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Benthic copepod guts as a selective microbial microhabitat in marine sediments

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ABSTRACT: The gut microbiome of the benthic copepod *Platychelipus littoralis*, a key species in the intertidal mudflats of western Europe, was characterized throughout a 1 yr period. It was hypothesized that benthic copepods living in sediment would have core microbial taxa in their gut microbiome, in accordance with the gut microbiomes of pelagic copepods living in the water column, but that this community might change rapidly. Copepods were isolated from sediment, and after a starvation period, the guts were microdissected. The copepod gut was found to be a selective microbial microhabitat, significantly different in microbiome composition from the sediment, with lower species richness and evenness. Although microbial cell counts were low in copepod guts, the gut microbiome was stable between 24 and 48 h of egestion. Diatoms were the main food source of the copepods, as confirmed by fatty acid biomarkers. Core bacterial species in the gut belonged to *Rhodobacteraceae*, *Flavobacteriaceae* and *Saprospiraceae*, known as degraders of complex organic compounds. *Bacteria* were not a significant food source themselves, but core bacterial taxa were potentially involved in food-assisted degradation. This study elucidated bacteria–copepod interactions, relevant for the food web ecology of benthic systems and potentially the optimization of copepod culturing.

KEY WORDS: Gut microbiome · Copepods · Intertidal sediment · Microhabitats

1. INTRODUCTION

Copepods comprise a diverse and abundant group of crustaceans in marine ecosystems. It is estimated that one-quarter of all crustacean species are copepods (Boxshall & Halsey 2004). Non-parasitic copepods can be predatory, omnivorous or detrivorous and, within their size classification (retained on 32/38 µm but passing a 1 mm sieve), are the major prey for higher trophic levels, e.g. fish larvae. They play an important role in energy transfer in marine food webs (Skovgaard et al. 2015, Baguley et al. 2019). Harpacticoid copepods have a benthic lifestyle and form the largest meiofaunal group next to free-living nematodes in estuaries, comprising 10–40% of fauna in sediments (Coull 1999). Their diet can be broad, ranging from consuming microalgae, flagellates, ciliates, mucoid substances and fungi to bacteria (Brucet et al. 2008, De Troch et al. 2010, Skovgaard et al. 2015). With the use of trophic biomarkers, the main food sources of copepods can be identified, as food uptake is hard to observe due to their small size. Fatty acid (FA) analysis is used to quantify the assimilation of carbon from the diet; e.g. palmitoleic acid is a biomarker for feeding on diatoms (El-Sabaawi et al. 2009). Copepods assimilate around 30–60% of ingested food, releasing a significant part as faecal pellets or dissolved organic carbon (Thor et al. 2007). They also show coprorhexy and coprophagy, the breaking apart and reingestion of their own faecal pellets and associated microbiome (Iversen & Poulsen 2007).

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Here, we present a study of the gut microbiome of a marine benthic copepod, the sediment-dwelling *Platychelipus littoralis* (Harpacticoida, family Laophontidae), a key species in the intertidal mudflats of western Europe. Bacteria can be found on both the outside surface of the copepods and living within the copepod's orifices, including the intestinal tract. The gut microbiome of copepods develops through the ingestion of micro-organisms from seawater and those attached to detritus particles, phytoplankton and other prey items (Tang et al. 2010). Gut micro-organisms represent both transient and residential communities. The transient community passes through the gut together with food items and is as variable as the food ingested. The residential community forms stable populations in the gut. They survive ingestion and have an adaptive mechanism to avoid being flushed out by egestion, possibly by continuous growth or attachment to gut tissue (Tang 2005, Peter & Sommaruga 2008). Bacterial densities per copepod range from around 10^7 to 10^{11} ml^{-1} body volume, while their faecal pellets can reach up to 10^{10} to 10^{11} cells ml^{-1} (Jacobsen & Azam 1984, Tang et al. 2010). Pelagic copepod guts are microbial hotspots in oceans, providing a microbial microhabitat that differs from the surrounding seawater (Møller et al. 2007, Wäge et al. 2019). The gut is an organic-rich, low-oxygen environment with a lower pH than seawater. Microelectrode gut profiling of the pelagic copepod *Calanus* sp. even showed the presence of fully anoxic zones in the gut (Tang et al. 2011). Furthermore, pathogenic and parasitic bacteria of copepods constitute mainly fish-pathogenic *Vibrio* species, as copepods are often pathogen vectors for commercially harvested and cultured fish species (Skovgaard et al. 2015, Bass et al. 2021).

