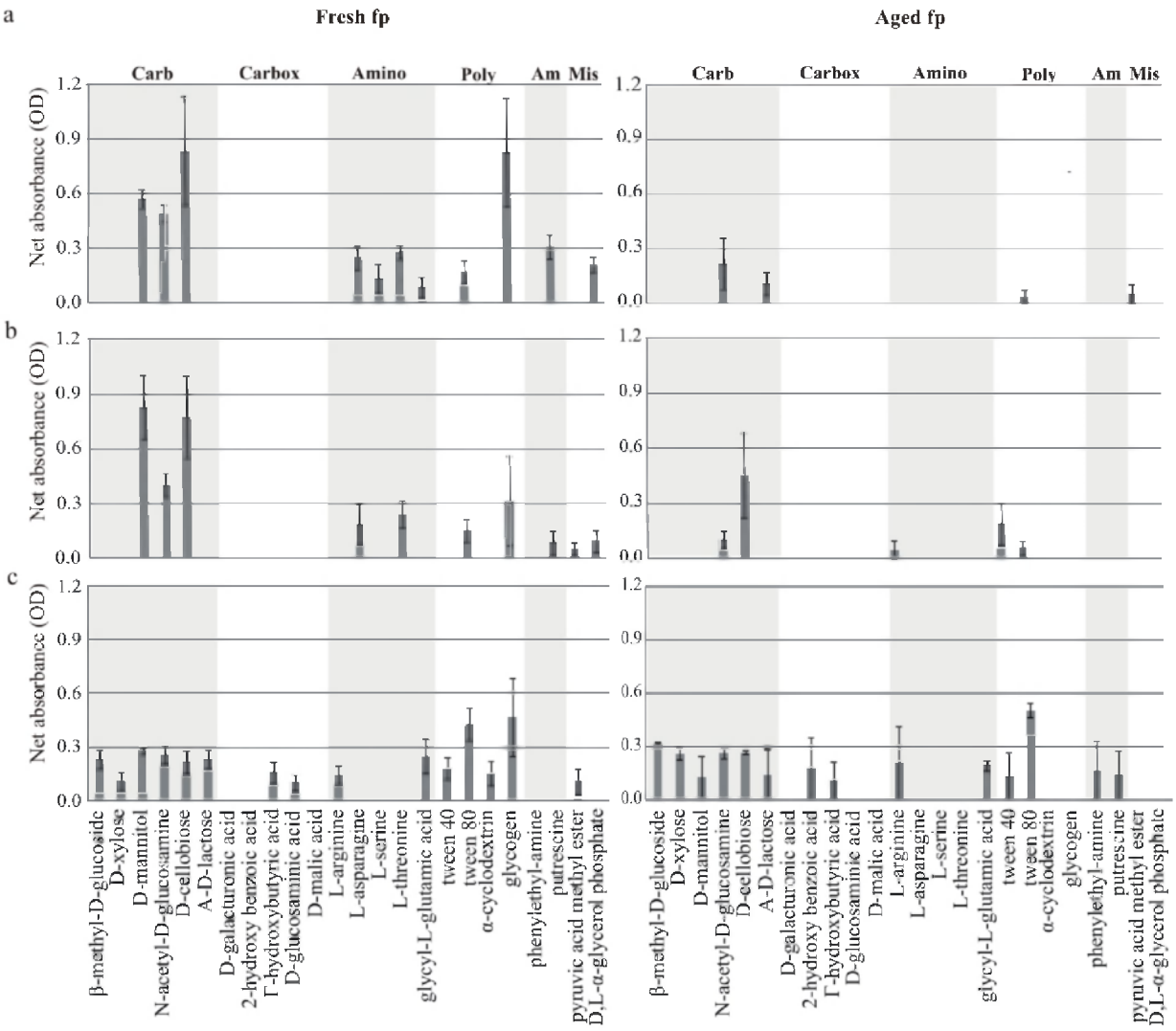


Fig. 5 Carbon substrate utilization patterns (CSUP) of bacteria associated with fresh (left) and 60-h aged (right) fecal pellets (fp): (a) natural *Platychelipus* fp, (b) laboratory *Platychelipus* fp and (c) laboratory *Paramphiascella* fp. When the substrate was positive in at least one replicate, OD values of all replicates were averaged and represented by bars (average ± SE).
Carbohydrates (Carb), carboxylic acids (Carbox), amino acids (Amino), polymers (Poly), amines (Am), miscellaneous (Mis).

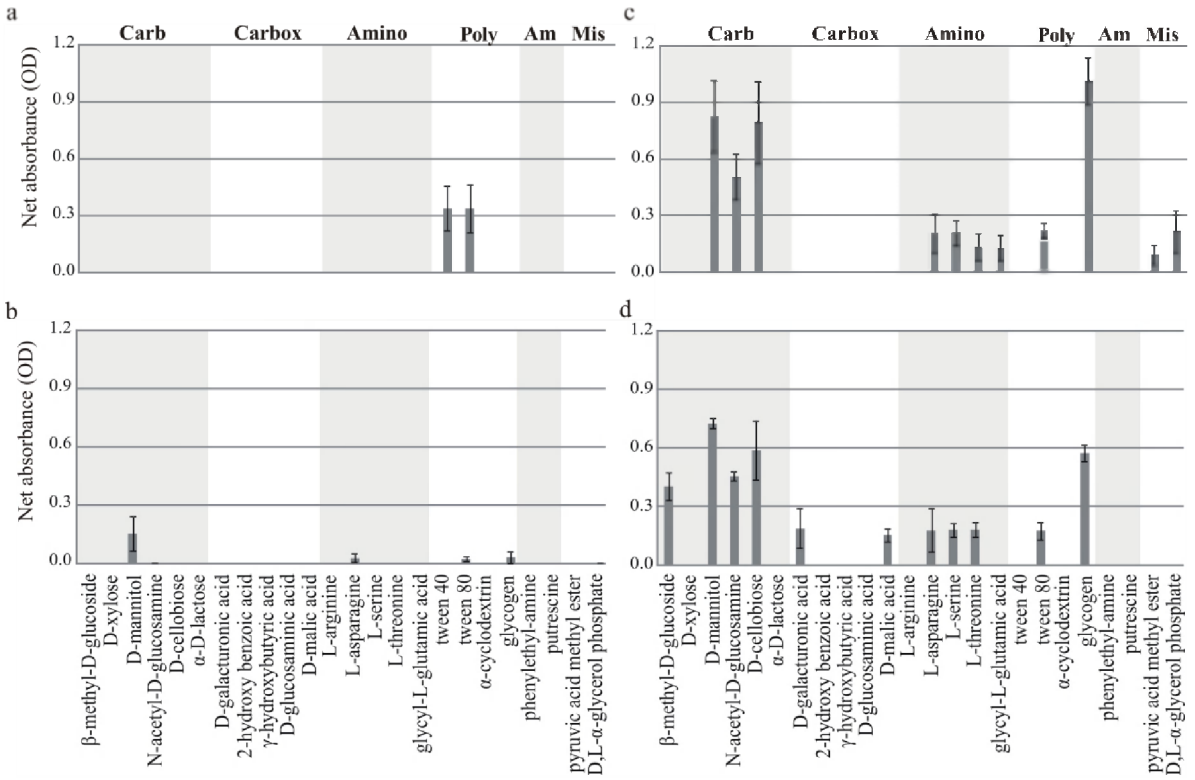


Fig. 6 Carbon substrate utilization patterns (CSUP) of bacteria associated with (a) *Seminavis*, (b) field-caught *Platychelipus* copepods, (c) laboratory-fed *Platychelipus* copepods and (d) natural seawater (NSW). When the substrate was positive in at least one replicate, OD values of all replicates were averaged and represented by bars (average ± SE). Carbohydrates (Carb), carboxylic acids (Carbox), amino acids (Amino), polymers (Poly), amines (Am), miscellaneous (Mis).

DISCUSSION

Microbes (protozoa and bacteria) are key degraders of copepod fp. For understanding the process of bacterial fp degradation, it is important to investigate the degradation taking place in the interior and on the exterior of the fp.

Many studies have proven the bacterial presence inside copepod fp as well as on the fp exterior (e.g. Gowing & Silver 1983, Lawrence et al. 1993). Few studies have focused on the metabolic activity of fp bacteria (Tang et al. 2001, Thor et al. 2003), which is of primary interest for investigating fp degradation, since intact bacterial cells observed in fp are not necessarily in an active status (viable but dormant, or inviable and harmed during gut passage but without cell lysis). Therefore, this study reports on bacterial fp degradation and the successive role of 'internal' and external bacteria, using RNA-based DGGE and carbon substrate utilization patterns, in which strictly the active fp bacteria are analyzed and thus excludes the fraction of viable but non-active 'internal' bacteria or the bacterial cells which may have died during the degradation process but are still associated with the fp.

Internal fp bacteria

Assemblages of active bacteria packed within fp were variable in species diversity and abundances and were more diverse for fp originating from field-caught copepods (natural fp) than for fp from cultured copepods. This is not surprising, given the trophic plasticity of benthic copepods allowing them to switch from one food source to another (Hicks & Coull 1983) . These 'internal' fp bacteria must originate from the

copepod's gut flora or from transient food bacteria. Long-term rearing of copepods in the lab on a single food source resulted in a more constant bacterial community than on fp of copepods from the field. Even for field-caught copepods, a reduction in fp bacterial diversity was found after 1 to 2 days of laboratory feeding.

Thus, differences in bacterial assemblages on natural fp can be explained by natural variability in copepod's feeding behavior which in turn shapes copepod gut flora. Variability in copepod gut microflora is well-known, with resource composition but also host feeding activity and copepod life history as potential regulating factors (Harris 1993, Tang 2005, Grossart et al. 2009, Tang et al. 2009, Cnudde et al. 2011). The food source with its 'microbial coat' delivers bacteria to the gut, which are voided shortly after (transient bacteria), or become part of the gut flora (resident bacteria) (Tang et al. 2009, De Troch et al. 2010), or food source ingestion can change bacterial dynamics inside the gut and stimulate bacterial growth of the gut flora (Tang 2005, Tang et al. 2009). Yet, the precise origin of gut bacteria, i.e. transient vs resident, is difficult to establish. The resident gut flora is actually composed of bacteria which are ingested through grazing and/or through 'drinking' of seawater. For copepods reared on *Seminavis*, the majority of active 'internal' fp bacteria as derived from DGGE band intensity (but not in terms of diversity of OTUs) related to the food source. Even in field-caught copepods which had been fed *Seminavis* for 24 h, at least one third of the active 'internal' fp bacteria originated from the food source. This strongly indicates that food bacteria have an immediate signature on the 'internal' bacterial community. This immediate and drastic impact of food on copepod flora explains why we did not find direct evidence of copepod-specific (resident) bacteria in fresh fp. Some genetic diversity of fresh fp bacteria may also originate from copepod exoskeleton bacteria, not only through colonization after fp egestion but through deposition of bacteria positioned around the anus (Carman & Dobbs 1997) during fp egestion.

Note that some OTU's (OTU 7, OTU 18) of laboratory fp which we considered to originate from *Seminavis*, were shared with natural fp. These OTU's may represent general diatom-associated bacteria which are not specific for *Seminavis* but also occur on other diatoms. In addition, caution is due when comparing specific OTUs on DGGEs of bacterial communities from different environments, because an OTU can represent more than a single bacterial species. We did not perform repeated sequencing of single bands and can therefore not completely exclude that particular OTUs may have represented more than a single bacterial species.

The presence of a diverse 'internal' active bacterial community in freshly produced fp, independent of their origin (i.e. laboratory or natural), underlines the general occurrence of 'internal' fp degradation. Due to the close link between food source bacteria and copepod bacteria, it is difficult to exactly determine the ratio transient : resident fp bacteria.

Structural shifts in bacterial communities during fp degradation

Incubation of fp in seawater rapidly and drastically changed the active fp bacterial communities (within the first 40 h), which is in agreement with the bacterial community shifts on planktonic fp (Jing et al. 2012). However, apart from this rapid early colonization, the latter study is in strong conflict with the current one, as it reports a high significance of bacterial diversity colonizing the copepod fp, while in the current study, the initial bacterial diversity of fresh fp was most spectacular and not the added diversity from seawater bacteria. Although no other studies have evaluated the importance of bacterial colonization in terms of diversity, the limited colonization of free-living bacteria from seawater on fp is in agreement with Gowing and Silver (1983), who found substantially higher bacterial abundances on the surfaces of laboratory-incubated fp than on those of field-collected planktonic fp. Nevertheless, the number of seawater-derived phylotypes colonizing the fp may have been underestimated for the following reasons. Some common seawater bacteria may regularly be ingested by copepods and thus become part of their gut flora, so that they also end up as 'internal' fp strains. Alternatively, fp colonization may be a selective

process in which only certain groups of seawater bacteria participate, but the trigger for bacterial attachment to copepod fp is yet unknown (Jacobsen & Azam 1984). We can, however, only compare our results with those of planktonic fp, since bacterial colonization rate for benthic copepod fp has not been studied.

However, during fp aging prominent changes in community structure were related to 'internal' bacteria due to a loss or elevated contribution of certain bacterial phylotypes and shifts in dominance. 'Internal' bacterial phylotypes participated almost equally to the 60-h aged bacterial community as external phylotypes (based on DGGE band intensity), indicating that 'internal' bacteria were not outperformed by external bacteria. As long as fp matter is tightly packed and surrounded by the peritrophic membrane, 'internal' and invasive external bacteria supposedly easily co-exist (limited competition for resources and space). Moreover, the progeny of attached fp bacteria is released into the seawater (Jacobsen & Azam 1984, Thor et al. 2003). Poulsen and Iversen (2008) reported that up to 59 % of the degradation rate of small planktonic copepod fp is due to fp-associated bacteria, but the applied experimental setup was not designed to make a distinction between 'internal' fp bacteria or surface-attached fp bacteria. Most studies report high 'internal' bacterial abundances (Honjo & Roman 1978, Gowing & Silver 1983, Jacobsen & Azam 1984, Tang 2005) and bacterial activity (Olsen et al. 2005, Ploug et al. 2008, Poulsen & Iversen 2008).

Functional diversity

Functionality of specific ecological microbial communities has been deduced from Lorenz curves in multiple studies (Dejonghe et al. 2001, Mertens et al. 2005, Wittebolle et al. 2008). In spite of a genetic shift in bacterial community during fp aging, fp of different ages showed a similar functional organization, characterized by a high level of dominance (Lorenz curves, on average 65 %), suggesting a specialized community (Marzorati et al. 2008). A stable functional organization could imply functional redundancy (Fernandez et al. 2000). However, dissimilar metabolic potential of fresh fp and aged fp communities (see further) rather suggests that the concept of functional redundancy is not valid for these environmental bacterial assemblages. Moreover, in the case of fp bacterial communities, community structure is not a good indicator for community functionality. Nevertheless, these results have to be carefully interpreted since DGGE-based diversity estimates may be biased at each single step involved in the molecular analysis (cell lysis, RNA extraction and degradation, PCR amplification and DNA fragment separation) (von Wintzingerode et al. 1997). Furthermore, the additional incubation period of fresh fp to increase bacterial abundances may have affected the bacterial community structure. For example, differential bacterial growth rates among species or complex bacteria-bacteria interactions may yield differences in community composition.

In marine environments, Biolog plates have already successfully been applied for the assessment of bacterial metabolic diversity of e.g. mollusks (Smith et al. 2001), estuarine bacterioplankton (Schultz & Ducklow 2000) and marine bacterioplankton (Jellett et al. 1996, Sala et al. 2008). Carbon substrate utilisation profiles of such culture-dependent assays do not necessarily reflect the functionality of the community under natural conditions (Smalla et al. 1998), due to the loss of the uncultivable fraction and due to shifts in the composition and density of the remaining fraction. Moreover, Biolog assays measure bacterial metabolic potential under aerobic conditions, while fp bacteria may be primarily facultative anaerobes or microaerophiles (Gowing & Silver 1983). Typically, the environment fp are exposed to after egestion, i.e. the intertidal sediment, is often hypoxic or even anoxic. At the site where *Platychelipus* was collected, for instance, oxygen penetration depth in the sediment is ca. 3 mm (Van Colen et al. 2012). Our lab incubations deviated from these *in situ* conditions in exposing the pellets to oxic conditions. Therefore, Biolog patterns should be interpreted with caution.

A predominant use of carbohydrates and polymers by external as well as 'internal' fp bacteria suggests that both may be able to degrade the fp peritrophic membrane, which is composed of polysaccharides such as chitin (N-acetyl-D-glucosamine sugar units) (Ferrante & Parker 1977, Kirchner 1995). Through the bacterial degradation of the peritrophic membrane and other complex substrates in the fp, carbon and nitrogen are recycled. At first sight, the reduced functional diversity of 60-h aged fp is unexpected given the increased genetic diversity of active bacteria compared to fresh fp, as shown by our DGGE profiles. This can perhaps be explained by metabolic specialization of the bacterial assemblage on aged fp, and by a low availability of high-quality substrate remaining in 60-h aged fp. For example, zooplankton fp contain high concentrations of amino acids (Poulet et al. 1986). These amino acids are selectively utilized by marine heterotrophic bacteria (Amano et al. 1982, Bright & Fletcher 1983), and in combination with spontaneous DOM release during fp aging, fp are rapidly depleted in amino acids, depending on temperature, within 3 to 5 days (Roy & Poulet 1990). Reduced functional diversity can also result from a reduced bacterial cell density associated with 60-h aged fp. It has been reported that bacterial cell abundance diminishes during degradation of diatom-based fp of the planktonic *Acartia tonsa* (Hansen et al. 1996), but we did not determine bacterial abundances on fp.

The metabolic profiles yield only limited information on the origin of fp bacteria due to the large overlap in substrate utilization between bacteria from *Seminavis*, copepods and seawater. The common occurrence of tween 40 utilisation by bacteria on *Seminavis* and on fresh and aged fp bacteria, supports the statement that undigested food bacteria may contribute to fp degradation.

The dominant presence of main groups Gamma- and Alphaproteobacteria and particularly the strong occurrence of *Vibrio* sp. on copepod fp is in line with earlier studies describing fp bacterial diversity (Delille & Razouls 1994, Hansen & Bech 1996, De Troch et al. 2010, Jing et al. 2012). Although *Vibrio* sp. is a well-known free-living seawater bacterium, some of these studies showed their enriched presence on copepod fp. Based on the high number of different OTUs retrieved from the fp Biolog assays and identified as *Vibrio* sp., our findings suggest their primary contribution to the observed substrate utilization pattern and thus their general importance to the fp degradation process.

General conclusion

Based on bacterial community dynamics, this study proves the significant role of 'internal' bacteria to fp degradation compared to bacteria which colonize fp from the outside. 'Internal' bacterial diversity is variable but significantly contributes to the total bacterial diversity of aging fp. 'Internal' fp bacteria consist of ingested food-associated bacteria, copepod gut bacteria and probably seawater bacteria. Copepod diet is the overall regulator of fp 'internal' bacteria: it delivers active bacteria directly to the fp and indirectly, it shapes the copepod gut flora, hence fp degradation by 'internal' bacteria may vary with copepod feeding ecology. Exterior colonization by ambient seawater bacteria occurred rapidly but by a rather limited number of bacterial phylotypes. Free-living seawater bacteria but also 'internal' fp bacteria are able to utilize a wide range of substrates, primarily carbohydrates and polymers. In terms of bacterial diversity and functional potential, there is little reason to believe that bacterial degradation of copepod fp on the interior is inferior to degradation from the outside.

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Chapter 7

Atomic Force Microscopy - Laser Scanning Confocal Microscopy imaging protocol for copepod fecal pellets

Under revision:

Francesca Malfatti, Clio Cnudde* and Marleen De Troch. Atomic Force Microscopy - Laser Scanning Confocal Microscopy imaging protocol for copepod fecal pellets. Scientific Reports*

* Equal contribution

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ABSTRACT

Within the marine carbon and nitrogen biogeochemical cycles, fecal pellets produced by pelagic and benthic copepods are important microbial activity hotspots as particle substrate for bacteria colonization and as sources of particulate and dissolved organic C and N. We developed a protocol combining Atomic Force Microscopy (AFM) and Laser Scanning Confocal Microscopy (LSCM) to study the peritrophic membrane structure and associated bacteria of fecal pellets produced by a benthic copepod, *Paramphiascella fulvofasciata.* AFM imaging revealed a fibrillar network structure of the peritrophic membrane, 0.7-5.9 nm thick similar to marine polysaccharides and α-chitin. Bacterial cell volume range was 0.006-0.117 μm^3 in liquid. LSCM imaging showed a 3D-heterogeneous microenvironment. This protocol would allow high-resolution interrogation of structural changes and bacterial dynamics within the copepod fecal pellets and other heterogeneous particles such marine snow under environmental conditions.

INTRODUCTION

Copepod fecal pellets significantly contribute to marine carbon and nitrogen cycles ((Tang et al. 2010) and references therein). As fecal pellets leach dissolved organic carbon and nitrogen (Thor et al. 2003), they can be colonized by heterotrophic bacteria (Gowing & Silver 1983) that further degrade the particulate fecal matter. Beside microbial degradation, other organisms contribute to their recycling, such as dinoflagellates and copepods (Poulsen et al. 2011). In the marine ecosystem, fecal pellet export fluxes are relatively small if compared to their production (Sampei et al. 2004) suggesting that pellet degradation is an important and efficient process. Microbial pellet degradation strongly depends on copepod species, copepod diet, fecal pellet surface-volume ratio, bacterial species and on the presence of the peritrophic membrane. Bacterial diversity on fecal pellets has been characterized (De Troch et al. 2010) and researchers have studied bacterial colonization and degradation rate of fecal pellets by SEM and TEM

(Köster et al. 2011). High bacterial abundances on the outer surface and the presence of internal metabolically active bacteria (Jacobsen & Azam 1984) and high rate of hydrolytic enzyme activities (Lawrence et al. 1993) indicate two ways of degradation that could occur simultaneously: outside-in and/or inside-out. Innovative high-resolution imaging tools can now visualize fecal pellet bacteria and follow their microbial dynamics. In the current era of molecular techniques, imaging analysis remains of value since "seeing is believing" especially if it is quantitative!