The gut microbiome can play an important role in copepod health as well as local biogeochemical cycling in marine sediments and marine food webs, but the specific functionality of copepod–bacteria interactions remains largely unknown. This includes the drivers and stressors of bacterial composition, the exact ecophysiological roles of gut bacteria and the microbial decomposition processes in faecal pellets (Tang et al. 2010, Moisander et al. 2015, Shoemaker & Moisander 2017, Datta et al. 2018). The microbiomes of several pelagic copepod genera have been characterized and core bacterial microbiomes were identified (reviewed in Sadaiappan et al. 2021). For benthic copepods, however, the study of faecal pellet microbiomes was only recently conducted for *Tigriopus kingsejongensis* living in Antarctic tidal pools (Oh et al. 2022). The genus *Tigriopus* has large potential as live aquaculture feed (Fleeger 2007). The genus, occurring in tidal rock pools, has been studied for the bacterial attachment and control of *Vibrio* spp. (Carli et al. 1993, Sahandi et al. 2023).

The present study explored factors shaping bacterial composition in the guts of temperate copepods, with a focus on those inhabiting intertidal mudflats. Unlike previous research that has primarily examined copepods in seawater, we investigated whether benthic copepods living in bacteria-rich sediment can host a distinct microbial community. The combination of microbiome analysis and FA profiles as trophic biomarkers allowed us to specify links between the copepod diet and gut microbiome composition and functionality. We tested whether the bacteria itself was a food source or merely acted as a facilitator for the assimilation of other food sources. Copepod guts are small and potentially dynamic in microbial composition; therefore, the gut microbiome was studied during 48 h of starvation.

This study aimed to determine whether (1) the gut of *P. littoralis* is selective towards the sediment microbiome, (2) the gut community is stable over a prolonged starvation period and (3) core gut microbial taxa can be identified and linked to environmental drivers of microbiome composition. These research questions were studied using amplicon sequencing, microscopy, flow cytometry and FA analysis. This allowed us to unravel the linkage between copepod feeding behaviour and the microbial food web.

2. MATERIALS AND METHODS

2.1. Field sampling

Sampling took place over a 1 yr time frame, with samples taken every other month (August 2022 to September 2023) at the intertidal mudflat of the Paulina salt marsh (Westerscheldt estuary, The Netherlands; 51° 21′ 5.2′′ N, 3° 44′ 21.8′′ E). The harpacticoid copepod species *Platychelipus littoralis* was field-caught and extracted alive from the top layers of sediment through sieving (1 mm top sieve, 250 µm bottom sieve). Adult copepods were hand-picked, indiscriminate of sex, using a glass pipette under a stereomicroscope (Wild M5–72230 Wild Heerbrugg). Additional sediment was collected for microbiome analysis by scraping only the top 2 cm of sediment and subsequently storing at −20°C.

Sea surface water temperature was obtained from a nearby field measuring station (Terneuzen westsluis zeezijde; www.rijkswaterstaat.nl). Water temperature

data from the sampling period was incomplete; therefore, data from the past 3 yr (21 November 2020–20 September 2023, daily read-out) was included to give an accurate view of seasonal temperature patterns.

2.2. Experimental lab setup

The experiment required 2 lab setups: (1) a starvation treatment for microbiome analysis and (2) gut clearance for FA analysis.

In the starvation experiment, adult copepods were washed in filter-sterilized natural seawater (FNSW, 0.22 µm filters, Millipore) with a salinity of 32 and then grouped per 50 individuals in a Petri dish (Ø = 55 mm; Novolab). A total of 4 dishes, each containing ca. 10 ml FNSW, were kept at 15°C in a climate room (temperature-controlled ±1°C). Copepods were kept in a 12 h light:12 h dark regime for 2 d without food. Gut samples were distinguished based on their starvation period, between 24 and 48 h. Dissections of living individuals were performed at 24 and 48 h, allowing the guts to clear prior to their collection for further analyses (for further details see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m756p019_supp.pdf). After 24 or 48 h, copepods were washed 3 times in filtered phosphate-buffered saline (0.22 µm filters; Millipore) to remove any contamination from epibionts or surrounding water. Guts were dissected from the copepod body under a stereomicroscope by means of 2 microdissecting needles armed with insect pins. One needle was positioned in the last segment (telson, anal somite, in between both furcas) while the second needle was used to gently pull the rest of the body away from the caudal end. The extracted guts of 10 randomly selected copepods were pooled in one sample per replicate collected in a DNA/RNA-free vial via minute pieces of Whatman filter paper (GE Healthcare Life Sciences) and stored at −20°C prior to sequencing. Sampling during July and September 2023 included 4–5 replicates, to allow for characterization of within-species variability, whereas the other months were represented by 1–2 replicates to allow feasibility of the experiment. Samples from the surrounding water were prepared by centrifuging 9–10 ml of the seawater from each Petri dish in which the copepods were incubated, in DNA/RNA-free vials (15000 × *g*, 2 min). The supernatant was then removed and samples were stored at −20°C. For the field sediment samples, 250 mg of sediment was collected and stored at −20°C.

A second set of randomly collected adult copepods was used for FA analysis. Again, gut clearance was allowed for 24 h prior to sample collection. Adult copepods were washed in FNSW and grouped, with 50 individuals in a Petri dish (Ø = 55 mm; Novolab) representing one replicate. A total of 4 replicates were kept at 15°C in a climate room. Copepods were kept in a 12 h light:12 h dark regime overnight without food. This enabled gut clearance, thus limiting bias from ingested food on lipid composition (De Troch et al. 2010, Couturier et al. 2020). After gut clearance, copepods were washed in FNSW and egg sacks were removed. Per sample, 50 copepods were grouped and stored at −80°C.

This study used amplicon sequencing to characterize the microbiome in copepod guts (samples consisting of 10 grouped guts with n = 1–2 for August 2022–May 2023 and n = 4–5 for July 2023 and September 2023), field sediment (n = 3 per sampling) and incubation water; flow cytometry to count microbial cells in copepod guts (n = 20 per sampling); microscopy to measure gut size (n = 5 for males and gravid females) and FA analysis (samples consisting of 50 copepods, n = 3–4 per sampling) to characterize the biochemical content of copepods and assimilation from the diet.

2.3. 16S rRNA gene amplicon sequencing

16S amplicon sequencing was performed on copepod gut samples, incubation water and field sediment samples. Genomic DNA was extracted from all samples using a DNeasy Powersoil Pro Kit (Qiagen) according to the manufacturer's protocol. PCR was performed using Phusion Plus DNA polymerase (Thermo Fisher Scientific) in 20 µl reactions containing 10 µl Phusion Plus green PCR Master Mix, 1 µl of 10 µM Primer 27F: AGA GTT TGA TCM TGG CTC AG, 1 µl of 10 µM Primer 1492R: TAC GGY TAC CTT GTT ACG ACT T, 7 µl of PCR water and 1 µl sample. For amplification, gut samples required 35 cycles, while other samples were run for 30 cycles. Amplification was run including initial denaturation for 30 s at 98°C, followed by 30–35 cycles of 10 s, denaturation at 98°C, 10 s, annealing at 55°C and 1 min extension at 72°C. A final elongation step was included at 72°C for 5 min. The obtained PCR product was run on a 1.5% agarose gel for 30 min at 100 V. The same protocol was performed with Archaeal primers, 340F: CCC TAY GGG GYG CAS CAG, 1000R: GGC CAT GCA CYW CYT CTC nested with U341F: TCC TAC GGG NGG CWG CAG, U806R: GGA CTA CVS GGG TAT CTA AT (Gantner et al. 2011, Klindworth et al. 2013).

The original genomic DNA extract (10 µl) was sent out to LGC genomics for library preparation and

sequencing on an Illumina MiSeq platform with v3 chemistry. The V3–V4 region of the 16S rRNA gene was amplified through PCR using bacterial primers (341F: TCC TAC GGG NGG CWG CAG, 785R: TGA CTA CHV GGG TAT CTA AKC C) and archaeal primers mentioned above. To assess the sequencing quality, a mock community was included in the sequencing run, matching the lowest and highest DNA concentrations of that run. Amplicon data were processed with the DADA2 R package (v.1.30.0) according to Callahan et al. (2016). Taxonomy was assigned using the Silva database (v.138) (Quast et al. 2013). All sequences that were classified as Eukaryota and mitochondria were removed. For the first sampling time point, DNA was extracted from individual guts and later results were merged *in silico*. For subsequent samples, DNA was extracted from grouped guts to increase DNA detection. 16S rRNA gene sequences from this study were deposited in the European nucleotide archive under accession number PRJEB72878.