We investigated the structure of the fecal pellets of a benthic copepod, *Paramphiascella fulvofasciata,* and their associated bacteria by Atomic Force Microscopy (AFM) and Laser Scanning Confocal Microscopy (LSCM) (Fig. 1a). We coupled AFM-LSCM to image at high-resolution the surface and the inside of the pellets. AFM, invented in 1986 (Binnig et al. 1986), can examine the nanoscale topography and nanomechanical properties of live cells and biological material (Dufrene 2002) under environmental conditions, while achieving atomic resolution. In the marine microbial ecology field, AFM has been used so far to study diatom nano-structures and their organic matter surface layer (Higgins et al. 2002, Pletikapić et al. 2012), gel particles and colloids (Santschi et al. 1998, Misic Radic et al. 2011) and bacteria (Nishino et al. 2004, Malfatti & Azam 2009). LSCM with 3D Z-sectioning is a powerful tool to map the pellet internal structural organization. LSCM has been used in the study of marine snow that presents a very heterogeneous composition and structure (Holloway & Cowen 1997, Malfatti & Azam 2009). Our goals were (1) to establish an AFM-LSCM protocol for imaging pellets, (2) to image the ultrastructure of the peritrophic membrane and (3) to visualize in 3D the internal fecal pellet micro-environment.

METHODS

Fecal pellet sample preparation

Adults of the benthic marine copepod *Paramphiascella fulvofasciata* (Harpacticoida, family Miraciidae) were fed the pennate diatom *Seminavis robusta* (~ 35 µm in length) and the flagellated microalga *Isochrysis* sp. (~ 5 µm in diameter) as previously described (De Troch et al. 2010). Fecal pellets were egested in 0.2 µm filtered autoclave seawater and were individually picked within 24 hours and fixed with a solution of 2 % formaldehyde (1h at 4°C). Then, the fecal pellets were deposited on an ethanol cleaned glass slide and left to dry completely at RT (protected from dust particles). In order to remove the salt crystals, the slides were then rinsed by dipping the slide in MilliQ water in a petri dish and dried again prior to imaging. We adapted this protocol from the method developed by Beardsley and colleagues for the preservation of marine bacteria samples on polycarbonate filters for microscopic analysis (DAPI staining and FISH – Fluorescence In Situ Hybridization) (Beardsley et al. 2011).

Atomic force microscopy

Atomic force microscopy (AFM) imaging was performed with a MFP-3D BIO (Asylum Research) as described by Malfatti and Azam (Malfatti & Azam 2009). We imaged the fecal pellets under hydrated condition (hydrated in sterile MilliQ water, called liquid from here on) and in air (dried conditions). AFM in liquid was necessary in order to evaluate image artifacts (such as organic matter deposition during the drying process), the hydrated dimension of the peritrophic membrane and the presence of deliquescent water surrounding the bacterial cells (Su et al. 2012). We imaged the pellets in AC mode. In liquid, we used a silicon nitride cantilever (TR400PB Olympus) with a spring constant of 0.02 N/m and scan rates of 0.5-0.8 Hz. In air, we used a silicon cantilever (AC160TS; Olympus), with a spring constant of 42 N/m and scan rates of 1 Hz. Given the scan rates used to generate an image it took from 4 to 12 minutes. The scan rate is adjusted to achieve the best image resolution and consequently AFM is not a high-throughput instrument (please note that new AFM can indeed achieve video-rate capture, but not our system nor our sample

type). The height, amplitude channels were recorded since the height channel gives quantitative data on sample topography, the amplitude channel, an error mode, offers high-resolution details of the sample surface. Topography images were processed with Planfit and Flatten functions in order to create 3D images.

Bacterial cell volume estimation by AFM

We have used two equations for computing bacterial cell volume: 1) $V=1/4\ (\pi/4)W^2 \times (L-W/3)$, where L is the length and W is the width and W is assumed to be equal to Z (height) (Bratbak 1985). This formula is widely used in the field of marine microbial ecology especially since only L and W are measurable from a 2D epifluorescence microphotography and is used here for comparison purposes; 2) $V=4/3\pi \times a \times b \times c$, where a, b and c being the semi-axes of the 3D-ellipse and a (=W, width) is different than c (=Z, height) (Nishino et al. 2004, Malfatti et al. 2010). This formula can be used only if the third dimension (Z, height) is measured.

Laser scanning confocal microscopy

LSCM followed the AFM interrogation of the same pellet. We marked the position of the pellet on the slide by carving with a diamond pen the back of the slide. We used these scratches as guiding lines to find the target at 1000x at LSCM. We stained the pellets using DAPI ('4- 6'diamidino-2-phenylindole), solution. This dye binds to nucleic acids thus labeling bacteria and Eukaryotic nuclei (Porter & Feig 1980) and it can unspecifically stain organic matter (Mostajir et al. 1995). A drop of a solution of DAPI VECTASHIELD (Vector Laboratories, Burlingame CA, USA) was deposited on the fecal pellets and immediately imaged at the A1R LSCM (Nikon, Japan). The samples were excited using three lasers: 405 nm, 488 nm and the 638 nm. The 405 nm laser excites the DAPI fluorophore that is a nuclear stain for detecting bacteria and other DNA containing cells (e.g. algae); the 488 nm laser has been used to excite the chitin-like structure that forms the peritrophic membrane of the fecal pellets (Yoshikoshi & Ko 1988) and the 638 nm laser visualized the chlorophyll, present in the diatoms and microalgae that have been fed to the copepods.

RESULTS

We successfully developed a protocol that allows imaging of the same fecal pellet (Fig. 1a) by AFM and by LSCM. The fecal pellet is a very heterogeneous environment both on its surface as well as in its interior. AFM imaging of the fecal pellets has been a challenge due to their surface adhesive properties, the rough surface topography and structural heterogeneity. Twenty formaldehyde-fixed pellets were imaged but the surface organic material interfered with the scan thus causing poor image quality and probe contamination. We were successful at imaging and analyzing parts of six fecal pellets (Fig. 1 to 3).

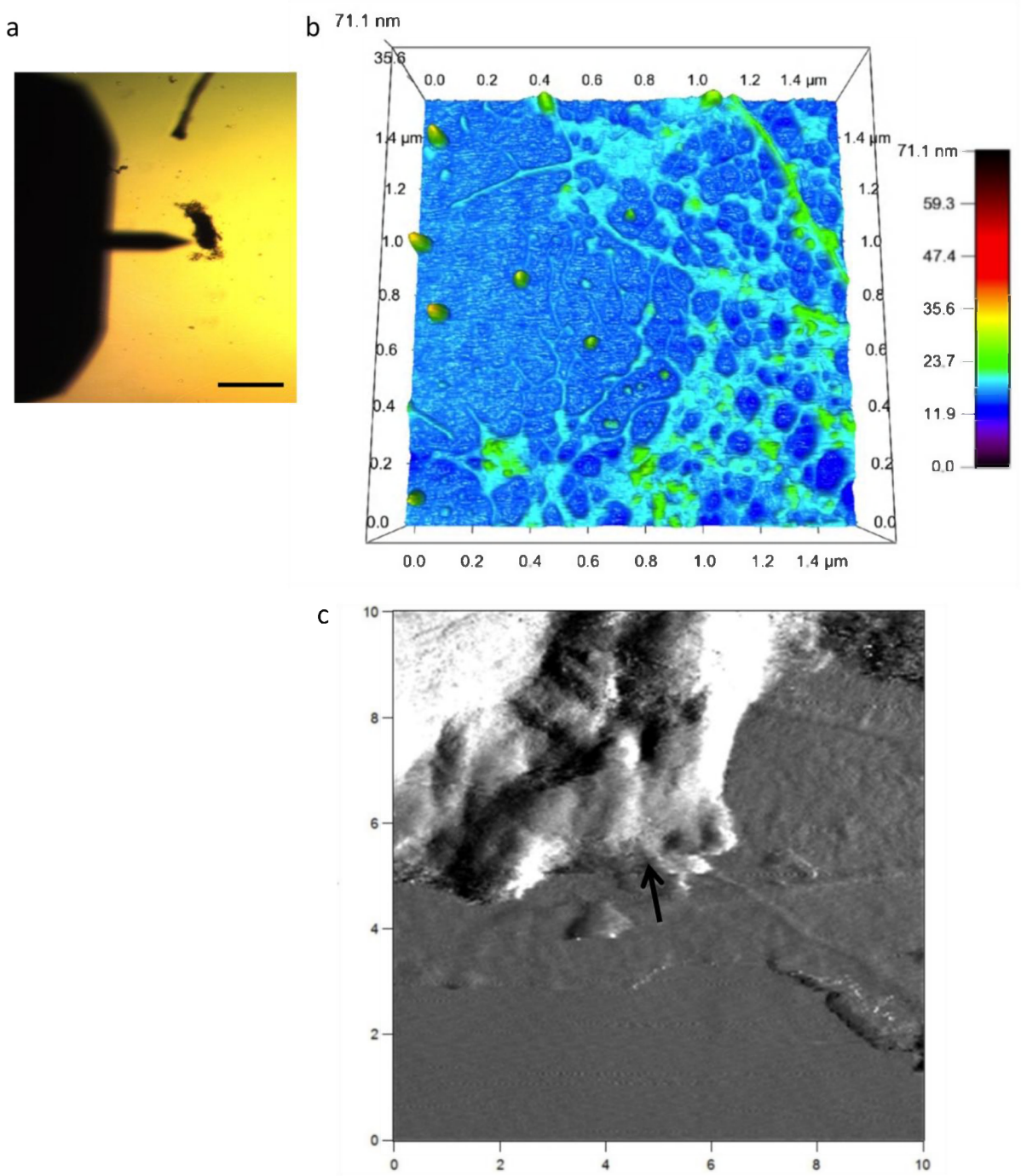

Fig. 1. Fecal pellet images by AFM. a) Transmitted light image of a fecal pellet at the AFM. The cantilever (arrow shape) is visible as a shadow on the left-side of the pellet. Scale bar 160 µm length. b) Topography image of the fine structure of peritrophic membrane in air. Color scale indicates Z (height), scan size is 1.5 x 1.5 μm^2. c) Peritrophic membrane in liquid (black arrow is point at the perimeter of the membrane). Amplitude channel, scan size is 10 x 10 μm^2.

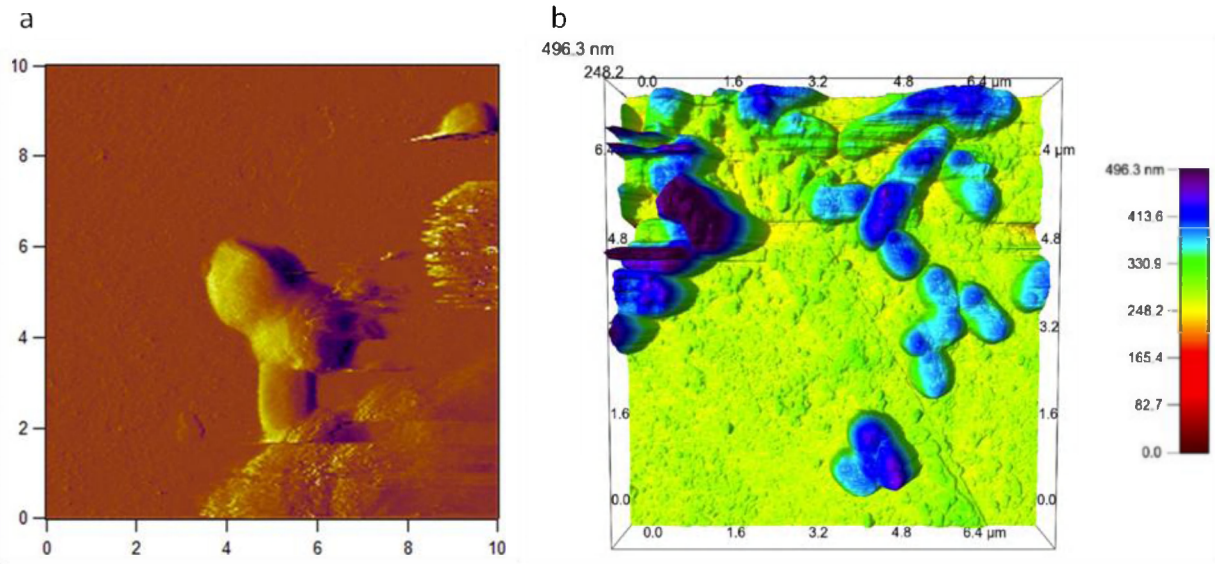

Fig. 2. Fecal pellets associated bacteria, images by AFM. a) Two bacteria imaged in liquid; a dividing cell adjacent to a rod shape cell (blue arrow is pointing to dividing cell). Amplitude channel, scan size 10 x 10 μm^2. b) Topographic image of bacteria in air. On the left side, some strips are visible indicating organic matter attaching to the cantilever tip thus influencing the scan quality. Color scale indicates Z (height), scan size 7 x 7 μm^2.

Peritrophic membrane

The fine structural organization of the peritrophic membrane was clearly visible at the very margin of the fecal pellet (Fig. 1b) where the membrane was not covered by particles and the thickness of the pellet was reduced. If the membrane is covered by particulate matter (*i.e.* bacteria, algae, uncharacterized debris) the AFM cannot distinguish this little difference in Z (height) on a very rough topography. In general, the fecal pellet surface showed large difference in topography of the order of tens of microns.

The image details of the peritrophic membrane in air were sharper than in liquid (Fig. 1b, c). In air, we measured the height of single fibrils whereas in liquid, since we could not identify any more the fibrils we measured the entire margin of the peritrophic membrane.

AFM showed that the structure of the peritrophic membrane was similar to a fine fibrillar network (Fig. 1b). The peritrophic membrane thickness ranged 20.0 -83.1 nm in the images acquired in liquid and 0.7 - 5.9 nm in the images acquired in air (Table 1).

Fecal pellet bacteria

By AFM, we visualized bacteria associated with the peritrophic membrane (Fig. 2 a,b). We measured width (W), length (L) and height (Z) of 20 cells in liquid and 33 cells in air and then we have computed their cell volume (Table 1). Cell volume estimates, with either W≠Z or W=Z (see Methods), showed similar results when comparing liquid versus air measurements. When using the assumption W≠Z the average values (0.07± 0.084 μm^3 in liquid and 0.04±0.342 μm^3 in air) were statistically significantly different than those computed with W=Z (0.30±0.266 μm^3 in liquid and 0.031±0.257 μm^3 in air).

	Peritrophic membrane			Bacterial cell volume			
	Z nm	# Measurements	Range nm	W≠Z (μm^3)	W=Z (μm^3)	# Cells	Range W≠Z (μm^3)
Air	2.2±1.28	30	0.70-5.86	0.07±0.084	0.30±0.266	30	0.003-0.322
Liquid	53.9±26.60	10	20.0-83.1	0.04±0.342	0.31±0.257	22	0.006-0.117

Table 1. AFM measurements of peritrophic membrane height (Z, in nm) and bacterial cell volume (in μm^3) in liquid and in air

Fecal pellets organization and bacterial distribution

By LSCM, we were able to 'see' the pellet 3D-organization (Fig 3 a, b). The pellet is a highly heterogeneous microenvironment. The fecal pellet interior was composed by food residues (algae), uncharacterized fluorescent debris and bacteria (as single cells and colonies). A very dim yellow fluorescent signal was visible at the pellet perimeter, i.e. the location of the peritrophic membrane (as reported by (Yoshikoshi & Ko 1988)) due to the N-acetyl-glucosamine residue fluorescence.

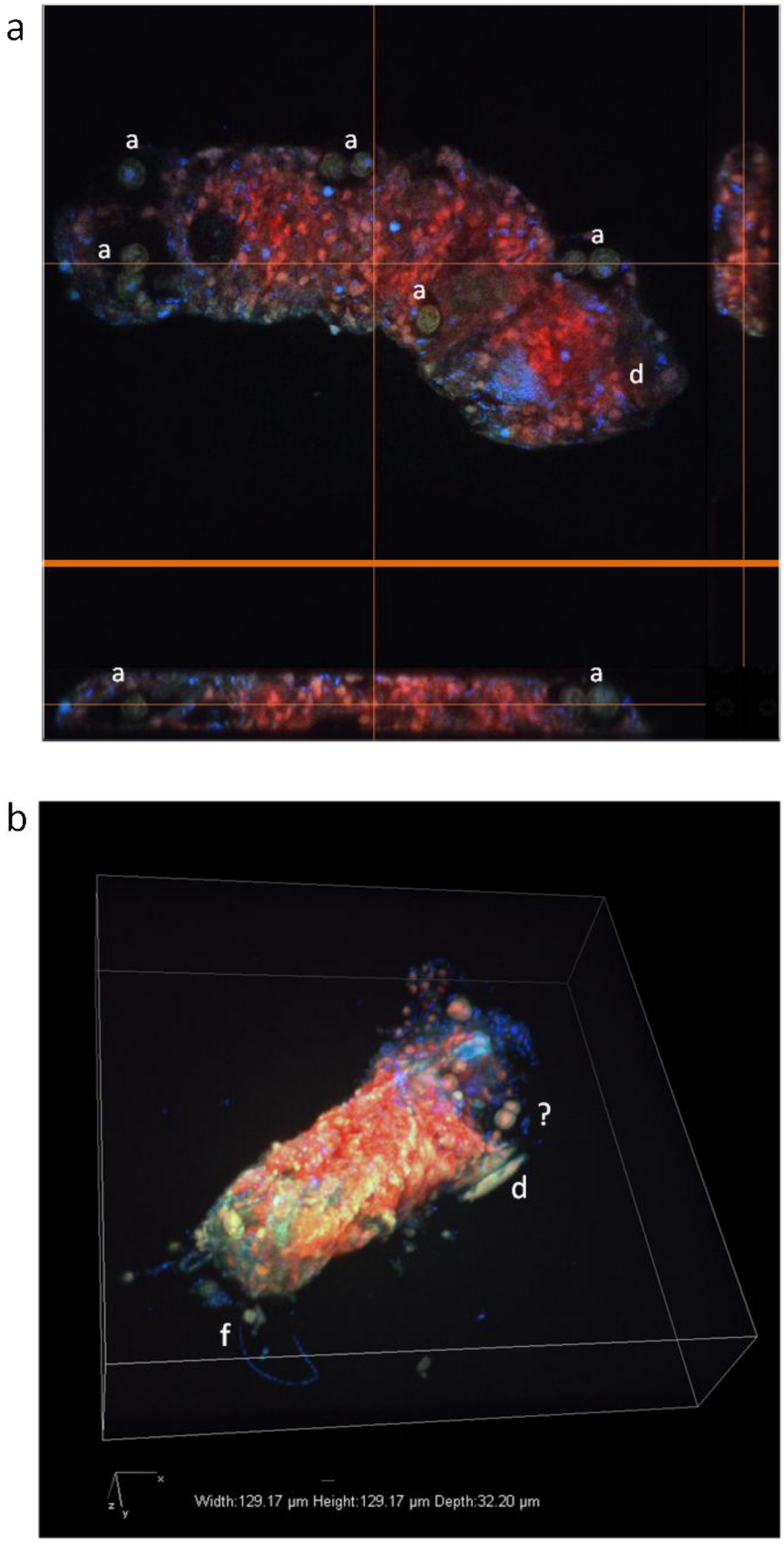

Fig. 3. Fecal pellets images by LSCM. a) 3D-sectioning of the pellet with top and side view details. Bacteria are stained with DAPI are blue, chlorophyll signal is red, microalgae (a) and diatom frustules (d) are visible; scale bar 5 µm. b) Volume rendering of a fecal pellet. Diatom frustule (d), microalgae (a) and filamentous bacteria (f) are visible.

DISCUSSION

The high-resolution combined visualization protocol has allowed us to investigate and quantify the structure of the fecal pellets and its associated bacteria. In our workflow, the AFM analysis needs to precede the LSCM imaging, since the AFM sample preparation does not requires any mounting medium in contrast to a high-power objective (100x oil immersion).

Our across scale analysis, from nm to µm, reveals that the fecal pellet is a very heterogeneous environment both on its surface as well as in its interior. Image details of the peritrophic membrane in air were sharper than in liquid (Fig. 1b,c). This could be due to the hydration layer (Hansma et al. 1993) of a highly packed supramolecular polysaccharide network part of the peritrophic membrane. The air-thickness values, measured on individual fibrils, and its structure suggest a supramolecular organization of polysaccharide moieties of the membrane. Our results are in agreement with the range found for natural marine polysaccharides (diameter 0.1 - 3 nm) (Santschi et al. 1998) and for shrimp derived α-chitin organization (Andrade et al. 2002). Moreover, a similar structural organization with fibrils and globules have been found in diatom polysaccharide matrix (Pletikapić et al. 2011) and in marine mucilage gel phase (Misic Radic et al. 2011).

According to the fecal pellet age, bacterial colonization can be very high. In our experiment, the fecal pellets were freshly egested in a sterile medium and we did not see an intense degree of bacterial colonization. The assumption W=Z (Bratbak 1985) gives larger volume than W≠Z, (Table 1). On the other hand, the average volume values in liquid and in air were similar i.e. 0.04 μm^3 and 0.07 μm^3 (assuming W≠Z), since the cells were dead. It has been shown that upon fixation and drying cell dimensions are deformed (Z collapses) but living cell volume has been found to be on average 3 time larger than dead cells (computed with W≠Z, Malfatti et al. 2010).

Bacteria distribution in the fecal pellet space, analyzed by LSCM, suggests two different ecological strategies: a solitary behavior and a social behavior. It has been proposed that in the ocean not all the bacteria will form colonies and biofilms on a solid substrate (e.g. marine snow, detritus, sediment grains, copepods carapace) but some bacteria will explore the environment as a single cell (Simu & Hagström 2004). Moreover, in the enclosed space of the fecal pellets bacterial community and distribution could be structured by antagonistic relationships (Long & Azam 2001) beside microscale resource availability(Azam & Malfatti 2007). An alternative explanation of the patchy bacterial distribution could be the difference in growth rate as function of phylogenetic diversity.

Fecal pellets can be considered as an "organism" (Johannes & Satomi 1966) thus they change over time. Fecal pellets are hot-spot loci of intense organic matter remineralization (high protease activity and low oxygen concentration) and production of dissolved organic matter in the surface ocean and deep-ocean (Alldredge & Cohen 1987, Urban-Rich et al. 1999). The establishment of this protocol will allow us to perform long-term investigations of bacterial colonization rate and bacterial metabolic activity on copepod fecal pellets. Microscale investigation on how bacteria are changing the matrix and the pellet chemical composition is pivotal to better quantify the pathways of carbon and other nutrients in marine food webs.