2.4. Flow cytometry

After dissection, individual guts were transferred to 0.5 ml of filtered-sterilized phosphate-buffered saline (PBS) (0.2 µm filters; Millipore). Gut and water samples were fixed in 2% glutaraldehyde (Carl Roth) for storage at 4°C. Cell counts were quantified within 2 mo, using an Attune™ NxT flow cytometer (Thermo Fisher) with BRxx configuration, equipped with a blue (488 nm, 50 mW) and red laser (638 nm, 50 mW), 7 fluorescence detectors with bandpass filters (BL1: 530/30 nm; BL2: 574/26 nm; BL3: 695/40 nm; BL4: 780/60 nm; RL1: 670/14 nm; RL2: 720/30 nm; RL3: 780/60 nm) and 2 scatter detectors on the 488 nm laser (FSC: 488/10 nm; SSC: 488/10 nm). The flow cytometer was operated with Attune™ focusing fluid (Thermo Fisher Scientific) as sheath fluid. Sample preparation was performed by sonication (20 min) in a sonication bath (Elmasonic S, 30 Hz), 2-time dilution in filter-sterilized PBS and subsequent second sonication (20 min). Sonication was used to separate the cells from each other and the gut tissue as much as possible. Samples were diluted in 96-well plates in filter-sterilized PBS and stained with 1 vol% SYBR® Green I (100× concentrate in 0.22 µm filtered dimethyl sulfoxide; Invitrogen) in the dark (20 min, 37°C) for total cell analysis. Quality control was performed daily using BDTM CS&T RUO beads (BD Biosciences). Samples were run in fixed volume mode (50 µl).

2.5. Microscopical measurements

As copepod size varies by sex, 5 males and 5 gravid females of *P. littoralis* were isolated per sampling campaign (see Fig. 1). Copepods were photographed using a Leica DMi1 inverted microscope connected to a FLEXACAM C1 camera (Leica) under 0.45× lens and 5× objective. Sexual dimorphism of copepods in adult stage (A1) of copepods allowed for sexual identification. Males and non-gravid females, respectively, were identified based on the presence and absence of a modified first antenna. Gravid females were defined by the presence of an egg sack. Gut length and diameter were measured using ImageJ (v.1.54g). Gut volume was calculated by assuming a cylindrical gut.

2.6. Fatty acid analysis

Lipid extraction, FA methylation and analysis of FA methyl esters (FAMEs) were performed according to De Troch et al. (2012). FAMEs were separated using a gas chromatograph (HP 6890N) coupled to a mass spectrometer (HP 5973) based on a splitless injection (5 µl of extract) at a temperature of 250°C using an HP88 column (Agilent). FAMEs were identified based on a comparison of relative retention time and on mass spectral libraries using the software MSD Chem Station (v.B.07.01, Agilent). FAME concentrations (µg FA per copepod) were calculated based on the internal standard nonadecanoic acid. Trophic biomarkers for bacteria, terrestrial input, diatoms and flagellates were used (El-Sabaawi et al. 2009, Gentsch et al. 2009), with palmitoleic acid as specific diatom biomarker; the sum of palmitoleic acid, eicosapentaenoic acid and all polyunsaturated FAs with a carbon length of 16 was used as a potential indication of feeding on diatoms.

2.7. Data analysis

Data analysis was performed in R (v.4.3.0). 16S sequencing data was processed using the 'phyloseq' package (v.1.46.0) and the 'vegan' package (v.2.6-4). Singletons were removed before analysis. Chloroplast sequences were filtered out before bacterial composition analysis. Normalization using total sum scaling was used to limit any bias when comparing samples run with different PCR cycles. Data exploration for diversity and evenness (Choa1, InvSimpson and Shannon indices) was performed using bar plots and a

principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity. Wilcoxon rank tests (alpha diversity) and PERMANOVAs with post hoc pairwise adonis (beta diversity) were used for statistical testing between different sampling times and types of samples (i.e. 24 and 48 h starved copepod guts, incubation water and sediment). PERMDISP was used to test within-group dispersion. Bonferroni-corrected *t*-tests were used to test between cell counts.

A core taxa analysis was performed using a cutoff for abundance at 50%, a more relaxed criterion to account for inadequate sampling efforts in accordance with earlier copepod microbiome studies (Moisander et al. 2015, Shoemaker & Moisander 2015, 2017, Cregeen 2016, Dorosz et al. 2016, Wäge et al. 2019, Sadaiappan et al. 2021, Velasquez et al. 2023). Similarly, occurrence cutoff was set at 1% relative abundance at the family level but specified further if the family was restricted to a single genus. Prior to core taxa analysis, sequences were grouped per sampling time point and type—starvation period (gut—24 h, gut—48 h) to limit bias caused by biological variability. The core taxon *Cutibacterium* was categorized as human contamination.

3. RESULTS

3.1. Comparison of sediment and copepod microbiome

Chloroplast sequences in sediments accounted for an average (±SD) of 23 ± 9% of total reads over the sampling campaigns, with the exception of an increase in July 2023 (65 ± 11%). In the guts of *Platychelipus littoralis* (Fig. 1), chloroplast reads were <1% for all except one gut sample. The guts were voided of any plant detritus or algae after 24 h of starvation, which corresponded to preliminary observations (Text S1, Figs. S1 & S2).

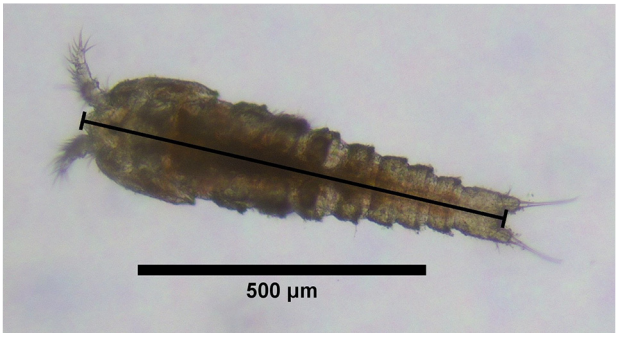

Fig. 1. The benthic harpacticoid copepod *Platychelipus littoralis* used in this study. Copepod length was determined from the top of the cephalothorax to the end of the telson using ImageJ

Sediments contained a wide range of bacterial phyla over the sampling time points (Fig. S3), with *Proteobacteria* being most abundant (34 ± 4% of chloroplast-filtered reads per sampling campaign), followed by *Actinobacteria* (16 ± 5%), *Bacteroidota* (15 ± 3%) and *Desulfobacterota* (11 ± 2%). *Cyanobacteria* were only observed in May 2023 (15 ± 2%) and September 2023 (4 ± 5%) and represented <1% during the rest of the year. Sediments contained a low variance presence of the bacterial families *Flavobacteriaceae* (10 ± 2%) and *Ilumatobacteraceae* (9 ± 2%) throughout the year. Along with *Bacteria*, *Archaea* were present at all sampling time points (Fig. S4).

The copepod gut microbiome consisted mainly of *Proteobacteria* (65 ± 20%), *Actinobacteria* (13 ± 8%) and *Bacteroidota* (15 ± 14%). *Desulfobacterota* were present at <2%. At the family level, copepod gut microbiomes showed high variability across sampling campaigns (Fig. 2). An extra sampling effort in July 2023 and September 2023 revealed high within-species variability. *Holosporaceae* were detected in half the samples of November 2022, July 2023 and September 2023, with relative abundances >45%. The presence of *Archaea* in copepod guts was checked in the January—May 2024 samples and not further analysed, as no positive PCR results were obtained.

Copepod gut and incubation water microbiomes had lower species richness and evenness compared to the sediment microbiome (Wilcoxon rank test for all alpha diversity indices, $p < 0.001$; Fig. 3). Species richness and evenness did not differ between the gut microbiomes of 24 and 48 h starved copepods. The PCoA (Bray-Curtis) indicated a difference in community composition between sediment and gut microbiomes (Fig. 4). PERMANOVA revealed significant differences between the groups of incubation water, 24 h starved gut, 48 h starved gut and sediment ($F_{3,57} = 6.3$, $p < 0.001$, $R^2 = 0.26$). Significant within-group dispersion was also detected (PERMDISP, $p < 0.001$), with dispersion for sediments being smaller than other groups (post hoc Tukey multiple comparison, $p < 0.001$ for all combinations with the group sediment). Pairwise PERMANOVAs showed significant differences between all groups except the gut microbiomes. Communities were different for sediment and the 24 h starved gut microbiome ($F_{1,29} = 11.4$, $p < 0.001$, $R^2 = 0.29$) and