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Chapter 8

General discussion

Interest of marine ecologists in the direct interaction between harpacticoids and bacteria originated from the following facts: (1) as a basal food source, benthic bacterial biomass is (equally) high compared to MPB biomass (van Oevelen et al. 2006b) while its general fate remains unclear, (2) in view of their high densities, meiofauna may play a significant role in the transfer of bacterial biomass to higher trophic levels and (3) the current lack of evidence of strong predation of harpacticoids upon protozoa relative to the more numerous observations of the high attractiveness of bacteria-rich substrates for harpacticoids (e.g. Hicks 1977, Ravenel & Thistle 1981, Dahms et al. 2007), might point towards a direct pathway of bacterial biomass transfer to harpacticoids.
Exposing linkages between the microbial and classic food web will significantly improve our understanding of energy flow in marine benthic food webs and the overall functioning of coastal ecosystems, which is desirable in the current era of rapid environmental change.

This PhD study aimed to explore the two-directional interaction between harpacticoid copepods and bacteria. The thesis dealt with three main topics, which will be discussed as such.

In the first part of the thesis, we focused on the overall trophic diversity of intertidal harpacticoids and revealed the presence of bacterivorous species belonging different harpacticoid assemblages (chapters 2 and 3). In this general discussion, the major findings are discussed from an ecological point of view, denoting the implications of the observed harpacticoid trophic diversity for the connectivity between benthic food webs, i.e. the classic, the microbial and the detrital food web with emphasis on the consequences for the transfer of bacterial biomass. Secondly, under laboratory conditions the mechanisms of bacterial uptake (substrate dependence, feeding selectivity) was analysed in combination with the nutritional role of bacteria for harpacticoid copepods (chapters 4 and 5). Bacterial feeding and impact on copepods' health status was overall inferred from their natural stable isotope signature and fatty acid profiles and from the change in these trophic markers resulting from laboratory feeding. This part of the discussion is more fundamental and from the perspective of the harpacticoid grazer, addressing harpacticoids' (species-specific) feeding behavior towards bacterial biomass and the nutritional role of bacteria and other consequences of bacterial feeding for harpacticoids. Additionally, some methodological considerations about the applicability of trophic biomarkers for studying bacterivory were raised.
In third, the thesis examined the mechanism of early copepod fecal pellet degradation by bacteria and introduced a new method for visualization of fp degradation. More specifically, the importance of internal fecal pellet bacteria, which originate from the copepod gut as undigested food-related bacteria or resident gut bacteria, were of significant importance to fp degradation (chapter 6 and 7). Exactly these fecal pellets form a link between the classic and detrital food web but as copepods excrete active bacteria with their fecal pellets, the focus of this part of the discussion involves the feedback between the classic and microbial loop.

In the end, some general conclusion are presented and some future perspectives are considered.

HARPACTICOID TROPHIC DIVERSITY IN AN ESTUARINE INTERTIDAL AREA AND THE LINK BETWEEN THE CLASSIC AND MICROBIAL FOOD WEB

To explore the trophic diversity of meiofauna, estuarine intertidal areas are excellent study areas as they cover a wide spectrum of resources, habitat types and meiofaunal communities. Based on the trophic

marker analyses (chapter 3), we concluded that microphytobenthos (MPB) grazing was undeniably the predominant feeding strategy for harpacticods. This is in different microhabitats, with characteristic abiotic variables, various food availability and carbon inputs and comprising distinct harpacticoid communities and difference food condition (e.g. food availability and food quality in tidal vs salt marsh).
A MPB-based food web is a general characteristic of intertidal benthic food webs (Chanton & Lewis 2002, Pinckney et al. 2003, van Oevelen et al. 2006b). The high-nutritional MPB, with a dominance of diatoms, was widely distributed in the Paulina intertidal area but salt marsh stations are at the same time also dominated by retention of deposited low-quality suspended organic matter (SPOM), vascular plant detrital matter and high bacterial densities. Despite these depositions, harpactoids did not show an increase in reliance on detritus at a certain moment. This is in agreement with Van Oevelen et al. (2006) stating that meiofauna and macrofauna, are highly selective for high-qualitative MPB and hence, detritivory in an interitidal system is nearly absent. As a consequence, this means that total organic matter is not an appropriate measurement of foodavailability.

Food availability in the sand flat was lower compared to salt marsh stations (cf. low OM, phytopigment and protein+lipid concentrations). We suggest that the species-poor and low-density harpacticoid assemblages from tidal flats are primarily structured by granulometry and tidal exposure (abiotic factors), while assemblages from salt marshes are more bottom-up controlled by food quality and quantity. The primary impact of granulometry on the copepod assemblage is highly visible in station H1. There, the change in granulometry resulted in a drastic change in copepod community. H1 copepod species (*Tachidius discipes*, *Amphiascus sp. 1*, *Asellopsis intermedia*) correlated to nearly the whole range of habitat factors, indicating the overall change of the habitat and the copepod community due to a change in granulometry. Only *Ectinosoma* sp. (Ectinosomatida) showed only a single correlation. This fits well with the idea of Ectinosomatidae being independent of MPB. Moreover, fatty acid profiles of this family were very poor in diatom-related fatty acids (EPA, 16:1ω7). For this harpacticoid taxon, dietary information was conform the variation in its distribution pattern. Exept for Ectinosomatidae, harpacticoid species distribution patterns and correlation with resource variables, revealed relatively little about the trophic structure of the harpacticoid community and the governing factors that regulate spatio-temporal variation. In contrast to nematodes, there is little interest for microscopic examination of species mouth parts due to the weak link between mouth part morphology and food source consumption (Marcotte 1977, De Troch et al. 2006). Nevertheless, mouth-morphology based feeding type classifications of nematodes in tidal flat areas are also often little informative on the main drivers of assemblage structure and on the main resources used. Spatial isotope studies on nematodes from the Paulina intertidal area have, however, confirmed that nematodes also predominantly rely on MPB-derived carbon, and this across habitats (Moens et al. 2002, Moens et al. 2005a, Bezerra & Moens unpubl.). However, the results gradually shift from a 'pure' MPB signature in sandy and hydrodynamically impacted stations like H2, to a more mixed isotopic composition which reflects an increased contribution of SPOM-derived carbon in accretory stations such as muddy bare tidal flat stations and, particularly, salt marsh gully stations (Moens et al. 2002). Nevertheless, $\delta^{15}N$ data demonstrate that a considerable proportion of nematodes obtain part or most of their MPB-derived carbon indirectly, i.e. through feeding on an intermediate trophic level which in turn utilizes MPB (Moens et al. 2005a, Moens et al. 2013). Other lines of evidence for a strong bottom-up effect of MPB on tidal nematode assemblages at the Paulina come from (a) a field experiment on the recolonization of defaunated sediment (Van Colen et al. 2009), and (b) from the spatial correlations of nematodes with phytopigment concentrations (or ratios of pigment concentrations) (Moens et al. 1999a). In the former study, strong MPB blooms on previously defaunated muddy tidal flat sediments triggered sudden population peaks of presumed diatom-feeding nematodes like *Ptycholaimellus, Chromadora* and *Daptonema* (Van Colen et al. 2009). To what extent the absence of 'normal' populations of bioturbating macrofauna, which may interfere with diatom grazing by nematodes (Braeckman et al. 2011), or even prey upon nematodes (Olafsson 2003), contributed to this increase, remains unknown. In terms of their nematode assemblages, sandier baretidal flat stations at Paulina are characterized by high abundances of (large) predacious nematodes (Gallucci et al. 2005), the activity of which has the potential to exert significant top-down controls over prey nematode (and perhaps also ciliate, see Hamels et al.

2001) assemblage abundance and composition (Moens et al. 2000, Gallucci et al. 2005). The importance of top-down controls, both from hydrodynamic impacts and from predation, is therefore likely much more pronounced in bare sandy stations than in salt-marsh associated stations. Whether harpacticoid copepods in bare sandy sediments are also more prone to suffer from predation is unclear. However, given the high nutritional quality of copepods for higher trophic levels (Gee 1989, Heath & Moore 1997) and the nursery function of salt marshes to a variety of hyperbenthic predators of copepods (Cattrijsse & Hampel 2006), we can not simply assume that top-down impacts from predation on harpacticoid copepods would be less in marsh stations than on bare tidal flats.

In view of the concept of species co-occurrence and biodiversity maintenance (Arroyo et al. 2007), resource partitioning is expected to support the species-rich harpacticoid communities of the salt marsh stations. In fact, based on dietary markers (^{13}C and FA), we found that a few species (*Paronychocamptus nanus*, *Microarthridion littorale*, *Amphiascus sp. 1*) temporally relied on SPOM. For none of the species a considerable utilization of *Spartina alterniflora* detritus was denoted. Such a partial or temporary reliance on SPOM also occurs in nematodes from accretory station (see previous §). Utilization of *Spartina* detritus is difficult to demonstrate with natural carbon isotopes, since the *Spartina* signature tends to overlap with that of MPB. Studies which have suggested that *Spartina* may contribute to the diets of salt marsh nematodes and copepods (Couch 1989, Carman & Fry 2002) have indeed highlighted this problem. Nevertheless, the low nutritional quality of *Spartina* detritus, and the high similarity in carbon isotopic signatures of nematodes inside and away from *Spartina* vegetation, all suggest that MPB rather than *Spartina* detritus is the main carbon source for intertidal meiofauna in and nearby small salt marshes such as the Paulina marsh.

The above-mentioned partly indirect reliance on MPB carbon by a variety of tidal flat nematodes belonging to different trophic guilds (i.e., in essence, omnivory) (Moens et al. 2013, Bezerra & Moens unpubl.) suggests that many nematodes have some degree of flexibility in their diets, and may adapt feeding depending on the availability of different resources. Similarly, harpacticoids are generally considered being dietary flexible (Hicks and Coull, 1983). They can cope with a temporary depletion of MPB, for instance, in summer (despite high MPB productivity, Herman et al. 2001) resulting from extensive benthic grazing (Herman et al. 2000, Pinckney et al. 2003, Weerman et al. 2011). Even so, it is hard to explain why some of these species exhibit a dietary shift to the low-quality SPOM in the winter period since (1) still a considerable portion of MPB was available based on peaking of chlorophyll a in November and (2) SPOM deposition was typical in June. Copepod grazing rates adjust to changing MPB production (Pinckney et al., 2003) but that is not necessarily a reason for a drastic dietary shift. Moreover, in the salt marsh harpacticoid assemblages, bacterial consumption was documented for Cletodidae species and *Delavalia palustris* (family Miraciidae). For the other species of the copepod assemblage, small dissimilarities among species and small temporal variations in carbon isotopic signatures suggested selective feeding by copepod species or dietary shifts among similar food sources. Selective feeding by harpacticoids on different food sources was shown by Decho and Fleeger (1988) and more fine-scaled resource partitioning among different sources from the same food type was illustrated by e.g De Troch et al. (2006, 2012b) and Rieper (1982). However, *in situ* harpacticoid resource partitioning remains a rather unexplored topic.

Less expected was the trophic distinctness of species from the species-poor harpacticoid assemblage in the sand flat, comprising a diatom feeder (*Asellopsis intermedia*), a potential dinoflagellate feeder (*Tachidius discipes*) and also a bacterial feeder (*Paraleptastacus spinicauda*). In this case, resource partitioning does not function as biodiversity regulator but is presumably out of necessity due to relatively low food availability in physically stressed coarse sediments). Furthermore, field ^{13}C signatures showed that Harpacticidae, *P. spinicauda* and Cletodidae were confined to a single food source. Additionally, for the latter two taxa, their consistent ^{13}C-enriched and extremely depleted signature, respectively, combined with remarkable FA profile characteristics (bacterial FA, no diatom or MPB

markers) prove a high dependency on bacteria even when abundant diatoms are available (specialist feeders) and underlines their discriminate feeding on bacteria (fig. 1).

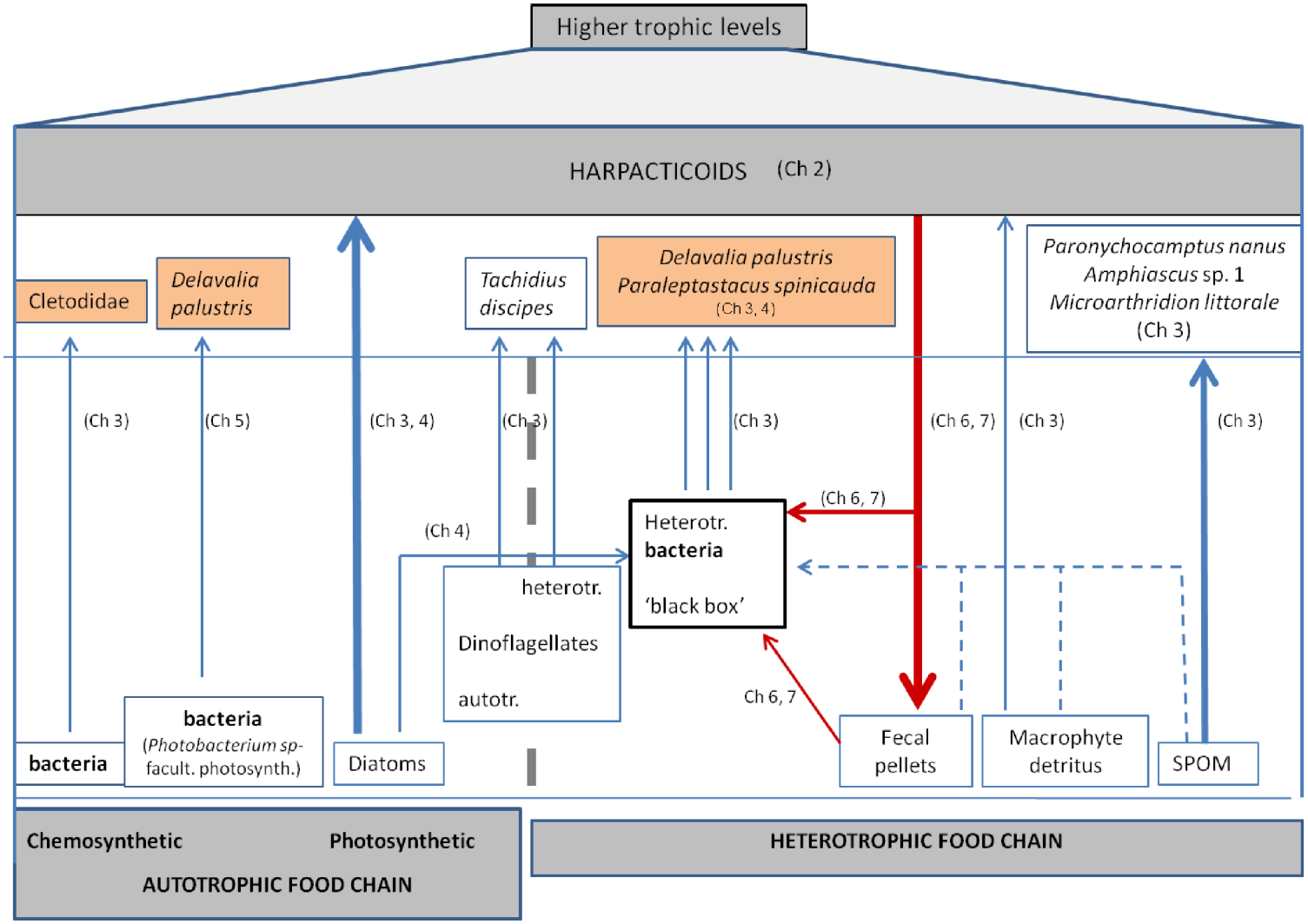

Fig. 1. Summary of major trophic linkages between harpacticoid and food sources that were covered in this PhD research. Specific chapters are indicated by numbers. Arrows represent observed interactions or potentially occuring interactions (dotted line). Line width illustrates the relative strength of the interaction (deduced from the number of literature reports, not quantified in this PhD thesis). Blue lines represent upwards fluxes of energy and red lines show the downward fluxes.

The combined use of dietary biomarkers, i.e. stable isotopes and fatty acids (FA), proved to be very useful to reveal differences in *in situ* food source consumption. However, we did encounter ambiguous results owing to the restrictions of stable isotopes and FA for *in situ* use (see further). Additional dietary biomarkers are needed to increase the resolution and to unravel food utilization, for example the use of FA biomarkers in the apolar fraction of lipids in consumers.

With respect to the ecological importance of bacterial feeding, our study covered a wide range of habitats and species and clearly showed limited assimilation of bacteria in terms of number of copepod species applying this feeding strategy. When compared to MPB, this implies a negligible contribution of bacterial production to the overall benthic trophic fluxes. However, the obtained results did not allow to make any strong suggestions on the relative importance of bacterial feeding opposed to some other food sources such as SPOM and protist feeding (dinoflagellates) (Walters & Moriarty 1993). The latter two were observed for only a few copepod species. Also the minute contribution of *Spartina* detritus to the diet of some species, suggests a poor energy flux of detrital matter (detritivory *sensu stricto*).

Van Oevelen et al. (2006a) and Epstein (1997) estimated that bacteria contribute 2-3% to a meiofaunal diet and bacterial carbon flux through meiobenthos grazing is merely 9% of total bacterial productivity. Van Oevelen et al. (2006a) found bacterial uptake by meiofauna to be high enough to be more than merely random uptake, but still too low to classify it as substantial bacterivory. It was concluded that transfer of bacterial carbon to grazers was limited (Kemp 1988, Epstein & Shiaris 1992) and that bacterial production still function as a carbon sink in food webs.

The few harpacticoid taxa with clear indication of trophic bacterial dependence were Cletodidae, *P. spinicauda* and *Delavalia palustris* (fig. 1). Highly depleted ^{13}C values typically points at the utilization of chemoautotrophic carbon (energy obtained from inorganic components), produced by bacteria. The lack of bacterial fatty acids, could imply that consumption of chemoautotrophic-derived bacterial-related carbon without actual assimilation of the bacterial cells, through assimilation of extracellular polysaccharide substances or bacterial derived DOM. Bacteria consumed by *P. spinicauda* are most likely epipsammic, autotrophic or heterotrophic bacteria while *D. palustris* consumed heterotrophic bacteria associated with diatoms. Despite the low harpacticoid diversity contributing to bacterial carbon transfer, the three copepod taxa showed to transfer bacteria between the chemoautotrophic, the heterotrophic and potentially also the photoautotrophic pathway in the estuarine food web (fig. 1).

HARPACTICOID BACTERIAL FEEDING: INGESTION, SELECTION AND ASSIMILATION OF BACTERIA

Harpacticoids are well-known substrate feeders and occasionally filter feeders. The high substrate-dependence for bacterial consumption reported in chapter 4, albeit only on a substrate with nutritional importance, therefore was expected. However, in addition to the consumption of bacterial biofilms associated with a high-nutritional substrate i.e. diatoms (mechanism 1) (Decho & Fleeger 1988, Souza-Santos et al. 1999), also two other mechanism have been recorded, namely grazing on bacteria associated with low-nutritional substrates (mechanism 2) e.g. copepod fp (De Troch et al., 2009, Arroyo et al., 2007), mucous substances (Hicks & Grahame 1979) and detritus (Perlmutter & Meyer 1991) and on bacteria associated with a non-nutritional substrate (coarse sediment grains) (mechanism 3)(Gray 1968). For the latter, the harvest of bacterial biofilms on sediment occurs via substrate scraping of coarse sediment grains or microbial stripping of ingested smaller grains inside the gut. In our experiment, the presence of silt grains as a pure physical substrate, however, did not enhance harpacticoid feeding on bacteria. However, we do not reject the *in situ* occurrence of this bacterial feeding strategy ('sediment grain grazing'). Particularly, a pure physical use of a bacterial-rich substrate is presumably applied by bacterial specialist feeders like Cletodidae from silty sediments and *P. spinicauda* from the sand flat. The latter most

likely applied grain scraping rather than grain ingestion considering the small copepod body volume:grain size ratio. Cletodidae may ingest silt grains followed by 'microbial stripping' in the gut. On the other hand, ingestion of a nutritional substrate with only the specific assimilation of the bacterial component is plausible and was once observed by Decho and Castenholz (1986), who noted copepod bacterial assimilation while co-ingested diatom cells were passed undigested to the fecal pellets. Finally, for *Delavalia palustris*, bacterial consumption is based on co-ingestion of bacteria and diatoms (mechanism 1), followed by co-assimilation, confirmed by both *in situ* (chapter 3) and experimental (chapter 4) fatty acid data showing considerable proportions of bacterial and diatom-specific FA. In contrast to *D. palustris*, other diatom feeders in the experiment (*Platychelipus littoralis*, *Microarthridion littorale*) did not show bacterial FA incorporation. Potentially, the signal of bacterial assimilation was too low compared to that of diatoms due to the large difference in biovolume. This would suggest that *D. palustris* obtained its bacterial signal by additional feeding on bacterial cells independently from diatom grazing. Based on the knowledge that copepod species, including *D. palustris*, are incapable of targeting bacteria in absence of a substrate, another explanation is needed. The difference in bacterial signal between *D. palustris* and other copepod species then points at a differential mechanism of food source assimilation. Copepods are able to actively enhance food digestion by means of regulating the gut transit time (see further, Tirelli & Mayzaud 2005). Moreover, little is known about the difference in digestibility of both food sources, diatoms and bacteria. Furthermore, we have no data at hand to exclude bacterial consumption through symbiosis. A third underlying mechanism of the trophic interaction with bacteria (in the case of *D. palustris*), can include the use of mucoid substances (mechanism 2). Similar to other mucus-producing harpacticoids inhabiting silty sediment (Hicks & Grahame 1979, Williams-Howze & Fleeger 1987) and e.g. mucus production by *D. palustris* (Nehring 1993)can be a strategy to 'garden' its own bacterial food. All this highlights the diversity in bacterial feeding strategies by intertidal copepods at the level of bacterial ingestion and of bacterial assimilation.
Finally, the inability of harpacticoid copepods to target 'loose' bacteria (chapter 4) is in line with the idea of harpacticoids' inability to capture and consume small-sized particles, independent of a substrate.

Considering the low assimilation efficiency of bacteria ingested by diatom grazers, only *Delavalia* showed to be a relatively efficient bacterivore. Although diatoms were assimilated by multiple intertidal harpacticoids (*Platychelipus littoralis*, *Microarthridion littorale*, *Nannopus palustris*), associated bacteria were not. Therefore, the fraction of random/co-ingested bacteria by most copepods does not contribute to the transfer of bacterial carbon through the food web.

Further, bacterial selectivity was proven by means of *in situ* observation of bacterial specialized feeders i.e. for Cletodidae and *P. paraleptastacus* (chapter 3). Similar to the selectivity of harpacticoids towards diatoms at the level of species, growth phase or cell size (De Troch et al. 2006, 2012b), it seems that harpacticoids discriminate among bacteria. Under experimental conditions, selective bacterial feeding was shown by Rieper (1982). However, this study is based on ingestion, while further assimilation was not estimated. In our experiment there was a slightly higher uptake of *Photobacterium* sp. by *D. palustris* (Chapter 5, Fig. 1). Bacteria offered were considered to be of different nutritional values (based on dissimilarity in fatty acid and protein composition). The experimental setup is rather restricted to draw general conclusions about harpacticoids' ability to select among bacteria and the possible causative factors for that e.g. nutritional content, depending copepod-species specific ability to process certain fatty acids.

The low and rather similar bacterial ^{13}C assimilation among copepod species pointed towards indiscriminate feeding on bacteria and random co-ingestion during diatom grazing (1996, Souza-Santos et al. 1999), but the higher bacterial fatty acid incorporation of *D. palustris* (bacterial bacterial constituting up to 23 % of total fatty acids) compared to the near lack of bacteria FA in other species (<5 %) (chapter 4) suggests copepod-specific differences in assimilation efficiency of bacteria. Harpacticoids may regulate gut transit time depending on the food quality to increase assimilation (Tirelli & Mayzaud 2005). The existence of differential digestion of resource among different copepod-species has not been reported for

meiofauna but was refuted for macrofaunal detritivores by Plante and Schriver (1998a, b): digestive fluid of multiple macrofaunal species showed equal bacterial cell lyses of 'ingested' sediment bacteria, but instead the food source characteristics determined their susceptibility to digestion (see also further).

NUTRITIONAL ROLE OF BACTERIA

Laboratory feeding experiments reveal assimilation of bacterial carbon and fatty acids but also confirmed the poor nutritional contribution of bacteria for harpacticoids relative to that of diatoms (chapter 4, 5). The majority of experimentally tested copepods, except for *Delavalia palustris*, clearly did not attain any nutrition from bacteria and copepods lived of their FA reserves in presence of bacteria as sole food source. However, feeding on a purely bacterial diet was not always found to result in a nutritional deficiency in the harpacticoid, since copepods were capable of normal reproduction (Rieper 1978). For *D. palustris*, the secondary importance of bacteria relative to diatom feeding, suggest bacteria to serve as a complementary food source, which is in line with the findings of Souza-Santos (1999) that supported the general idea that bacteria are co-assimilated with a high-qualitative food source without having a confounding influence on grazer nutrition, and may merely provide some dietary supplements such as vitamins.

However, a higher and constant dependence on bacteria and limited consumption of another food source found for Cletodidae and *Paraleptastacus spinicauda* (bacterial FA: up to 15 % of copepod FA content) suggest that the bacteria might be more than a complementary food sources. Moreover, in spite of the highly characteristic carbon isotopic signal of Cletodidae linking this species to a chemoautotrophic food source, the absence of bacterial FA in Cletodidae, might indicate the use of extracellular released compounds of chemoautotrophic bacteria (EPS, DOM), as mentioned before, instead of actual bacterial cell lysis in the gut and subsequent assimilation of bacterial cell biomass.

The poor nutritional value of bacteria is related to the lack of PUFAs and sterols, which are essential compounds for copepods (Ederington et al. 1995) as they contribute to somatic growth and cell membrane functioning and any shortage of these essential FA may limit zooplankton productivity. In addition to the FA composition of the food, copepods are selective towards protein-rich resources (Cowles et al. 1988) and other nutritionally important components such as amino acids, elemental composition (e.g. C:N:P ratios), vitamins and other trace elements (Dauwe et al. 1999, Touratier et al. 1999). Another remarkable finding is the differential biochemical response of *D. palustris* compared to the other species to poor-food-value conditions. In absence of a high-quality food source, all copepods including *D. palustris* lost FA within the first 4 days but the latter was able to recuperate during the following days of incubation and even biosynthesize essential FA (PUFA), through FA biosynthesis using bacterial-derived FA as precursors (chapter 3, 4). Clearly, in the absence of diatoms, also for *D. palustris* a limited nutritional contribution of bacteria is observed. As a potential stress response, a PUFA-production is thought to increase the survival rate of this species when it occurs in poor-food conditions.

DIETARY BIOMARKER ANALYSIS FOR THE STUDY OF BACTERIVORY: METHODOLOGICAL CONSIDERATIONS AND METHOD EVALUATION

Techniques used for quantifying copepod feeding are based on gut analysis, removal rates, fecal pellet production and pigment content of fecal pellets (e.g. Harris 1994, Azovsky et al. 2005, de Souza Santos & Castel 2013). However, neither of these are suitable for inferring bacterial grazing. Many studies promote the use of stable isotopes and FA for disclosing trophic interactions and carbon fluxes in the lower food web (Peterson & Fry 1987, Leduc et al. 2009). These dietary biomarkers are a relatively new and powerful

tool for direct measurements of assimilation. However, this approach has some drawbacks with respect to bacterivory (see below).

In the microcosm experiments, both types of dietary markers, stable carbon isotopes and fatty acids, provided direct information on bacterial uptake by harpacticoids. For revealing *in situ* food source consumption, copepod fatty acid signatures were highly suitable for inferring bacterial feeding while isotopic analysis informed on utilization of non-bacterial resources, demonstrating the added value of complementary use of fatty acids and stable isotopes.

In situ application of stable isotopes

Carbon isotopic signatures of bacterial biomass and their substrate (e.g. algae, detritus) overlap (Boschker & Middelburg 2002). For nitrogen, isotopic signatures are little predictable since bacterial ^{15}N fractionation depends on the molecular nature of the organic nitrogen source (see chapter 3) (Macko & Estep 1984, McCarthy et al. 2007).

Moreover, during simultaneous feeding on diatom and bacterial biomass (e.g. for *Delavalia palustris)*, diatom assimilation exceeded bacterial assimilation and copepod isotopic signatures will therefore resemble to those of herbivores, masking the bacterial uptake. Hence, the use of dual isotope analysis for discriminating between bacterial feeding copepods (e.g. *Paraleptastacus spinicauda* and *Delavalia palustris*) and copepods with another feeding strategy (e.g. herbivorous) was insufficient. In this respect, results from controlled experiments using ^{13}C-prelabelled bacterial food are less confounding.

In situ application of fatty acids

In situ copepod fatty acid (FA) profiles are characterized by a certain extent of natural variability, which obscures clear patterns of food utilization (detritus, MPB, SPOM). This results from spatio-temporal variability in resource FA, by copepod feeding on a mixture of resources and by copepod FA variability resulting from copepod species-specific behavior (breeding pattern) and FA metabolism (see chapter 3). This was more a concern for inferring feeding on non-bacterial resources based on indicator FA such as EPA and DHA. The highly specific odd-chained and branched bacterial fatty acids (15:0, 17:0, 15:1ω5, 17:1ω7) provided clear evidence of bacterivory in harpacticoid species. Additional use of the bacterial characteristic FA 18:1ω7, which is often proportionally more abundant than the odd-chained bacterial FA, would reinforce the bacterial signal of consumers markedly. In our study, this FA could not be identified due to restrictions in the detection resolution of the GC-column hampering the separation of 18:1ω7c and 18:1ω9c.

In this work, FA from total lipid extracts (polar and apolar lipids) were analysed. The use of the apolar fraction is a more ideal method for inferring dietary information since the other fraction i.e. the polar fraction (membrane FA) is highly dependent on genetic and environmental factors (e.g. temperature). The presence of FA derived from polar lipids thus reduces the value of the analyzed FA pool (from the total lipid extract) as trophic markers to reveal the nature of consumed food sources.

BACTERIAL FECAL PELLET DECOMPOSITION

Independent of their origin (field or lab conditions), **benthic fecal pellets are carriers of a diverse set of bacteria** that has survived gut passage, as visualized by AFM-LSCM. Internal bacteria maintained their activity, hence breaking down the fecal pellet content. The additional efforts made to include natural fecal

pellets from field captured copepods in this study proved to be of value, since they illustrate even better the diversity of active bacteria that is shunted to the fecal pellet and the natural variability in bacterial assemblage composition. Fecal pellets tested differed in food content, i.e. composed of unknown natural and likely mixed food sources versus a laboratory diatom food source composed of a single diatom species. Fecal pellets also originated from two different copepod species, i.e. a field copepod species and a second, laboratory-reared copepod species. Hereby, we excluded the possibility of only an artificial effect of fecal pellet enrichment with active bacteria.

Although a majority of bacterial phylotypes could not be unambiguously linked to the ingested food source or to copepod associated bacteria (i.e. in the gut or externally on the body), at least a fraction are undigested food bacteria. Furthermore, the easy and rapid addition of food bacteria to the gut flora of *Platychelipus littoralis* stresses the major influence of the type of grazed food source on both gut flora and bacteria fecal pellet content. These results underline the impact of copepod feeding behavior, including food source preferences and temporal dietary shifts, and the co-ingested bacterial fraction on the genetic diversity of active bacteria. Internal fecal pellet bacteria had a relatively wide metabolic potential, well suited to decompose a wide range of substrate types such as polymers, carbohydrates, etc. Corresponding to the genetic variability in fecal pellet bacteria induced by copepods' feeding behaviour, the metabolic potential of the bacterial assemblage might change, provoking differential fecal pellet degradation rates.

Moreover, benthic fecal pellets were less subjected to bacterial colonization from the surroundings, which could imply that recycling of benthic fecal pellets differs from that of planktonic fecal pellets. As a consequence of limited 'external' bacterial enrichment of the fecal pellet in terms of bacterial diversity, colonizing bacteria only limited contributed to the catabolic traits of the active bacterial community of the fecal pellet. This stresses the profound contribution of internal bacteria to fecal pellet degradation. This contrasts with the strong significant bacterial enrichment observed for planktonic fecal pellets (Jacobsen & Azam 1984, Jing et al. 2012). Reports on benthic fecal pellets are lacking but (natural) fecal pellets in sediment traps are in a way representative for benthic fecal pellets due to their static state. Also on these fecal pellets bacterial coating was poor (Gowing & Silver 1983). Consequently, degradation of benthic fecal pellets (or pellets from sediment traps) is not comparable to the degradation process of planktonic fecal pellets. Due to the static position of benthic fecal pellets and the concomitant limited exchange of oxygen, DOM or other chemical cues (produced by bacterial activity or leaked from the fecal pellet) (Ploug & Grossart 1999) compared to sinking fecal pellets, bacterial colonization is supressed. On the other hand, a more efficient bacterial exchange between the environment and the benthic fecal pellets would be expected as benthic fecal pellets are in close contact with other bacteria-rich matter such as sediment biofilms or detrital particles. Hence, undigested bacteria from the copepod gut and ingested food sources which are channeled to the fecal pellet, and not those from the seawater colonizing the fecal pellet, are the primary degraders of benthic fecal pellets. A considerable fraction of ingested bacteria has the potential to survive gut passage. For example, for macrodetritivores ingesting large amounts of sediment bacteria, 74% of sediment bacterial strains were resistant to digestion. The susceptibility of sedimentary bacteria to lysis by digestive fluid relates mainly to the cell wall type of bacteria (Plante & Shriver 1998a, b).

For a better comprehension of fecal pellet degradation, molecular tools for screening the bacterial diversity involved in the degradation process can be complemented with a visualization tool, for instance AFM-LSCM. AFM-LSCM allows more precise measurement of bacterial cell volumes (due to depth measurement) and the persistence of internal bacteria compared to the invasion by external bacteria can be closely followed and can visualize bacteria-bacteria interactions or the state of the peritrophic membrane. With the use of appropriate fluorescent markers, the changes in biochemical content can be mapped recording whether biomolecules (carbohydrate, chitine, lipids, etc.). Biochemical change of the copepod fecal pellet als fits in the topic of throphic upgrading.

GENERAL CONCLUSIONS

Trophic diversity of intertidal harpacticoids in the Paulina intertidal area was low in respect of the heterogeneity in habitats. The dominating role of microphytobenthos, in particular diatoms, as a food source for intertidal harpacticoid copepods compared to bacteria, suggests a negligible connectivity between the grazer food web and the microbial loop and denotes bacteria as a carbon sink in the intertidal ecosystem. Nevertheless, clear evidence of bacterivory for field harpacticoids has been found, giving us a wake-up call regarding their excistence. By revealing Cletodidae as a consumer of chemoautotrophic bacteria or bacterial-derived exudates, a new trophic link has been introduced for intertidal harpacticoids. From the few earlier observations of a chemoautotrophic pathway (see discussion chapter 3), copepod identification was still missing. In addition, consumption of bacteria by *Delavalia palustris* was continuously confirmed, from field and experimental data. Moreover, of four tested species, it was the only one that had the ability to biosynthesize PUFA during starvation. It remains unclear if this is some sort of adaptation related to feeding on bacteria but it can be expected that this strategy will not support *D. palustris* for a longer time period. Finally, contrasting to the previous two copepod taxa, *Paraleptastacus, spinicauda* is a bacterivore in the sand flat. These observations of bacterivory among harpacticoids suggest that (1) bacteria from different origin can be consumed (chemoautotroph, heterotrophy and potentially photoautotroph), that (2) bacterial utilisation is not confined to a single habitat type (muddy salt marsh and sand flat) and that (3) bacteria can be an additive to the diet of a herbivore or be the primary food source a a specialist feeder. Other dietary information from field copepods, such as the poor reliance on *Spartina* detritus, fits well with the study of Van Oevelen et al (2006b). Dietary information, was of no surplus value for explaining harpacticoid distribution patterns.

The bacterial assemblage of fresh copepod fecal pellets, located at the inside of the fecal pellet as confirmed by AFM-LSCM, denoted a reverse interaction between the grazer food web and microbial-detrital food web. A proportion of ingested bacteria, for most harpacticoid species co-incidentally ingested during grazing on a primary food source, returns to the microbial loop and this contributes to the concept of bacteria being a sink. Furthermore, the absence of intensive external colonization contrasts with observations from the planktonic system. It raises questions about the efficiency of degradation processes in the benthic ecosystem compared to the pelagic microbial-detrital food web

FUTURE RESEARCH

- Although the transfer of bacterial biomass seems at first limited and restricted to a few harpacticoid species, it is still interesting to continue this research and **exposing more bacterivorous (omnivorous) harpacticoid species** since the discovery of bacterivorous Cletodidae and Paraleptastacidae was also not expected. We need more exact data on the amount of bacterial biomass transfer and we currently might still underestimate this food web link. The bacterivore species from this thesis were fairly abundant, if not the dominant species from the harpacticoid assemblage (e.g. *Paraleptastacus spinicauda*, dominant in the sand flat community). Locally, depending habitat type, bacterial transfer can be higher than we would currently expect.

- No strong indication of **selective bacterial uptake** was observed, but more experimentation is needed, testing a broader range of nutritionally different bacterial strains.

- At the same time, I belief it is interesting to start investigating another way of bacterial biomass transfer, i.e. the interaction between **bacterivorous protists and harpacticoids**. This food web

link is nearly unexplored and it contributes to the still urging question of what the fate of bacterial production is. Protists are of higher food quality (high C content, through trophic upgrading) (Breteler et al. 1999) but can also be toxic to harpacticoids (Colin & Dam 2003). Omnivorous harpacticoids would benefit from protozoan grazing e.g. during periods of limited MPB.

- In addition, if indeed the microbial loop is a dead end in bacteria (van Oevelen et al. 2006b), we should start exploring the DOC-pathways, the direct link between **harpacticoids and DOC** (Cletodidae, feeding on DOC of symbiotic bacteria?) or through the microbenthos as intermediates. Van Oevelen et al (2006b) state that most protozoans from the intertidal feed on DOC rather than on bacteria.

- We introduced in this thesis (chapter 7) **AFM-LSCM** as a potential tool for visualizing **fp degradation**. It would be interesting to further exploit this technique, applying it to follow-up some fecal pellet characteristics during fp degradation: the state of the peritrophic membrane, the bacterial biomass on the inside and outside of the fecal pellet, the change in biochemical composition (carbonates, chlorophylls, chitine, …).

- I'm curious whether the limited colonization of seawater bacteria on benthic fecal pellets is a general phenomenon for benthic particles. This could have implications for the **degradation rates of benthic material**. To my knowledge, no studies have yet compared degradation rates of the planktonic and the benthic system.

ADDENDA

Addendum I

Effect of food preservation on the grazing behaviour and on the gut flora of the harpacticoid copepod *Paramphiascella fulvofasciata*

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ABSTRACT

Harpacticoids owe their 'reputation' as primary consumers in aquatic food webs to their substantial grazing on diatoms, thus assuring an efficient energy flow to higher trophic levels. Due to the complex feeding behaviour of harpacticoids, the nature and dynamics of diatom-harpacticoid trophic interactions remain poorly understood. In addition, there is a growing interest from aquaculture industry in mass-culturing harpacticoids with algal foods but the labour costs of maintaining algal stock cultures are high. This study focuses on the palatability of preserved diatoms for copepods and considers the possible role of bacterial mediated effects on diatom food uptake.

The grazing of *Paramphiascella fulvofasciata* on a preserved freeze-dried diatom diet was tested and compared to the grazing on fresh cells. *P. fulvofasciata* assimilated the preserved diet, but assimilation of fresh cells was higher. When both cell types were mixed, no selective feeding was observed. Community fingerprinting of the bacteria associated with diatoms and fecal pellets suggests that the copepod gut flora was modified depending the food source. Furthermore, the results suggest that the egestion of gut bacteria enriches the microenvironment and this can have an additional influence on the feeding behaviour of the copepod.

Experimental research using preserved foods must take into account that copepod grazing assimilations of fresh foods are likely to be significantly higher. Yet, the stated high assimilation of the mixed diet, encourages further exploration of the application of preserved balanced foods for harpacticoid mass-culturing.

KEY WORDS: Harpacticoida, selective feeding, preserved diatoms, fecal pellet, bacterial community composition

INTRODUCTION

Meiobenthic harpacticoid copepods (Crustacea) are distributed worldwide and play a pivotal role in marine benthic food webs. Harpacticoids can feed on a wide spectrum of food items (Hicks & Coull 1983),

but their trophic link with diatoms has received most attention (e.g. Decho 1986, Montagna et al. 1995). Based on their grazing on microalgae, they are assigned the position of 'primary consumers', and the meiobenthos-algae link is considered essential for the energy flow at the basis of marine food webs (Buffan-Dubau & Carman 2000). Harpacticoids showed a positive response, as expressed by their densities and grazing rates, to microphytobenthos availability, validating the dependent relationship with its autotrophic food source (Montagna et al. 1995). Harpacticoids graze on living cells, but whether and to what extent they also utilize and participate in the recycling of dead diatoms, e.g. after the natural decline of a diatom bloom, remains unknown.

As primary consumers, harpacticoid copepods transfer primary production to higher trophic levels (Coull 1990). Copepods are important prey for a variety of other invertebrates and for juvenile or small bottom-dwelling fish (Gee 1989, Tsubaki & Kato 2009). Despite their clear significance as a food source in natural environments, utilization of harpacticoid copepods in aquaculture is limited. The superior nutritional value of copepods, mainly ascribed to their natural essential fatty acids content (McEvoy et al. 1998, Evjemo et al. 2003), renders copepods a promising live feed (Stottrup 2000). Fish cultures are typically maintained on nutritionally enriched live feeds, such as rotifers or *Artemia* (Kraul et al. 1992, Hanaee et al. 2005, Kotani et al. 2009), but supplemental feeding on copepods has shown to increase fish production yields (Heath & Moore 1997, Olivotto et al. 2008).

However, limited effort has hitherto been devoted to the development of intensive harpacticoid cultures for aquaculture applications due to their benthic life style, diverse feeding habits and complex life history. While most harpacticoid species were cultured for experimental purposes, generally using diatoms as food (Matias-Peralta et al. 2005, De Troch et al. 2006), successful attempts to scale up harpacticoid cultivation (e.g. Sun & Fleeger 1995, Stottrup & Norsker 1997, Rhodes 2003) resulted from trial-and-error application of artificial food sources (shrimp pellets, a lettuce/*Mytilus* paste, vegetable juices, yeast, fish flakes) and eventually cultivated algae. These studies illustrate the need for detailed information on the feeding requirements of harpacticoids and stress the importance of ecological research on feeding. Moreover, harpacticoid mass-rearing requires optimisation to reduce economic costs. One of the main obstacles is the need for a large-scale algal/diatom production with a sufficient supply and low labour costs. Preserving the algal food stocks through lyophilisation could partially circumvent these difficulties and would minimize the risk of contaminating the algal cultures.

The implications of preservation for feeding selectivity and efficiency remain unknown. In general, preservation (drying, freezing and freeze-drying) of microalgae is associated with a reduction of food quality but the extent of quality loss and its consequences for aquatic herbivores is still controversial (Albentosa et al. 1997, Dobberfuhl & Elser 1999). Preservation processes can modify cell morphology, the external epiflora and/or the presence of exudates and the biochemical content of cells. Considering the discriminative abilities of copepods (Rieper 1982, Cowles et al. 1988, DeMott 1988), even a minor modification of the food source can have an impact on their feeding behaviour. Acquiring knowledge about these effects on harpacticoid feeding is relevant for assessing the functional role of harpacticoids in benthic food webs, i.e. the transfer of primary production by harpacticoids, which could differ at and during diatom bloom conditions.