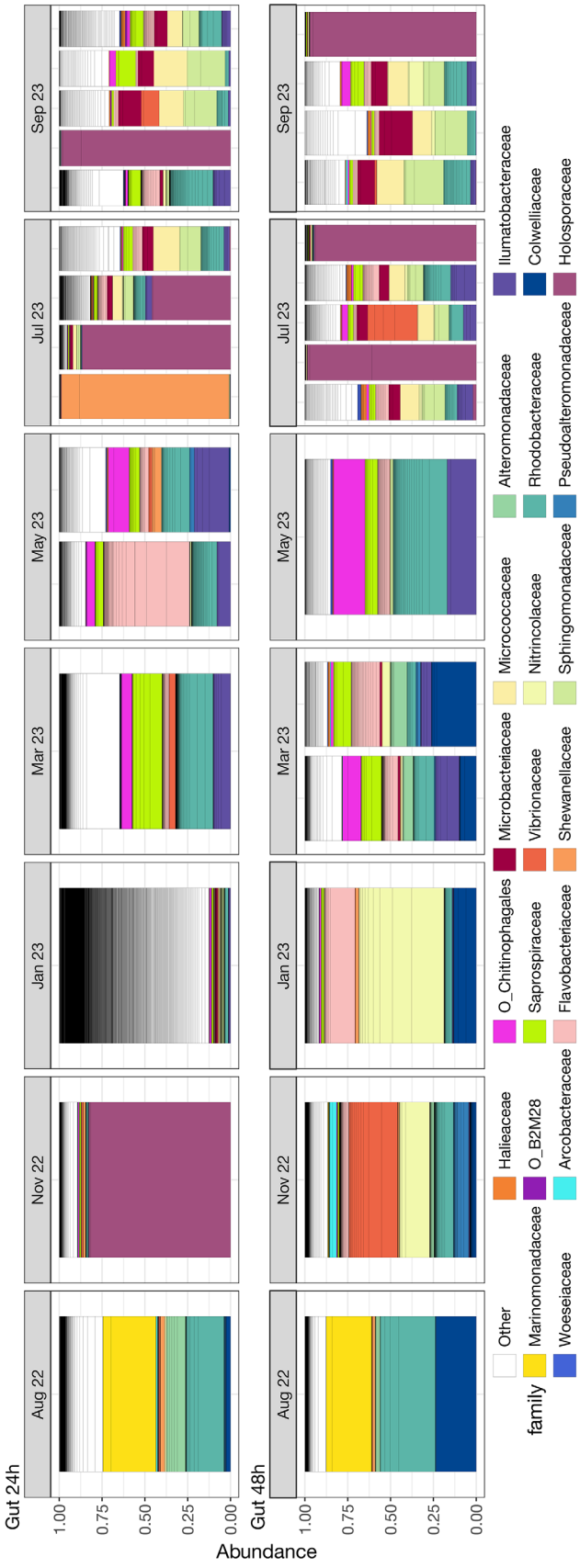

Fig. 2. Relative abundance of amplicon sequence variants (ASVs) identified at the family level from grouped copepod gut microbiomes (10 guts grouped per sample; n = 1–2 for August–May 2023 and n = 4–5 for July 2023 and September 2023 to allow observations on within-species variability)

the 48 h starved gut microbiome ($F_{1,29}$ = 12.1, p < 0.001, R^2 = 0.30).

Core taxa were defined at the family level including amplicon sequence variants (ASV) identified as core taxa at the genus level. In the 24 h gut community, core members belonged to *Rhodobacteraceae* (including ASV80), *Shewanellaceae* (including *Shewanella*), *Saprospiraceae*, *Ilumatobacteraceae* (including *Ilumatobacter*) and *Flavobacteriaceae*. In the gut 48 h starved gut community, identified core members belonged to *Rhodobacteraceae* (including ASV64), *Colwelliaceae* (including *Colwellia*), *Saprospiraceae* and *Flavobacteriaceae*.

The most abundant phylum in the incubation water was *Proteobacteria* (77 ± 22%), together with *Bacteroidota* (10 ± 7%). *Campylobacterota* showed high relative abundance (62 ± 34%) only in samples from September 2023.