The importance of other potential food sources such as fecal material and associated bacteria remains underexplored. Harpacticoid copepods have the ability to discriminate between bacterial strains (Rieper 1982) and selectively assimilate a microbial diet (Decho & Castenholz 1986). Additionally, previous studies indicated that fecal pellets and associated bacterial flora may play a role in their feeding ecology. An increase in daily fecal pellet production after the removal of fecal pellets from the microcosms is an indication that harpacticoids need fecal pellets in their vicinity (De Troch et al. 2009). As fecal pellets were not actively fragmented by the copepods, attached bacteria rather than fecal pellet content may have accounted for the trophic value of the fecal pellets (De Troch et al. 2009, 2010).

This study aimed to assess the suitability of lyophilized diatom cells as compared to living cells as a food source for the harpacticoid copepod *Paramphiascella fulvofasciata*. In view of the above-mentioned nutritional links between diatoms, fecal pellets, bacteria and copepods (De Troch et al. 2005), changes in the microbial flora on the fecal pellets produced after feeding on fresh and freeze-dried cells were analysed.

MATERIALS AND METHODS

Laboratory stock cultures

The copepod species *Paramphiascella fulvofasciata* (family Miraciidae) was cultured as described by De Troch *et al.* (2005). Laboratory stock cultures were maintained in glass beakers with artificial seawater (ca. 32 psu, Instant Ocean® salt, Aquarium Systems, France) and were regularly provided with fresh benthic diatoms, the epipelic pennate species *Navicula phyllepta* and *Seminavis robusta*. The cultures were incubated at 17 ± 1 °C under a 12:12h light:dark regime with 25-50 µmol photons m^{-2} s^{-2}. Copepod specimens from the cultures reached up to 0.83 mm in adult size (body length) and a mean carbon content of 1.3 ± 0.2 µg C (including gut content).

For the experiment, the benthic diatom *Seminavis robusta* strain 84B (diatom culture collection of the Laboratory for Protistology and Aquatic Ecology, Ghent University) was grown as a food source for *P. fulvofasciata*. Diatom cells were grown in cell tissue culture flasks with f/2 culture medium (Guillard 1975) based on autoclaved artificial seawater (32 psu) and under identical light and temperature conditions as the copepod stock cultures. At the beginning of the experiment, the cells measured 32.9 ± 0.3 µm (mean ± SE) in length.

To trace food uptake, diatoms were grown in a ^{13}C-enriched f/2 medium by adding $NaH^{13}CO_3$ as described by De Troch *et al.* (2005). At the start of the experiment, the growth medium was replaced by artificial seawater (ASW) and the density of the diatom cells was estimated under an inverted light microscope.

In addition, a portion of the fresh unlabeled and labeled diatom cultures, henceforth annotated as F and F* respectively, were lyophilized. The resulting freeze-dried cells, henceforth designated as D and D* respectively, were used as a second type of food source. To remove salt crystals resulting from the ASW, the cells were washed after lyophilization with MilliQ water over a GF/F Whatman filter (pore size 0.7 µm) and briefly freeze-dried again.

The labeling technique resulted in an increase in $\delta^{13}C$ value from -17.46 ‰ ± 0.07 ‰ (mean ± SE) to 7720.98 ‰ ± 84.73 ‰ for the fresh cells and from -2.51 ‰ ± 0.21 ‰ to 8148.57 ‰ ± 19.81 ‰ for the freeze-dried cells (see further standardization of the data).

Experimental design

To determine the effect of diatom preservation on the feeding behaviour of *P. fulvofasciata*, a grazing experiment was conducted in small petri dishes (polystyrene, surface area = 21.2 cm^2). The microcosms were provided with 4 different *S. robusta* diets: labeled fresh cells (treatment F*), labeled freeze-dried cells (treatment D*), a 1:1 mix of labeled fresh cells and unlabelled freeze-dried cells (F*D) and a 1:1 mix of unlabelled fresh cells and labeled freeze-dried cells (FD*).

For each of the above treatments, 3 diatom cell densities were applied: 0.5 x 10^6 cells petri $dish^{-1}$ (low density, 'l'), 1 x 10^6 cells petri $dish^{-1}$ (medium density, 'm') and 2 x 10^6 cells petri $dish^{-1}$ (high density, 'h'), resulting in a total of 12 treatments. Each food combination (diatom type x diatom density), was

replicated 5 times. All replicates within each concentration level were standardized with respect to biomass e.g. the mixed diets contain equal biomass as the single-food diets.

After settlement of the diatom cells on the microcosm bottom surface, 20 adult specimens of *P. fulvofasciata* were collected from the laboratory stock cultures, washed multiple times in artificial seawater and starved overnight were added to the microcosms. This number of specimens guarantees at least 15 µg C copepod biomass, which is well above the detection limit of carbon isotopic measurements.

The experimental units were placed randomly on a shelf under identical temperature and light conditions as described for the stock cultures of copepods and diatoms. The units were left undisturbed for 5 days. The experiment was terminated by freezing the experimental units at -20 °C which were subsequently stored, until further processing. Prior to freezing, the mortality of the copepods was assessed in each microcosm and was found to be limited to 5.2 ± 0.3 % in all treatments.

Fecal pellets

Before freezing the experimental units, fecal pellets produced by the copepods during the 5-day period were collected for analysis of the associated bacteria.

For each food combination, fecal pellets were hand-picked, rinsed in autoclaved and filtered artificial seawater and collected in an eppendorf tube. Fecal pellet sample size was standardized by putting in 100 fecal pellets per tube. Because some microcosms contained less than 100 pellets, 2 fecal pellet samples of each food combination treatment were prepared by pooling fecal pellets from 2 or 3 replicate microcosms. The fecal samples were stored at -20°C until further bacterial DNA extraction.

Stable isotope analysis

Frozen microcosms were thawed at room temperature and copepods were collected and rinsed several times in MilliQ water to remove adhering particles. The copepods were transferred into a drop of MilliQ water in tin capsules (8 x 20 mm). The overall procedure was executed within 2 hours after thawing to minimize leakage of ^{13}C from the copepod body (Mourelatos et al. 1992, Moens et al. 1999d). Subsequently, the tin capsules were oven-dried overnight at 60 °C, pinched closed and stored under dry atmospheric conditions until analysis. Samples of the experimental food sources, i.e. fresh and freeze-dried diatom cells from the laboratory stock culture and from the ^{13}C-enriched cultures, were also analysed to check the initial labeling of the food sources.

Delta ^{13}C values ($\delta^{13}C$) were measured using an isotope ratio mass spectrometer (type Europa Integra) at the Davis Stable Isotope Facility (University of California, USA).

Uptake of ^{13}C is expressed as specific uptake ($\Delta\delta^{13}C = \delta^{13}C_{sample} - \delta^{13}C_{control}$). The control signal refers to the $\delta^{13}C$ value of *P. fulvofasciata* from the laboratory cultures, representing the isotope signal of the copepods prior to the start of the experiment. These data were further standardized according to Middelburg *et al.* (2000) by calculating excess (above background) ^{13}C and expressed as total uptake (I) in milligrams of ^{13}C per individual, calculated as the product of excess ^{13}C (E) and mean individual copepod biomass (organic carbon) per sample. Excess ^{13}C is the difference between the fraction ^{13}C of the control ($F_{control}$) and the sample (F_{sample}), 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 $R_{VPDB} = 0.0112372$ as $\delta^{13}C$ is expressed relative to Vienna Pee Dee Belemnite (VPDB).

As labeled diatoms had different initial $\delta^{13}C$ signatures (see 'laboratory cultures'), the total uptake per individual was further standardised taking into account the atomic % of ^{13}C in each food type. Differences

in diatom uptake by copepods between the various treatments were analysed with two-way analysis of variance (ANOVA) with the fixed factors 'food type' and 'food density', using the software package R, version 2.10.0 (R Development Core Team 2009) . *A posteriori* comparisons were carried out with the Tukey test using 95 % confidence limits. Prior to ANOVA, the Shapiro-Wilk test and the Levene's test were used to check for normality of the data and homogeneity of variances, respectively.

Bacterial community analysis by DGGE

Bacterial DNA was prepared through alkaline lysis (Baele et al. 2000) from subsamples of the original diatom stock culture (F cells), of the labeled culture (F* cells) and of both diatom cultures after lyophilization (D and D* cells). Bacterial DNA was also extracted from the fecal samples of each microcosm treatment (cell type x cell density). For the latter, the procedure from Baele *et al.* (2000) was slightly modified by reducing volumes of the reagents fourfold.

From each DNA extract an internal 194 bp fragment of the V_3 region of the 16S rRNA gene was amplified using the primer set 357f and 518r (Yu & Morrison 2004) (Sigma Aldrich) with a GC-clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3′) (Temmerman et al. 2003) coupled to the forward primer. PCR mixtures were prepared according to Temmerman *et al.* (2003). Respectively, 1 µl and 4 µl of DNA template from the diatom and fecal samples were included to achieve a successful PCR reaction. A touchdown PCR (Van Hoorde et al. 2008, 2009) with 10 cycles of decreasing annealing temperature (0.5 °C $cycle^{-1}$ decrement, from 61 to 56 °C) followed by 25 cycles of regular PCR was performed with a Bio-Rad DNA thermal cycler. Fecal samples were subjected to a 'nested' PCR, i.e. the resulting PCR products were subjected to a second identical PCR. Subsequent denaturing gradient gel electrophoresis (DGGE) analysis using a 35-70 % gradient and staining of the gel were done as described by Van Hoorde *et al.* (2008, 2009).

Digitized DGGE gels were normalized and analysed by means of the BioNumerics programme (version 4.61, Applied Maths, Sint-Martens-Latem, Belgium). Calculation of the Jaccard correlation coefficient and application of Unweighted Pair Group Method with Arithmetic Mean (UPGMA) resulted in a dendrogram visualizing similarity between the band patterns of diatom and fecal pellet samples. Clusters were determined by visual inspection and by using the cluster cut-off method in the Bionumerics programme, a statistical tool to select most significant groups. Band profile similarities were also analysed by means of non-metric multidimensional scaling (nMDS).

RESULTS

Stable isotope signatures

Overall, specific diatom uptake by the copepods (expressed as $\Delta\delta^{13}C$; Fig. 1A) differed significantly among the various diatom diets and densities (2-way ANOVA, food: $p < 0.001$, density: $p < 0.01$, interaction: $p < 0.01$).

Between the single-food diets (Fig. 1A; Table 1; comparison of F*-D*), there was a highly significant difference in $\Delta\delta^{13}C$ values, except for F*l-D*h and F*l-D*m. Copepods clearly preferred the fresh cells as illustrated by the higher $\Delta\delta^{13}C$ values in comparison to the copepods of the corresponding freeze-dried treatments.

Table 1. Results of Tukey *post-hoc* tests for all treatment combinations. *: p<0.05, **: p<0.01, ***: p<0.001, -: not significant.

		single diet						mixed diet					
	food:conc	F*l	F*m	F*h	D*l	D*m	D*h	F*Dl	F*Dm	F*Dh	FD*l	FD*m	FD*h
single diet	F*l		-	*	***	-	*	***	***	*	***	***	***
	F*m			-	***	***	***	***	***	***	***	***	***
	F*h				***	***	***	***	***	***	***	***	***
	D*l					-	-	-	-	-	-	-	-
	D*m						-	-	*	-	-	***	-
	D*h							-	-	-	-	*	-
mixed diet	F*Dl								-	-	-	-	-
	F*Dm									-	-	-	-
	F*Dh										-	*	-
	FD*l											-	-
	FD*m												-
	FD*h												

Diatom cell density within a single-food diet did not strongly affect diatom assimilation by the copepod (Table 1; comparisons within F* and within D* treatments). For the fresh diet, a significant difference in diatom grazing was only recorded between the low and high cell density (F*l-F*h). In the case of freeze-dried cells, however, there was no effect of diatom density on the uptake by copepods.

Between the mixed-food treatments (Fig 1A; Table 1; comparison F*D-FD*), irrespective of the concentration, there were no differences in uptake of fresh and freeze-dried cells (except for F*Dh-FD*m). No selective grazing was observed, in contrast to the selectivity towards fresh diatoms in the single-food treatments. In comparison with the single-food diets (Table 1; comparison F*-F*D* and D*-FD*), grazing on fresh cells decreased when both cell types were offered simultaneously, whereas the uptake of freeze-dried cells in the single diet did not differ significantly from the uptake in the mixed diet (except for D*m-FD*m and D*h-FD*m). Within each mixed diet (Table 1; comparisons within F*D and within FD* treatments), diatom density did not interfere with diatom uptake, as was observed for the single foods.

After correction for the initial ^{13}C-enrichment levels of the food sources, the total carbon uptake per individual (Fig. 1B) was similar to the $\Delta\delta^{13}C$ pattern (Fig. 1A) except for the FD* treatment, due to the higher carbon content of the analyzed copepods in that treatment.

By summing the total carbon uptake per individual of the 2 mixed diet treatments, i.e. total carbon uptake of fresh cells (Fig. 1B; F*D) and total carbon uptake of freeze-dried cells (Fig. 1B; FD*), the overall uptake in the mixed diet was estimated (Fig. 1B; FD). Carbon uptake by copepods increased by one fifth when offered a mix of both cell types compared to assimilation of the fresh diet.

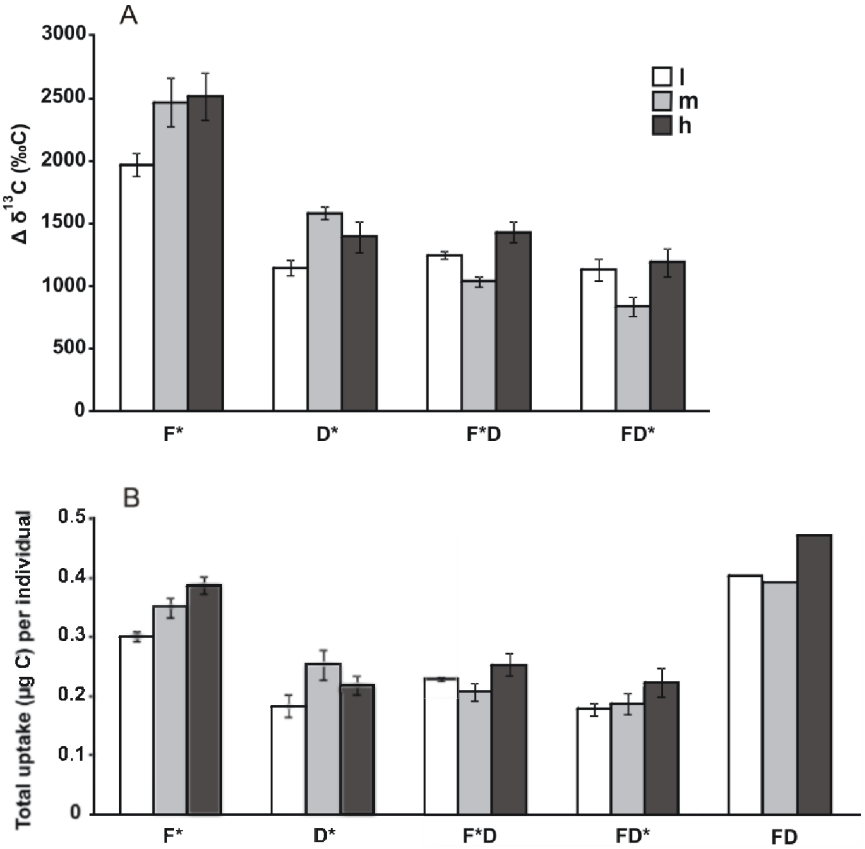

Fig. 1. Assimilation of ^{13}C (mean ± standard error) by *Paramphiascella fulvofasciata* after grazing on 4 enriched diatom diets (F* = fresh cells, D* = freeze-dried cells, F*D = mix of both where the fresh cells were enriched and FD* = mix of both where the freeze-dried cells were enriched) provided in 3 diatom cell densities (l = low, m = medium, h = high). Diatom uptake within each treatment is expressed as (A) $\Delta\delta^{13}C$ and (B) total uptake per individual relative to biomass and to stable isotope signature of the enriched food sources. FD shows the overall uptake of the mixed diet, after summing the assimilation from the treatments F*D en FD*.

Bacterial community on diatoms and fecal pellets

In the DGGE dendrogram representing the microorganism communities, three distinct clusters were defined (Fig. 2; cluster 1-3). Cluster 1 consists of fecal samples collected from the freeze-dried treatment (D*). Cluster 2 groups the bacterial communities on the fresh and freeze-dried diatoms. The bacterial communities on fecal pellets after feeding on fresh diatoms (F* or F*D) clustered together in cluster 3. Similarity between the clusters was ≤ 30 % and within the clusters ≥ 44 %. The same groups were retrieved in the nMDS plot (Fig. 3). The 'diatom cluster' (cluster 2) was more similar to the 'fresh fecal pellet cluster' (cluster 3) than to the 'freeze-dried fecal pellet cluster' (cluster 1).

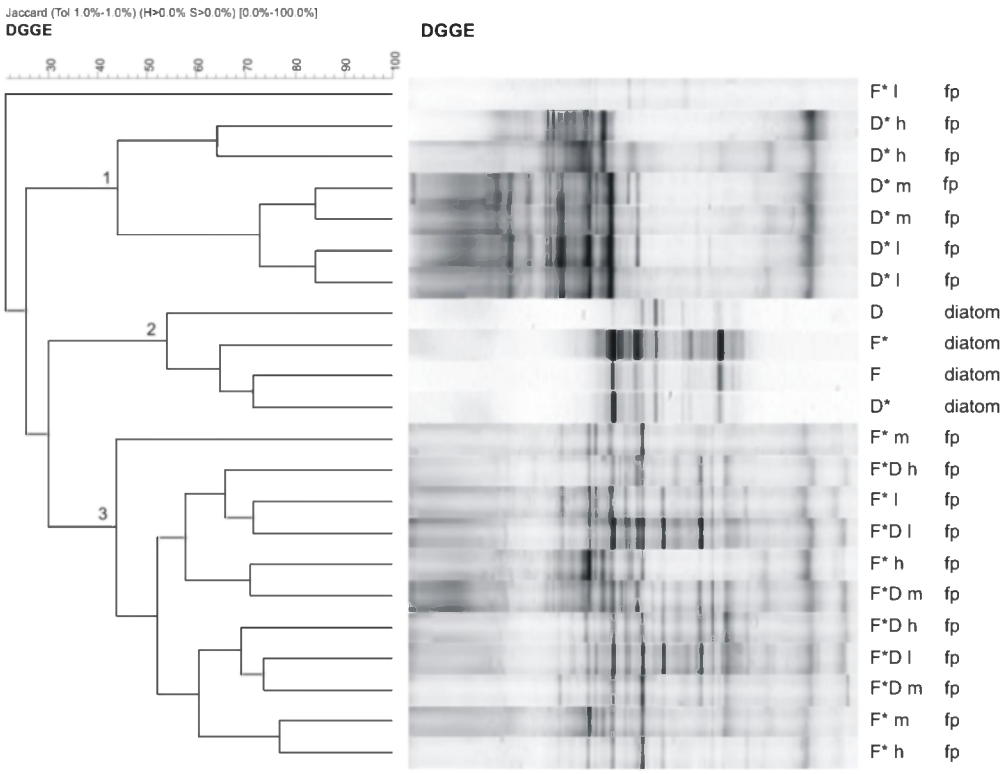

Fig. 2. Dendrogram (UPGMA) based on DGGE fingerprints of V_3 region amplicons (fragment of the 16S rRNA gene) representing bacterial communities associated with the diatom *Seminavis robusta* and with copepod fecal pellets (referred to as fp) collected from the experimental treatments containing diets F*, D* and F*D in densities l, m and h.

Bacterial assemblages on the diatoms clustered together (cluster 2), regardless of the freeze-drying and labeling of the diatom cells, with a similarity between 54 and 71 %. Differences in DGGE band profile intensities were notable. Labeled cells (F*, D*) showed more intense bands than the unlabeled cells (F, D). The samples of freeze-dried cells (D, D*) showed a weaker profile than its fresh equivalents (F, F*).

On the other hand, bacterial communities associated with fecal pellets were not at all alike, as illustrated by the presence of two fecal pellet clusters (cluster 1 and 3), with a similarity of 25 %. Within cluster 1, treatments were grouped according to the food densities as samples of the high density treatment differed from the two other densities. In cluster 3, however, no clear grouping of the different food densities and their replicates was observed.

One sample, F*l, was found isolated in the dendrogram due to the poor quality of the banding profile (top lane of Fig. 2). This sample will be ignored in further discussion, its replicate in cluster 3 is, however, still considered.

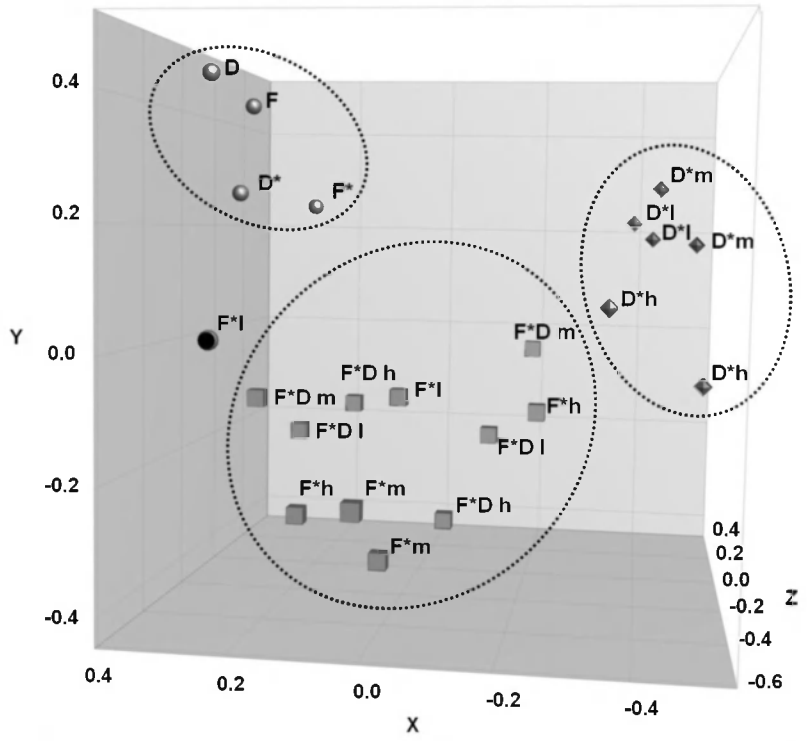

Fig 3. MDS ordination plot based on the relative band positions of the DGGE profiles.

DISCUSSION

Effect of freeze-drying on diatom palatability

P. fulvofasciata assimilated both fresh and freeze-dried *S. robusta* cells. The measured $\Delta\delta^{13}C$ values after 4 days of foraging on either of the diatom cell types were within the range of previous studies (De Troch et al. 2007, 2008) and together with the low copepod mortality rate confirms the suitability of this epipelic diatom species as a diet for *P. fulvofasciata*, even after preservation of the diatom.

A clear effect of the preservation status (fresh vs. freeze-dried) of the diatom cells on the feeding rate of *P. fulvofasciata* was, however, found. Fresh cells were significantly more assimilated by this harpacticoid copepod than freeze-dried cells. This selectivity toward a fresh diet was unlikely to be caused by morphological traits of the diatom, but presumably it was driven by chemical (and qualitative) traits of the cells and could be the result of active, i.e. ingestion of cells, as well as passive, through differential assimilation or digestibility, feeding preferences. To our knowledge, no morphological effect of freeze-drying on microalgae cells has been documented. Microscopic observations of the diatoms after freeze-drying and rehydration in seawater, showed that diatom frustules did not burst and frustules appeared intact. Frustules still contained the protoplast. The increase of the $\delta^{13}C$ value after freeze-drying, which is indicative of diffusion of the lighter ^{12}C from the diatom, may point toward the presence of small injuries of the diatom cell wall. Furthermore, lyophilisation is known as a better preservation technique than drying as it stabilizes biochemicals with limited damage thus maintaining food quality. Nevertheless, Albentosa *et al.* (1997) and Dobberfuhl and Elser (1999) reported a minor diminution of microalgae quality due to the freezing process, mainly attributed to modifications of protein content and elemental ratios of C, P and N. However, the elemental ratios were still in a range considered indicative of a favourable food quality.

The current study suggests that external characteristics of the diatom cell contributed to the difference in copepod assimilation of fresh and freeze-dried cells. The exterior of the diatom cells may have been modified by lyophilisation, for instance by the loss of exudates (e.g. extracellular polymeric substances) and the limited revival and/or loss of bacteria associated with the diatom frustules. A change in the external 'appearance' could have reduced ingestion (active selection) or inhibited digestion of the freeze-dried diatoms (passive selection) by the copepod. The high selective ability of copepods has been illustrated by their discrimination between live and dead cells of the same diatom species (DeMott 1988). That study further reported that calanoid copepods discriminate against axenic diatoms, in line with our hypothesis that the bacterial community associated with diatoms may be a more important determinant for diatom selection by copepods than the intrinsic nutritional value of the diatoms (Cowles et al. 1988, Cotonnec et al. 2001). Barofsky *et al.* (2010) and Cowles *et al.* (1988) found that copepods discriminate diatom cells depending on growth phase, which is associated with differences in both internal and external metabolite composition. Studies have repeatedly presumed food selection and ingestion rates of copepods to be driven by chemoreception or the presence of bacteria (Hicks 1977, Cowles et al. 1988, Moore et al. 1999, Jiang et al. 2002) and we believe that this is also the cause of the low assimilation of freeze-dried diatom carbon. Beside active selection by the harpacticoid, freeze-dried diatoms might have a lower digestibility. For clams it appeared that growth rates were coupled to the ingestion and absorption rates of the preserved diet rather than to the biochemical content of the food sources (Albentosa et al. 1997), suggesting that acceptability of the preserved diet is of greater importance in determining the nutritional quality of a preserved food.

In the mixed diet of preserved and living cells, no selective feeding was observed. *P. fulvofasciata* equally assimilated both cell types. Moreover, total assimilation of the mixed diet was much higher than assimilation of the preserved diet and even the fresh diet. This is consistent with our suggestion that the external traits of diatoms were important in determining active food selection by the copepods. Substitution of 50 % of the freeze-dried food by fresh diatoms, compensated for the lack of chemical stimuli of freeze-dried cells. A similar observation on reduced chemical-mediated selectivity (Cowles et al. 1988) of dead diatoms after mixing with living cells have been documented for the calanoid copepod *Acartia clausi* and ingestion rates increased substantially (Mayzaud et al. 1998).

Bacterial communities on diatoms and fecal pellets

The DGGE profiles of the bacterial communities differed between the fresh and the lyophilized diatoms, although both originated from the same culture. Bacterial richness (number of bands) on the freeze-dried cells was similar to that on the living cells. However, taking into account the semi-quantitative properties of DGGE, bacterial abundances, represented by band intensities, were different.

Part of the dissimilarity may have been caused by the additional washing step of the diatoms after lyophilisation to remove salt crystals. In addition, an artificial effect of the added carbon (^{13}C labelling) on the bacteria is observed (i.e. higher intensity and higher number of bands). The increased level of carbon sources in the diatom growth medium could have stimulated bacterial growth, directly or indirectly. However, DGGE band intensities should be interpreted with caution since the lack of standardization toward diatom biomass during bacterial DNA extraction could account for differences in band intensities. Moreover, viability of the diatom-associated bacteria after freeze-drying and rehydration is expected to be extremely low since no protective medium was used (Berner & Viernstein 2006).

Bacterial composition on fecal pellets depended upon diet and was very distinct from the bacterial communities on diatoms. Copepod fecal pellets are a growth substratum for a variety of heterotrophic bacteria (De Troch et al. 2010). Fecal pellet bacteria have been suggested to originate from the environment through colonization after egestion or to consist of transient or resident gut bacteria, i.e.

ingested bacteria that survived gut passage (Harris 1993, Hansen & Bech 1996). Based on our DGGE profiles, bacteria associated with diatoms contributed only a minor part of the bacterial richness found on fecal pellets, thus indicating that there was a substantial release of gut bacteria by the harpacticoid and not just an exchange of bacteria between copepod and the environment, irrespective of the experimental diet. The bacterial diversity on the fecal pellets and thus on the copepod gut lining, however, did differ according to the type of food ingested by the copepod. Hence, the ingestion of the diet, not its presence in the environment, was the main factor shaping the bacteriflora in the copepod digestive tract, as was documented already in terms of bacterial abundance (Tang 2005) and diversity (De Troch et al. 2010).

Fecal pellets produced after feeding on the lyophilised cells harbored a specific and relatively stable bacteriflora. The variability of the bacterial communities on fecal pellets after feeding on fresh diatoms was, however, pronounced. The introduction of metabolically active bacteria associated with the fresh diatoms into the gut likely gave rise to complex bacterial dynamics (e.g. competition for nutrients, space), diversifying the gut microflora. This assumption is in agreement with previous studies (Tang 2005, De Troch et al. 2010) and our fecal pellet analyses are complementary to the study of Tang *et al.* (2009) who deduced an effect of food on the gut biota indirectly through examination of the copepod body, using DGGE.

Bacterial enrichment due to grazing

At the beginning of the experiment, a similar bacterial community (i.e. the bacteria associated with the diatom cells) was introduced to all microcosms. Nevertheless, the bacterial richness on the fecal pellets at the end of the experiment deviated strongly from the original microcosm diversity, as shown by the bacterial diversity found on the fecal pellets. The egestion of fecal pellets introduced new bacteria into the experimental units (see also De Troch et al. 2010, who identified Alphaproteobacteria, Flavobacteria and Bacilli as bacteria solely found on fecal pellets). An increase in bacterial diversity was indirectly caused by the copepod grazing activity on diatoms (via fecal pellets) indicating that the presence of grazers induced shifts in environmental bacterial diversity.

Since harpacticoids seasonally switch between food sources (Lee et al. 1976, Hicks & Coull 1983), further research on copepod-bacteria interaction would be interesting. A top-down effect of copepod grazing on bacterial community composition in sediments is not inconceivable and can play a potential role in ecosystem functioning such as degradation processes and the biological pump.

Implications

The present study shows that the copepod *P. fulvofasciata* is able to discriminate between freeze-dried and fresh diatoms. This finding has important implications for laboratory experiments that use freeze-dried food. The use of preserved food for ecological experiments offers several advantages and in some cases may be required in case of limited access to living algae or for experiments in remote or extreme environments (e.g. deep sea) (Ingels et al. 2010). Based on our results, we can state that the grazing rates reported in studies using preserved food may deviate from the feeding rates on their natural food sources.

For aquaculture applications aimed at upscaling of harpacticoid cultures, our results suggest that a preserved diatom food may result in lower copepod growth rates. Long-term experiments are necessary to analyse the effects of preserved diets on copepod fitness, especially since adverse effects of a diet may not appear within the first week (Irigoien et al. 2002). The observation of a lower assimilation of the preserved food together with previously published indications that freeze-dried diatoms may have a lower nutritional value (Albentosa et al. 1997) urges some caution as to the use of preserved diatoms as food in aquaculture. However, this paper is the first to show an elevated assimilation of a mixed diet

consisting of preserved and fresh diatoms (at least as high as the uptake of a completely fresh diet). Changes in nutritional quality of freeze-dried diatoms should be profoundly studied on a biochemical level as this would provide baseline information for 'engineering' balanced preserved diets consisting of multiple freeze-dried food sources. In addition, information on the diversity, composition and abundance of the microbial assemblages associated with the copepod food should be included since bacteria may impact harpacticoid feeding behaviour. Our study underlines the potential of freeze-dried, or in general dead, diatom cells as food for copepods. This corresponds to what can be expected what happens during spring blooms when diatom cells of different status reach the benthos and where the cell condition may be important for the grazing selectivity of e.g. copepods. This all suggests that copepods, as well-known diatom grazers, have the potential to cope with live and dead diatom cells both under natural and experimental conditions.

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Addendum II

Supporting material – chapter 2

Table S1. Harpacticoid copepod species list
Overview of harpacticoid copepod taxa found in the Paulina polder marsh. Classification based on Boxshall and Halsey (2004).

Kingdom Animalia

Phylum Arthropoda

Subphylum Crustacea

Class Maxillopoda Dahl, 1956

Subclass Copepoda

Infraclass Neocopepoda Huys & Boxshall, 1991

Superorder Podoplea Giesbrecht, 1882

Order Harpacticoida Sars 1903

(Suborder Oligoarthra)

Family Cletodidae Scott T., 1905

Cletocamptus sp. 1

Enhydrosoma gariensis Gurney 1930

Enhydrosoma sp. 1

Family Ectinosomatidae Sars G.O., 1903

Ectinosoma sp. 1

Bradya sp. 1

Pseudobradya sp. 1

Family Harpacticidae Dana, 1846

Harpacticus flexus Brady & Robertson D., 1873

Harpacticus sp. 1

Family Laophontidae Scott T., 1904

Asellopsis intermedia Scott T., 1895

Paronychocamptus nanus Sars G.O., 1908

Paronychocamptus curticaudatus Boeck, 1886

Platychelipus littoralis Brady, 1880

Family Leptastacidae Lang 1948

Paraleptastacus spinicauda Scott T. & Scott A. 1895

Family Miraciidae Dana, 1846

Delavalia palustris Brady 1868

Robertsonia diademata Monard 1926

Amphiascus sp. 1

Amphiascus sp. 2

Family Huntemanniidae Por, 1986

Nannopus palustris Brady, 1880

Family Tachidiidae Boeck 1865

Microarthridion littorale Poppe, 1881

Tachidius discipes Giesbrecht, 1881

Table S2 Results from PERMANOVA analysis for differences in the environmental variables (multivariate matrix with 22 variables) amongst stations (stat) and months (mo) based on an euclidian resemblance matrix

St				Mo				St x Mo			
df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
4	186.8	24.37	< 0.0001	3	86.4	11.27	< 0.0001	12	22.6	2.95	< 0.0001

Groups of stations	June		August		November		February	
	t	P(perm)	t	P(perm)	t	P(perm)	t	P(perm)
H1, H2	4.01	0.026	2.44	0.0281	2.38	0.031	2.16	0.030
H1, H3	2.47	0.032	1.93	0.0305	1.59	0.029	1.64	**0.145**
H1, H4	4.00	0.030	2.59	0.0276	2.51	0.025	3.61	0.027
H1, H5	3.13	0.028	3.05	0.0309	2.52	0.030	4.00	0.029
H2, H3	2.09	**0.055**	2.42	0.0279	2.95	0.027	2.84	0.028
H2, H4	4.81	0.029	2.63	0.0273	5.84	0.028	6.39	0.025
H2, H5	4.07	0.031	3.10	0.0299	6.04	0.032	7.22	0.027
H3, H4	2.24	0.030	2.10	0.0262	2.52	0.026	3.02	0.028
H3, H5	2.22	0.030	1.77	0.0273	2.99	0.025	3.81	0.029
H4, H5	0.98	**0.455**	2.06	0.0293	3.37	0.028	2.74	0.031

Table S3. Results from PERMANOVA test for differences in copepod densities in the 0- 3 cm sediment layer (univariate matrix) amongst stations and months, based on an euclidian resemblance matrix. For the significant St factor, pairwise tests of stations were performed.

St				Mo				St x Mo			
df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
4	82747	5.08	0.003	3	19242	1.18	0.327	12	27499	1.69	0.094

Groups of stations	t	P(perm)
H1, H2	2.76	**0.009**
H1, H3	2.72	**0.003**
H1, H4	3.54	**0.0008**
H1, H5	8.39	**< 0.0001**
H2, H3	1.82	**0.043**
H2, H4	2.70	**0.007**
H2, H5	4.10	**0.0008**
H3, H4	0.76	0.468
H3, H5	0.18	0.932
H4, H5	1.16	0.290

Table S4. Results from PERMANOVA test for differences in copepod community structure (multivariate matrix) amongst stations and months, based on a Bray-Curtis resemblance matrix. For the significant St x Mo factor, pairwise tests of stations within levels of the factor months were performed and of months within each station. The significantly different stations or months are indicated with p-values drawn from Monte-Carlo samplings.

St				Mo				St x Mo			
df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
4	19355	27.49	< 0.0001	3	4709	6.69	< 0.0001	12	1992	2.83	< 0.0001

Groups of stations	June		August		November		February	
	t	P(MC)	t	P(MC)	t	P(MC)	t	P(MC)
H1, H2	7.18	0.0005	2.11	0.036	4.31	0.005	3.06	0.021
H1, H3	1.93	0.0571	2.86	0.010	4.00	0.005	2.43	0.019
H1, H4	2.93	0.0082	3.43	0.006	4.43	0.003	3.32	0.005
H1, H5	5.10	0.0015	3.79	0.005	4.98	0.003	3.98	0.004
H2, H3	2.43	0.0220	2.59	0.018	5.11	0.002	2.69	0.020
H2, H4	3.40	0.0128	2.82	0.018	5.94	0.001	4.41	0.002
H2, H5	8.44	0.0001	3.12	0.014	6.64	0.001	6.00	0.001
H3, H4	0.60	0.7761	1.67	0.092	2.75	0.021	1.23	0.248
H3, H5	1.29	0.2256	1.88	0.070	2.89	0.018	1.17	0.289
H4, H5	2.03	0.0560	0.78	0.633	1.52	0.140	1.18	0.289

Groups of months	H1		H2		H3		H4		H5	
	t	P(MC)	t	P(MC)	t	P(MC)	t	P(MC)	t	P(MC)
June, August	2.99	0.011	1.04	0.37	1.23	0.25	1.53	0.14	1.69	0.085
June, November	4.24	0.004	1.35	0.24	1.86	0.06	2.54	0.03	2.58	0.018
June, February	4.12	0.002	1.7	0.1	1.43	0.14	1.92	0.06	2.02	0.041
August, November	1.54	0.132	1.07	0.35	2.57	0.02	2.67	0.02	3.21	0.007
August, February	1.68	0.102	1.18	0.28	1.34	0.19	1.67	0.11	2.54	0.016
November, February	2.25	0.031	0.33	0.85	1.29	0.23	0.42	0.83	1.29	0.221

Table S5. Mean total densities (N = 3) per 10 cm^2 of harpacticoid species from the top cm, within each station and month

		H1				H2				H3				H4				H5			
Family	**Species**	June	Aug	Nov	Febr	June	Aug	Nov	Febr	June	Aug	Nov	Febr	June	Aug	Nov	Febr	June	Aug	Nov	Febr
Leptastacidae	*Paraleptastacus spinicaudus*	0	0	0	2	110	25	27	45	2	2	0	1	1	0	0	0	0	0	0	0
Tachidiidae	*Microarthridion littorale*	5	0	0	0	2	1	0	0	3	5	94	16	4	47	104	163	56	35	85	29
	Tachidius discipes	17	0	0	0	4	1	0	0	2	5	1	0	2	7	0	0	0	1	0	1
Laophontidae	*Asellopsis intermedia*	1	2	5	6	10	5	1	2	0	0	0	0	0	1	0	1	0	0	0	0
	Platychelipus littoralis	0	0	0	0	0	1	0	0	44	2	1	2	64	18	12	18	15	3	6	4
	Paronychocamptus nanus	3	2	2	1	0	1	0	1	2	8	273	58	0	5	17	15	1	3	40	19
	Paronychocampus curticaudatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cletodidae	*Enhydrosoma gariene*	0	0	0	0	0	0	0	0	3	8	4	1	10	37	1	2	12	21	3	2
	Enhydrosoma sp. 2	0	0	0	0	0	1	0	0	3	11	7	0	7	85	3	7	16	69	6	1
	Cletocamptus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Miraciidae	*Delavalia palustris*	9	1	0	0	0	1	0	0	3	2	4	0	2	27	12	16	14	7	15	6
	Robertsonia diademata	1	0	0	0	0	0	0	0	0	41	0	2	2	2	0	0	0	0	0	0
	Amphiascus sp. 1	14	0	0	0	0	0	0	0	0	6	27	6	1	3	52	100	13	1	6	5
	Amphiascus sp. 2	0	0	0	0	0	0	0	0	2	0	1	1	0	1	1	3	0	0	0	1
Huntemaniidae	*Nannopus palustris*	0	0	0	0	0	0	0	0	0	6	1	2	1	6	3	6	20	12	25	7
Harpacticidae	*Harpacticus flexus*	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	Harpacticus sp. 2	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
Ectinosomatidae	*Ectinosoma* sp.	3	2	10	1	0	0	0	0	2	2	9	1	4	1	6	17	0	1	7	1
	Pseudobradya minor	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
	Bradya sp.	1	2	1	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	0	0
Copepod abundance (0-1cm)		55	9	22	8	127	36	28	48	65	97	424	90	98	238	212	351	146	153	192	80

Table S6. Mean Relative abundances of harpacticoid species from the top cm, within each station and month

		H1				H2				H3				H4				H5			
Family	**Species**	June	Aug	Nov	Febr	June	Aug	Nov	Febr	June	Aug	Nov	Febr	June	Aug	Nov	Febr	June	Aug	Nov	Febr
Leptastacidae	*Paraleptastacus spinicaudus*	0	0	0	10.0	86.3	51.1	85.6	87.3	1.2	1.3	0	11.1	10,0	0	0	0	0	0	0	0
Tachidiidae	*Microarthridion littorale*	8.8	0	0	0	1.4	1.73	0	0	6.0	7.6	16.5	9.1	2.9	16.5	44.6	40.7	38.3	21.9	44.3	36.1
	Tachidius discipes	30.2	0	0	0	3.9	3.9	0	0	7,0	5.2	0.1	0	10.2	2.4	0	0	0	0.2	0	1.5
Laophontidae	*Asellopsis intermedia*	1.2	25.3	21.8	72.2	8.2	30.8	7.7	5.0	0	0.6	0	0	0	0.2	0	0.2	0	0.4	0	0
	Platychelipus littoralis	0	0	1.2	0	0	3.92	0	0	27.1	2.0	0.1	6.9	51.0	14.4	10.7	19.5	10.6	1.9	2.9	6.3
	Paronychocamptus nanus	5,0	21.9	8.7	11.1	0	0.8	6.6	6,0	0.9	11.0	71.6	35.5	0	1.8	10.1	4.1	0.7	1.5	20.4	22.2
	Paronychocampus curticaudatus	0.6	0	0	0	0	1.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cletodidae	*Enhydrosoma gariene*	0	0	0	0	0	0	0	0	39.1	9.9	0.4	0.2	13.6	16.6	0.5	0.4	8.3	13.3	1.2	2.99
	Enhydrosoma sp. 2	0	0	0	0	0	1.7	0	0	6.2	16.3	0.8	0	4.7	30.6	1.8	1.6	11.6	41.4	3.1	1.4
	Cletocamptus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.8
Miraciidae	*Delavalia palustris*	16.6	7.7	0	0	0	3.9	0	0	3.3	1.7	1,0	2.7	1.3	11.2	6.7	8.5	8.8	6.6	7.8	8.4
	Robertsonia diademata	1.2	0	0	0	0	0	0	0	0	31.4	0	13.8	1.1	0.5	0	0	0	0.4	0	0
	Amphiascus sp. 1	25.8	0	2,0	0	0	0	0	0	0.1	5.4	5,0	13,0	1.0	1,0	19.2	17.0	8.4	1,0	3,0	5.5
	Amphiascus sp. 2	0	3.7	0	0	0	0	0	0	5.4	0	0.3	0.2	0	0.2	0.2	0.5	0.2	0	0	0.7
Huntemaniidae	*Nannopus palustris*	0	0	0	0	0	0	0	0	0.1	4.8	0.15	6.2	1.0	3.7	2.4	3.0	12.8	10.7	13.3	9.8
Harpacticidae	*Harpacticus flexus*	0	0	16.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.7
	Harpacticus sp. 2	0	0	0	0	0	0	0	1.5	0	0	0	0.4	0	0	0	0	0	0	0	0
Ectinosomatidae	*Ectinosoma* sp.	5.8	17.8	42.3	6.6	0	0	0	0	2.9	2.1	2.6	0.2	2.7	0.2	3.05	2.9	0	0.2	3.7	0.9
	Pseudobradya minor	2.6	0	2,0	0	0	0	0	0	0	0	0.8	0	0	0	0	0	0	0	0	0
	Bradya sp.	1.9	23.3	5.1	0	0	0	0	0	0	0	0	0	0	0	0.3	0.9	0	0	0	0

Table S7. Results from PERMANOVA tests for differences in Hill's indices (unnivariate matrices) amongst stations and months, based on Euclidian resemblance matrices. For the significant St x Mo factor, pairwise tests of stations within levels of the factor months were performed and of months within each station. The significantly different stations or months are indicated with p-values drawn from Monte-Carlo samplings.

	St				Mo				St x Mo			
N_0	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
	4	79.74	28.84	< 0.0001	3	6.19	2.23	0.1006	12	10.37	3.75	0.001

	St				Mo				St x Mo			
N_1	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
	4	19.10	12.79	< 0.0001	3	4.96	3.32	0.0296	12	3.55	2.38	0.0215

	St				Mo				St x Mo			
N_2	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
	4	10.39	6.61	0.0005	3	3.73	2.37	0.0866	12	2.06	1.31	0.2417

	St				Mo				St x Mo			
N_{inf}	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
	4	3.25	4.26	0.007	3	1.39	1.83	0.153	12	0.7	0.92	0.539

N_0	Groups of stations	June		August		November		February	
		t	P(MC)	t	P(MC)	t	P(MC)	t	P(MC)
	H1, H2	5.66	**0.005**	Negative		3.16	**0.035**	1.00	0.3733
	H1, H3	0.52	0.642	6.72	**0.002**	0.94	0.391	2.77	**0.0503**
	H1, H4	1.25	0.277	5.30	**0.007**	3.48	**0.028**	3.94	**0.0175**
	H1, H5	0.90	0.415	4.43	**0.010**	3.18	**0.033**	12.5	**0.0004**
	H2, H3	5.15	**0.015**	4.25	**0.014**	3.06	**0.038**	2.46	0.0705
	H2, H4	2.43	**0.073**	3.35	**0.028**	8.57	**0.001**	3.71	**0.0216**
	H2, H5	5.81	**0.004**	2.98	**0.040**	9.50	**0.001**	16.26	**0.0003**
	H3, H4	1.38	0.269	1.07	0.345	1.22	0.284	1.41	0.2273
	H3, H5	1.30	0.279	1.25	0.283	0.85	0.442	2.68	0.0539
	H4, H5	0.67	0.547	0.25	0.823	1.00	0.379	0.35	0.7376

N_0	Groups of months	H1		H2		H3		H4		H5	
		t	P(MC)	t	P(MC)	t	P(MC)	t	P(MC)	t	P(MC)
	June, August	4.60	**0.011**	0.80	0.462	0.73	0.52	1.67	0.18	1.11	0.33
	June, November	2.67	0.053	2.00	0.111	1.10	0.35	1.61	0.18	0.89	0.42
	June, February	6.32	**0.003**	1.41	0.229	1.45	0.24	1.31	0.26	3.58	**0.02**
	August, November	1.06	0.351	1.75	0.154	2.08	0.11	0.32	0.76	0.63	0.56
	August, February	3.50	**0.025**	1.34	0.253	2.55	0.06	0.16	0.88	1.26	0.27
	November, February	3.16	**0.033**	1.00	0.375	0.32	0.76	0.34	0.75	4.24	**0.01**

N_2	Groups of stations	t	P(perm)
	H1, H2	4.27	**0.0012**
	H1, H3	0.05	0.9660
	H1, H4	0.18	0.8589
	H1, H5	1.42	0.1708
	H2, H3	3.09	**0.0098**
	H2, H4	4.97	**0.0004**
	H2, H5	5.38	**0.0006**
	H3, H4	0.18	0.8591
	H3, H5	1.06	0.3010
	H4, H5	1.75	0.1007

N_{inf}	Groups of stations	t	P(perm)
	H1, H2	4.25	**0.0005**
	H1, H3	0.27	0.7972
	H1, H4	0.66	0.5249
	H1, H5	0.51	0.6130
	H2, H3	2.79	**0.0129**
	H2, H4	3.91	**0.0014**
	H2, H5	4.33	**0.0008**
	H3, H4	0.23	0.8231
	H3, H5	0.67	0.5154
	H4, H5	1.14	0.2686

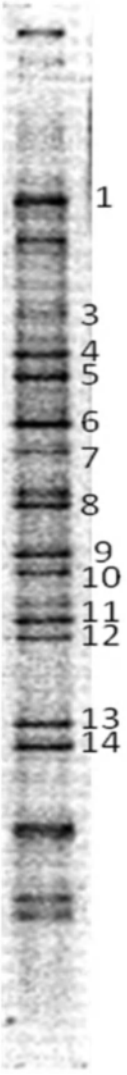
Appendix 1. Composition of DGGE marker

The reference for DGGE analysis was constructed from bacterial isolates of Paulina tidal flat-salt marsh sediment sampled from stations H2 and H5. Bacteria were isolated and cultured on Marine Broth (Difco) agar at 20°C in the dark.
To obtain a reference pattern with a sufficiently large band spread on a 35-70% gel and enclosing the expected spreading of sample patterns, strain composition of the reference was based on the vertical position of their individual V_3-DNA band(s) on the DGGE gel.
Six reference strains originated from the marsh gully (station H5) and their identification, RDP identification score and accession number were published in Cnudde et al. (2013) (addendum IV) and presented here in Table S2. Five reference strains originated from the sand flat H2. Their identification, RDP identification score and accession number is given in Table S2. Two reference strains remained unidentified (no sequence data).

Table S8. DGGE reference: strain composition.

DGGE reference band	Strain code	Origin	Genus identification	Taxonomic position	Identification scor	Access. number
'band 1'	Z45	sand	Unidentified			
'band 3'	Z107	sand	Staphylococcus sp.	Firmicutes	95%	awaiting
'band 4'	Z107	sand	Staphylococcus sp.	Firmicutes	95%	awaiting
'band 5'	Z100	sand	unidentified			
'band 6'	S19a	mud	Psychrobacter	Gammaproteobacteria	100%	HE999729
'band 7'	S19a	mud	Psychrobacter	Gammaproteobacteria	100%	HE999729
'band 8'	S109	mud	Erythrobacter sp.	Alphaproteobacteria	84%	HE999745
'band 9'	Z77	sand	Jannaschia sp.	Alphaproteobacteria	95%	awaiting
'band 10'	S145	mud	Photobacterium sp.	Gammaproteobacteria	100%	HE999728

DGGE reference



Addendum III

Supporting material – chapter 3

Table S1. Natural isotopic signatures of *Fucus vesiculosus*, *Spartina alterniflora* and epiphytes (collected at station H4 unless specifically noted) and suspended particulate organic matter (SPOM, collected at near station H1) (mean ± SD, n = 3).

Material	$\delta^{13}C$ (‰)		$\delta^{15}N$ (‰)	
Fucus (June)	-20.40	± 0.39	15.28	± 0.25
(Aug)	-18.16	± 0.20	13.74	± 0.62
(Nov)	-22.10	± 0.08	16.73	± 0.34
(Febr)	-22.26	± 0.45	14.14	± 0.19
Fucus - litter (Aug)	-22.91	± 0.43	16.2	± 0.54
Spartina - fresh tissue (June)	-13.45	± 0.10	14.73	± 0.49
(Aug)	-14.14	± 0.11	12.06	± 0.25
Spartina - decaying standing tissue (June, H3)	-13.25	± 0.28	10.64	± 0.36
(June)	-12.90	± 0.06	12.57	± 0.36
(Nov)	-12.95	± 0.08	14.52	± 0.13
Spartina - litter (Nov)	-13.25	± 0.12	14.29	± 0.26
Spartina - fibrous detritus (Nov)	-15.45	± 0.18	13.66	± 0.08
Epiphytes *Fucus* (June)	-21.15	± 0.25	7.49[3]	± 2.07
(Aug)	-19.31	± 0.10	10.8	± 0.77
(Nov)	-17.46[3]	± 0.08	14.2	± 0.36
(Febr)	-20.31	± 0.05	10.51	± 0.64
Epiphytes *Fucus* - litter (Febr)	-22.94	± 0.13	8.06	± 0.36
Epiphytes *Spartina* (Nov)	-19.46[3]	± 0.41	10.71	± 1.13
(Aug)	-21.14[3]	± 0.28	7.51	± 1.25
Epiphytes *Spartina* - decaying standing tissue (Febr)	-20.38	± 0.10	10.52	± 0.48
(Febr, H5)	-18.88	± 0.08	10.52	± 0.72
SPOM (June)*	-21.73	± 0.23	8.97	± 0.93
SPOM (Aug)	-23.88[2]	± 0.40	4.65	± 0.54
SPOM (Nov)	-22.87	±1.36	7.05[1]	± 1.64
SPOM (Febr)	-23.70	± 0.11	4.25	± 0.23

[1]n = 2; [2]n=5, [3]n = 4, * possibly contaminated with pelagic copepods

Table S2. $\delta^{13}C$ signatures of Paulina salt marsh harpacticoids (average ± SD, n = 3)

	H1				H2				H3				H4				H5			
	June	Aug	Nov	Febr	June	Aug	Nov	Febr	June	Aug	Nov	Febr	June	Aug	Nov	Febr	June	Aug	Nov	Febr
Amphiascus sp.	-16.0 0.6		-16.5								-18.6 2.3	-15.5 1.5	-16.0 0.6	-17.0 2.2	-16.9 0.4	-16.2 0.2			-20.3 0.7	
Asellopsis intermedia		-14.7 1.3	-16.0 0.4	-14.0 0.7	-15.1 0.4	-14.3 1.1	-13.9 0.4	-12.6 0.1	-14.7			-13.1								
Cletodidae									-40.3 1.1	-34.3		-30.6	-37.9 0.4	-34.8 2.8			-35.8 0.2	-34.6 1.2		
Delavalia palustis	-14.8 0.3			-15.7					-16.1	-16.7	-16.0	-16.1 0.4	-15.7 0.3	-15.6 2.8	-16.3 0.2	-15.9 0.1	-16.3 0.3	-18.2 0.6	-18.5 0.2	-17.9 0.4
Ectinosomatidae	-17.4 0.5	-18.4 1.2									-17.7	-16.3 0.4	-15.7 0.6	-22.0	-17.0 0.2	-16.5 0.0				
Harpacticidae			-15.9 0.8	-16.4					-17.0		-16.9 0.2	-16.4 0.6								
Microarthridion littorale	-17.0 0.9	-19.5 1.8	-18.0						-17.5		-17.0 0.6	-16.4 0.7	-16.0 0.4	-20.5 1.7	-16.6 1.0	-15.7 0.1	-17.2 0.7	-17.4 0.8	-18.3 0.1	-16.4 1.0
Nannopus palustris									-15.3	-15.6				-17.0			-16.3 0.4	-16.2 0.2	-18.1 0.7	-17.0 0.1
Paraleptastacus spinicauda					-15.1 0.9	-14.3 1.4	-14.0 0.6	-12.7 0.6	-14.4 1.2											
Paronychocamptus nanus	-21.0	-15.8 0.8	-15.7 0.2			-14.8 0.6					-15.9 0.7	-14.8 1.1			-15.8 0.9	-15.4 0.3			-23.5 1.2	-15.7 0.5
Platychelipus littoralis			-15.0							-15.2 0.2	-14.9	-14.5 0.2	-15.1 0.3	-15.0 0.4	-14.8 0.4	-14.7 0.1	-16.3 0.4	-16.2 0.2	-16.9 0.3	-16.4 0.4
Robertsonia diademata										-15.9 0.6	-18.3 0.5	-15.7								
Tachachidius discipes	-15.7 0.1	-15.6 0.5	-15.8 0.5	-16.1 0.2	-15.8 0.2	-14.7	-14.7 0.4	-14.9 0.1	-15.6 0.4	-15.6 0.5	-14.7 0.5	-15.1			-15.6 0.1	-15.2 0.0				

Addendum IV

Supporting material – chapter 4

Table S1. Results of main ANOVA tests for copepod mortality and carbon uptake in both the substrate experiment (2-way ANOVA, Cop x Sub) and the time-series experiment (3-way ANOVA, Cop x Sub x Time), with fixed factors copepod species ('Cop'), substrate ('Sub') and time ('Time'). Tukey post-hoc test (data not shown) were performed on the significant interaction factors or if not insignificant, on the single factor.

Substrate exp.	ANOVA mortality			ANOVA carbon uptake		
	MS	F value	p value	MS	F value	p value
Cop	10399	179.2	< 0.0001	0.6	47.9	< 0.001
Sub	5541	95.5	< 0.0001	309.5	219.7	< 0.0001
Cop:Sub	4544	78.3	< 0.0001	0.8	62.3	< 0.0001
Time-series exp.	**ANOVA mortality**			**ANOVA carbon uptake**		
	MS	F value	p value	MS	F value	p value
Cop	119.4	119.1	< 0.0001	73892	281.5	< 0.0001
Sub	0.6	69.3	0.012	292974	1116.4	< 0.0001
Time	117.4	117.1	0.001	86550	329.8	0.006
Cop:Sub	0.1	11.3	0.333	42005	160.0	0.038
Cop:Time	0.1	11.0	0.344	14444	55.0	0.010
Sub:Time	167.7	167.3	< 0.0001	51031	19.4	< 0.0001
Cop:Sub:Time	0.04	0.4	0.652	4719	17.9	0.187

ES Table 1. Relative fatty acid concentrations (%) in cultured food sources, in natural copepods (T_0) and in experimental copepods after 4 (T_4) and 9 days (T_9) grazing in B and BD treatments. Values between brackets are standard deviations (n = 3).

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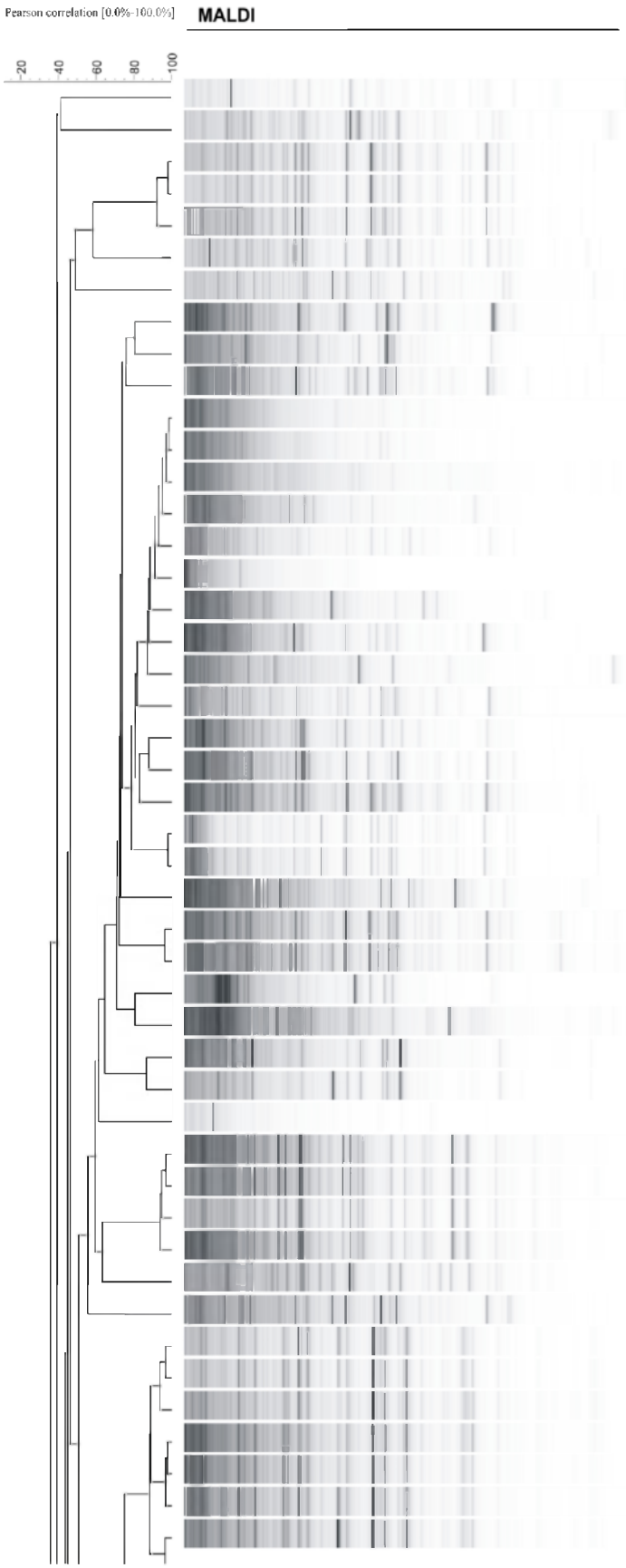
	FOOD				NATURAL COPEPODS						EXPERIMENTAL COPEPODS												
	Diatoms		Bacteria		*Platychelipus*		*Nannopus*		*Dalavalia*		*Platychelipus*				*Nannopus*				*Delavalia*				
											B		BD		B		BD		B		BD		
											T_4	T_9	T_4	T_9	T_4	T_9	T_4	T_9	T_4	T_9	T_4	T_9	
C14:0	**6.2**	(0.2)	1.8	(0.0)	3.0	(1.7)	2.9	(0.2)	2.6	(0.1)	2.3	2.2	2.7	2.6	2.3	2.2	2.3	2	2.3	2.3	2.8	2.8	C14:0
C15:0	0.5	(0.1)	0.6	(0.0)	1.0	(0.5)	1.9	(0.3)	**8.7**	(0.6)	1.5	1.3	0.6	0.4	2.6	2.6	2.2	1.5	**6.1**	4.9	1.9	0.8	C15:0
C15:1ω5	-		-		-		0.8	(0.1)	1.6	(0.2)	-	-	-	-	-	-	-	-	-	-	-	-	C15:1ω5
C16:0	**29.6**	(3.0)	**14**	(0.1)	**40.0**	(5.5)	**32.0**	(1.7)	**26.5**	(3.9)	**29.2**	**29.9**	**21.5**	**20.4**	**25.5**	**24.6**	**25.7**	**21.8**	**22.1**	**20.7**	**22.6**	**21.2**	C16:0
C16:1ω7	**36.1**	(3.4)	**29**	(0.1)	**15.4**	(1.6)	**20.7**	(1.1)	**9.8**	(1.3)	**19.7**	**14.6**	**24.6**	**26.8**	**19.4**	**17.4**	**23.2**	**21.5**	**9.2**	**13.7**	**25.4**	**29.8**	C16:1ω7
C17:0	0.4	(0.1)	4.3	(0.1)	0.8	(0.3)	1.1	(0.2)	**6.9**	(2.0)	1.6	1.7	0.5	0.4	1.7	1.73	1.3	1	**5.4**	4.5	1.7	0.8	C17:0
C17:1ω7	-		**11**	(0.2)	-		1.1	(0.2)	**6.3**	(1.1)	-	-	0.4	0.3	1.7	1.6	1.5	1.1	**5.0**	4.3	1.5	0.6	C17:1ω7
C18:0	4.4	(3.5)	2.1	(0.0)	**12.1**	(3.4)	**8.2**	(1.3)	**8.6**	(1.9)	**7.1**	**8.3**	3.4	3.2	**6.4**	**6.9**	**5.3**	4.7	**9.9**	**7.4**	4.6	3.2	C18:0
C18:1ω9	2.3	(0.6)	**38**	(0.4)	**5.2**	(1.7)	**7.1**	(0.6)	**7.7**	(0.7)	**9.0**	**8.2**	**6.0**	**6.5**	3.6	**10.3**	**11.2**	**9.2**	**6.9**	**10.8**	**5.5**	**6.0**	C18:1ω9
C18:2ω6c	0.3	(0.0)	-		0.2	(0.3)	0.5	(0.0)	1.0	(0.0)	-	-	0.5	0.4	-	-	-	-	-	-	**5.0**	-	C18:2ω6c
C18:3ω6	4.2	(0.9)	-		0.1	(0.2)	0.4	(0.0)	0.8	(0.8)	-	-	2.5	3.1	-	-	1.2	1.3	1.6	2.0	3.3	3.9	C18:3ω6
C20:1ω9	-		-		0.1	(0.1)	0.3	(0.1)	0.4	(0.1)	-	-	-	0.3	-	-	0.5	0.4	-	-	-	0.4	C20:1ω9
C20:4ω6	-		-		0.7	(0.6)	0.7	(0.2)	2.4	(0.5)	1.5	1.4	1	0.9	0.9	-	-	0.9	3.1	2.3	1.7	1.1	C20:4ω6
C20:5ω3	**14.3**	(1.9)	-		**14.4**	(6.2)	**16.6**	(0.7)	**13.4**	(4.1)	**22**	**20.8**	**27**	**25.4**	**21.4**	**17.9**	**18.5**	**20.3**	**20.0**	**19.3**	**21.8**	**23.2**	C20:5ω3
C22:6ω3	1.7	(0.3)	-		**6.9**	(4.7)	**5.8**	(0.2)	3.3	(0.8)	**6.0**	**11.6**	**9.4**	**9.3**	**14.5**	**14.7**	**7.1**	**14.2**	**8.3**	**7.8**	**6.8**	**6.2**	C22:6ω3
Σ bacterial FA	0.8	(0.2)	15.5	(0.3)	1.9	(0.7)	4.9	(0.5)	23.4	(2.5)	3.1	2.9	1.5	1.1	5.9	6.0	5.0	3.7	16.6	13.8	5.2	2.2	Σ bacterial FA
ΣPUFA	20.6	(1.9)	-		22.4	(3.6)	24.0	(0.9)	21.0	(6.1)	29.5	33.8	40.3	39.0	36.9	32.6	26.8	36.7	33	31.4	33.9	34.4	ΣPUFA

Addendum V

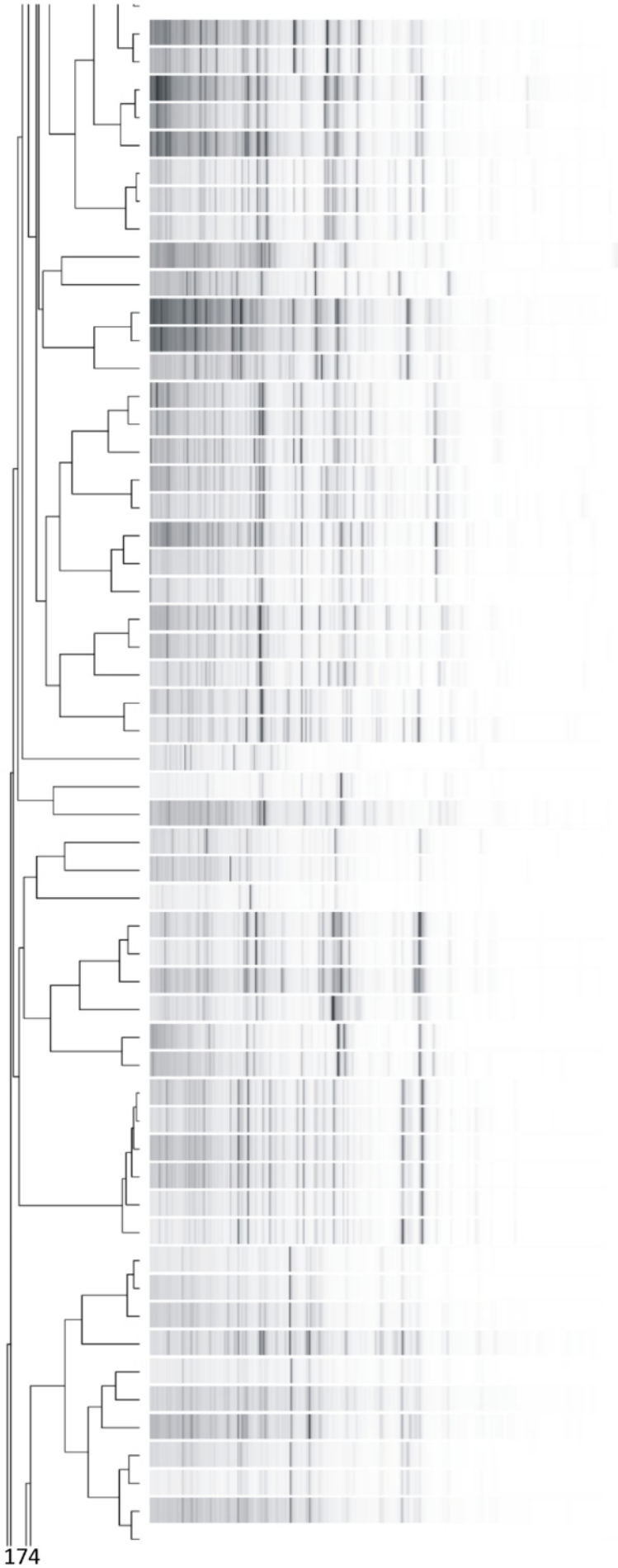
Supporting material – chapter 5

Fig. S1. UPGMA dendrogram of MALDI-TOF ribosomal protein spectra of all bacterial isolates collected from the Paulina salt marsh sediment. Spectrum quality was visually assessed based on signal intensity, number of peaks and background noise. *Gramella* (strain S105), *Jannaschia* (strain 130) and *Photobacterium* (strain S145) isolates are indicated by a box.

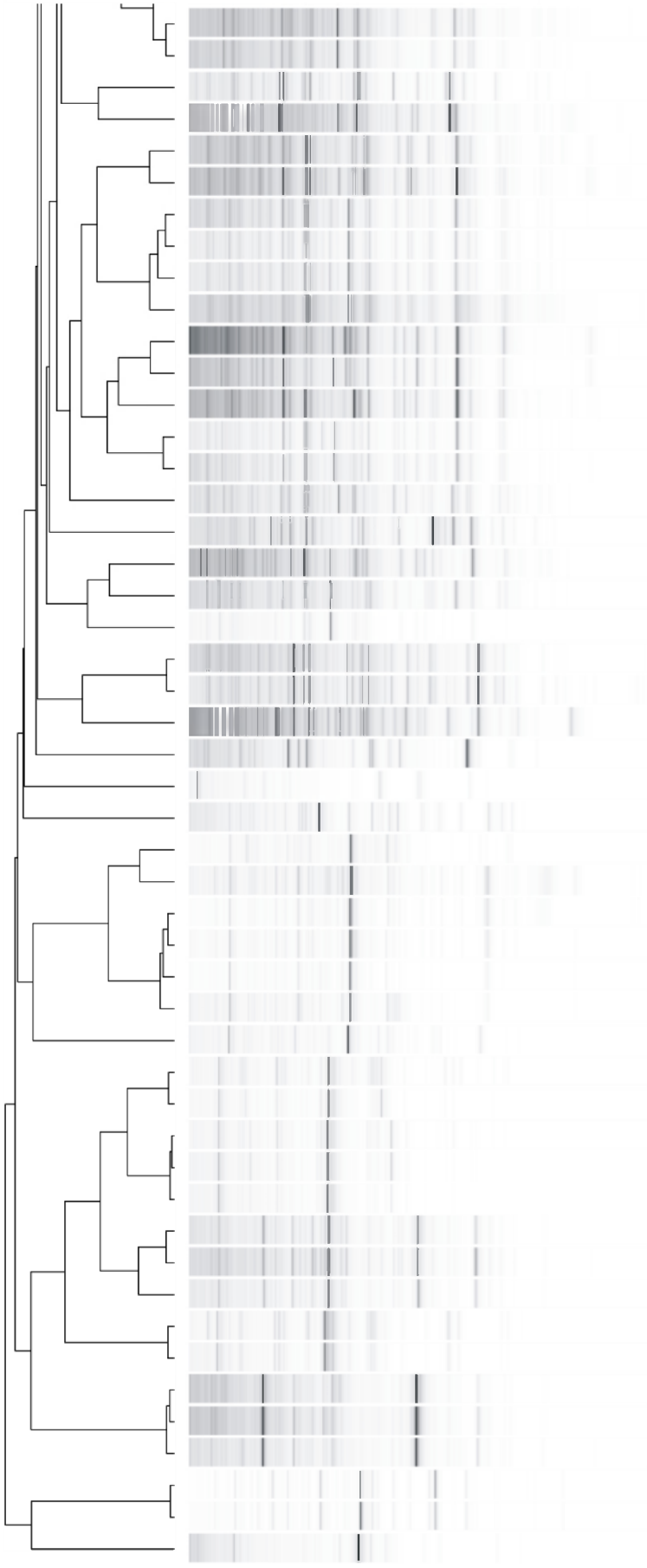
(*: agar medium from which colonies were isolated, followed by standardized cultivation on marine agar for MALDI-TOF analysis. MA: marine agar, MA/10: marine agar diluted ten times, SA: seawater agar)



Strain	Morphological charactersistics	Isolation medium*
S67	Yellow, translucent	MA/10
S151	Purple	MA
S60b	White-grey, mucous	MA/10
S61	White-grey-brown, mucous	MA/10
S20a	White-grey, mucous	SA
S35	White-grey, translucent	SA
S139	White- pink	MA
S132	White-grey	MA
S154	White	SA
S134	White	MA
S28	White, translucent	SA
S95a	Pink, translucent	MA
S157	Yellow, translucent	MA/10
S93	Orange	MA
S10	Pink, opaque	SA
S150	Yellow, opaque	MA
S144	Pink	MA
S97	Pink	MA
S50	Yellow	SA
S4	White-grey	SA
S13	White, opaque	SA
S94	Pink, translucent	MA
S20b	White, translucent	SA
S19a	White, translucent	SA
S19b	White-grey	SA
S114	Yellow, translucent	MA
S131	White	MA
S136	White-grey	MA
S14	White	SA
S118	Yellow, translucent	MA
S62	White, translucent	MA/10
S69	White-grey	MA/10
S11	White, translucent	SA
S9	Yellow	SA
S42	Yellow, translucent	MA/10
S109	Yellow, opaque	MA
S15	Yellow, translucent	SA
S113	Yellow, opaque	MA
S143	White, translucent	MA
S34	White	SA
S66b	White-grey, translucent	MA/10
S122a	White-grey	MA
S27	White, translucent	SA
S51	White, opaque	MA/10
S33	White	SA
S95b	White-grey	MA



S95b	White-grey	MA
S127	White-grey	MA
S70	White-grey	MA/10
S82	White, translucent	MA/10
S22	White-grey	SA
S68a	White-grey	MA/10
S68b	Slow-growing	MA/10
S85		MA/10
S105	Yellow	MA
S120	Yellow	MA
S21a	White	SA
S21b	White, translucent	SA
S63b	White, translucent	MA/10
S12	White, translucent	SA
S52	White, translucent	MA/10
S152a	White-grey	MA
S31a	White	SA
S31b	White-grey-brown	SA
S87	White-grey, translucent	MA/10
S124	White-grey, translucent	MA
S41	White-grey	SA
S161	White	MA/10
S8	White	SA
S101	Pink	MA
S30	Yellow	SA
S115	Yellow	MA
S32	White	SA
S130	White-grey	MA
S140a	White, translucent	MA
S17	Yellow	SA
S155	Yellow	SA
S16	Yellow	SA
S74a	White, translucent	MA/10
S91	White, translucent	MA/10
S77	White	MA/10
S84	White	MA/10
S102	Pink-red, translucent	MA
S125	Pink	MA
S145	White-grey-brown, mucous	MA
S146	White-grey-brown, mucous	MA
S148	White-grey	MA
S152b	White-grey, mucous	MA
S153a	White-grey, mucous	MA
S147	White-grey-brown	MA
S99	Orange	MA
S100	Orange	MA
S96	Orange	MA
S103	Orange, translucent	MA
S81	Orange, translucent	MA/10
S167	Orange, translucent	MA/10
S163	Orange, translucent	MA/10
S164	Orange, translucent	MA/10
S165	Orange	MA/10
S46	Orange	MA/10



S46	Orange	MA/10
S79	Orange, translucent	MA/10
S123	Yellow, translucent	MA
S129	Yellow	MA
S98	Orange	MA
S137	Orange	MA
S60a	Orange, translucent	MA/10
S65b	Orange, translucent	MA/10
S57	Orange, translucent	MA/10
S65a	Orange, translucent	MA/10
S89		MA/10
S166	Orange	MA/10
S83	Orange	MA/10
S40	orange	SA
S49	Orange, translucent	SA
S74c	Orange	MA/10
S45	Yellow	MA/10
S43	Yellow, translucent	MA/10
S44	Orange, translucent	MA/10
S63a	Yellow	MA/10
S106	White	MA
S119	Orange	MA
S58	Yellow	MA/10
S168	White	MA/10
S122b	Yellow, translucent	MA
S149	White-grey, mucous	MA
S53	White-grey, opaque	MA/10
S138	White-grey	MA
S107	Yellow, opaque	MA
S140b	White, slow-growing	MA
S88	Yellow	MA/10
S117	Yellow	MA
S74b	Yellow, translucent	MA/10
S47	Yellow-orange	MA/10
S159	Yellow, translucent	MA/10
S80	Yellow, translucent	MA/10
S162	Yellow	MA/10
S86	Yellow, translucent	MA/10
S54	White, translucent	MA/10
S126	White-grey, opaque	MA
S66a	White, translucent	MA/10
S55	Yellow, translucent	MA/10
S158	Yellow	MA/10
S26	White, opaque	SA
S39a	White, opaque	MA/10
S39b	White, opaque	MA/10
S72a	White	MA/10
S72b	White	MA/10
S135	Pink	MA

Table S1. Identification of sediment bacteria from the Paulina salt marsh based on partial 16S rRNA gene sequences and the RDP classifier tool (with minimum confidence threshold of 80%)

Strain designation	Accession number	RDP genus affiliation	Confidence treshold	Bacterial class
S105	HE999723	*Gramella*	100%	Flavobacteria
S17	HE999713	*Leeuwenhoekiella*	100%	Flavobacteria
S122b	HE999756	*Muricauda*	100%	Flavobacteria
S140b	HE999725	*Micrococcus*	100%	Actinobacteria
S55	HE999736	*Demequina*	100%	Actinobacteria
S74b	HE999743	*Demequina*	100%	Actinobacteria
S80	HE999754	*Demequina*	100%	Actinobacteria
S72a	HE999715	*Acinetobacter*	100%	Gammaproteobacteria
S67	HE999716	*Lysobacter*	100%	Gammaproteobacteria
S39b	HE999719	*Psychrobacter*	100%	Gammaproteobacteria
S63b	HE999726	*Pseudomonas*	100%	Gammaproteobacteria
S66a	HE999727	*Psychrobacter*	100%	Gammaproteobacteria
S145	HE999728	*Photobacterium*	100%	Gammaproteobacteria
S19a	HE999729	*Psychrobacter*	100%	Gammaproteobacteria
S98	HE999755	*Erythrobacter*	98%	Alphaproteobacteria
S168	HE999714	*Thalassospira*	100%	Alphaproteobacteria
S34	HE999717	*Labrenzia*	99%	Alphaproteobacteria
S52	HE999718	*Rhodobacteraceae*	100%	Alphaproteobacteria
S35	HE999720	*Loktanella*	86%	Alphaproteobacteria
S151	HE999721	*Rhodovulum*	100%	Alphaproteobacteria
S130	HE999722	*Jannaschia*	99%	Alphaproteobacteria
S101	HE999724	Rhodobacteraceae	100%	Alphaproteobacteria
S10	HE999730	Rhodobacteraceae	100%	Alphaproteobacteria
S69	HE999731	*Paracoccus*	100%	Alphaproteobacteria
S81	HE999732	Erythrobacteraceae	98%	Alphaproteobacteria
S44	HE999733	Erythrobacteraceae	93%	Alphaproteobacteria
S61	HE999734	*Loktanella*	82%	Alphaproteobacteria
S32	HE999735	Rhodobacteraceae	100%	Alphaproteobacteria
S94	HE999737	*Loktanella*	100%	Alphaproteobacteria
S117	HE999738	*Altererythrobacter*	94%	Alphaproteobacteria
S14	HE999739	Rhodobacteraceae	100%	Alphaproteobacteria
S57	HE999740	*Erythrobacter*	98%	Alphaproteobacteria
S149	HE999741	Rhodobacteraceae	100%	Alphaproteobacteria
S50	HE999742	Erythrobacteraceae	100%	Alphaproteobacteria
S131	HE999744	*Loktanella*	91%	Alphaproteobacteria
S109	HE999745	*Erythrobacter*	84%	Alphaproteobacteria
S139	HE999746	Rhodobacteraceae	100%	Alphaproteobacteria
S45	HE999747	*Erythrobacter*	100%	Alphaproteobacteria
S4	HE999748	Rhodobacteraceae	100%	Alphaproteobacteria
S123	HE999749	Erythrobacteraceae	98%	Alphaproteobacteria
S91	HE999750	Rhizobiales	100%	Alphaproteobacteria
S124	HE999751	Rhodobacteraceae	100%	Alphaproteobacteria
S106	HE999752	Sphingomonadales	100%	Alphaproteobacteria
S68a	HE999753	Rhodobacteraceae	96%	Alphaproteobacteria

Table S2. Characteristic fatty acids of the bacterial strains *Gramella*, *Jannaschia* and *Photobacterium*, expressed as relative abundances (%) (mean ± SD, n=3). Fatty acids specific for each strain are highlighted in dark grey, fatty acids in common between 2 strains are indicated in light grey

	Gramella	*Jannaschia*	*Photobacterium*
C14:0	7.29 ± 1.10	0	0.75 ± 0.11
C15:0	21.34 ± 6.91	0	0.93 ± 0.19
C16:0	40.52 ± 7.80	8.45 ± 6.24	14.98 ± 2.87
C16:1ω7	0	43.38 ± 8.00	52.89 ± 2.64
C17:0	0	0	1.96 ± 0.66
C17:1ω7	0	0	5.58 ± 5.12
C18:0	30.86 ± 3.32	23.54 ± 10.53	0.67 ± 0.10
C18:1ω9	0	24.63 ± 7.18	22.24 ± 3.21

Addendum VI

Supporting material - chapter 6

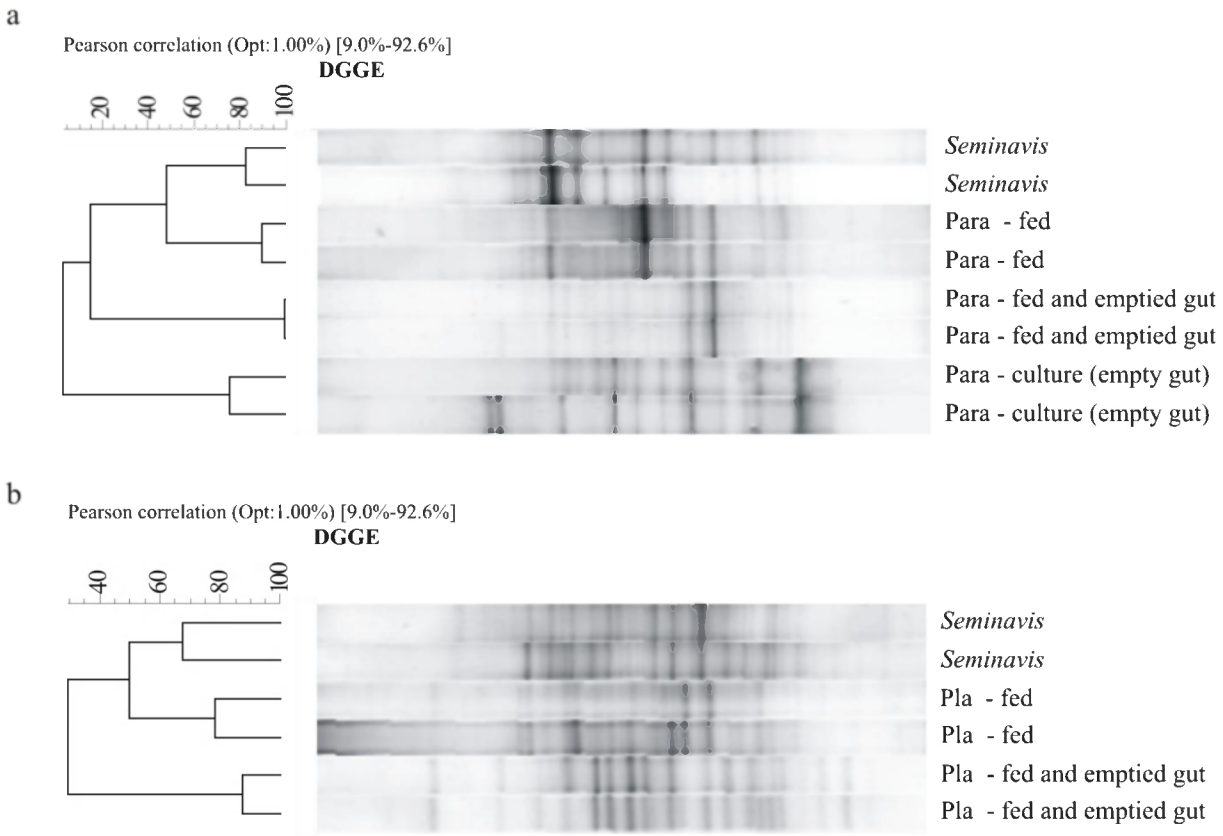


Fig S1. Cluster analysis of *Seminavis* and copepod bacterial communities to indicate the change in copepod bacteria resulting from *Seminavis* feeding, (a) for *Paramphiascella* ('Para') and (b) for *Platychelipus* ('Pla'). Copepods were sampled after feeding on *Seminavis* ('fed') and after feeding on *Seminavis* followed by gut clearance ('fed and emptied gut'). In addition, control copepods, i.e. copepods which have not been exposed to *Seminavis*, were sampled directly from the batch culture ('culture').

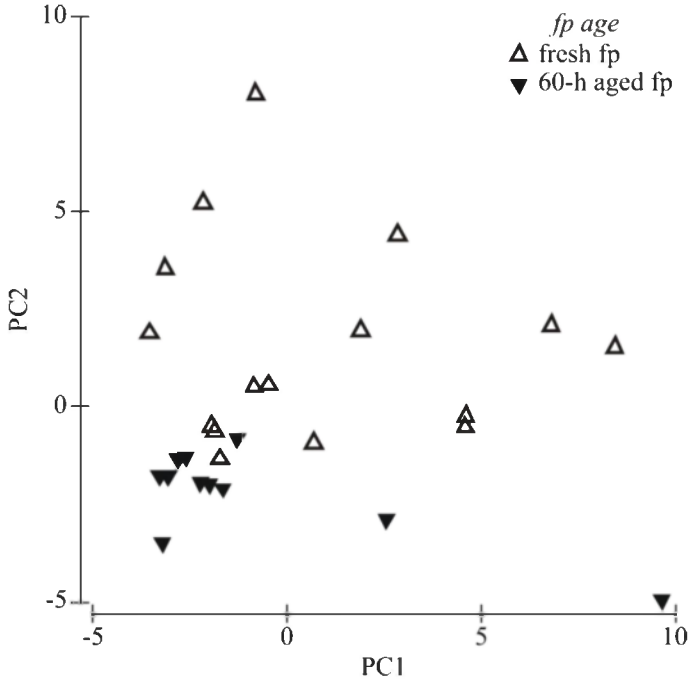


Fig S2. PCA based on carbon source utilization patterns of bacterial communities from fresh and aged copepod fp.

Table S1. Overview of all sample types analyzed with DGGE and Biolog and indication of the number of replicates.

				DGGE	Biolog
Sample type	Copepod	Origin	Treatment	# rep*	# rep**
Fecal pellet:	*Platychelipus*	natural	fresh	3	4
			20h	1	
			40h	1	
			60h	1	4
		laboratory-fed	fresh	4	6
			20h	2	
			40h	2	
			60h	2	6
	Paramphiascella	laboratory-fed	fresh	2	4
			20h	2	
			40h	2	
			60h	2	2
Copepod:	*Platychelipus*	field	defecated		6
		laboratory-fed	full gut	2	3
			defecated	2	
	Paramphiascella	culture	defecated	2	
		laboratory-fed	full gut	2	
			defecated	2	
Seminavis:				4	3
Natural seawater:					3

* biological replicates, originating from different copepod batches (1500 to 2500 specimens per batch)

** technical replicates

Table S2. Substrate richness S (number of positive substrate responses per sample) per substrate guild for bacteria from fresh and aged fecal pellets (fp) and for *Seminavis*, copepods and NSW. Differences in guild size (number of substrates per guild) were taken into account.

Sample type	Carbohydrates	Carboxylic acids	Amino acids	Polymers	Amines	Miscellaneous
Fresh fp	4.10	0.13	1.69	4.08	1.41	1.31
Aged fp	1.29	0.17	0.50	1.69	0.75	0.25
Seminavis	0	0	0	4.50	0	0
Copepod	1.86	0	1.67	2.75	0	1.33
NSW	5.14	0.67	2.00	3.00	0	0

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