The genus *Colwellia* was negligible in 24 h gut microbiomes (0.5 ± 1%). The genus increased to 15 ± 9% abundance in 48 h gut microbiomes for half of the sampling time points (August 2022–March 2023), while remaining negligible in the other samples. In the incubation water, the genus reached 40 ± 8% abundance for half of the samples (November 2022–May 2023) while being negligible in the other samples.

3.2. Microbial cell counts

The copepod gut microbiome was quantified by flow cytometry. Microbial cells were also counted in the seawater used during copepod starvation to qualify microbial growth on egested faecal pellets. The copepod gut microbiome showed multiple log variations in cell counts throughout the sampling period (Fig. 5). On average (±SD), a copepod gut contained 2 ± 4 × 10^5 cells per gut. The average gut length was 563 ± 88 µm, resulting in a cell density of 4 ± 7 × 10^5 cells mm^{-1} gut surface and 1 ± 3 × 10^{11} cells ml^{-1} gut volume.

A significant difference in cell counts between the 24 and 48 h starved gut microbiome was detected only for March 2023 (p < 0.001). After 24 and 48 h star-

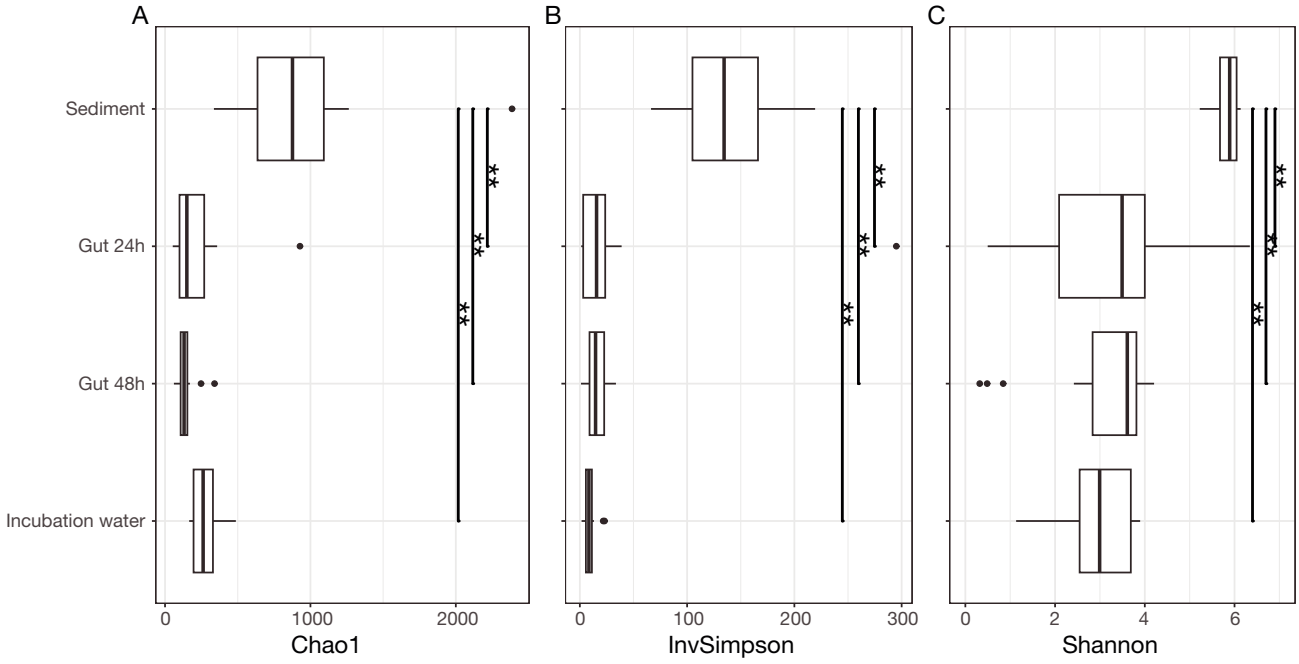

Fig. 3. Alpha diversity indices of the sediment microbiome, the 24 and 48 h starved copepod gut microbiome and the incubation water. Boxplots indicate first quartile, median and third quartile; whiskers indicate minima and maxima, with dots as outliers. Sediments had higher alpha diversity values compared to 24 and 48 h starved guts and the incubation water, indicated by asterisks (Wilcoxon rank test, $p < 0.001$)

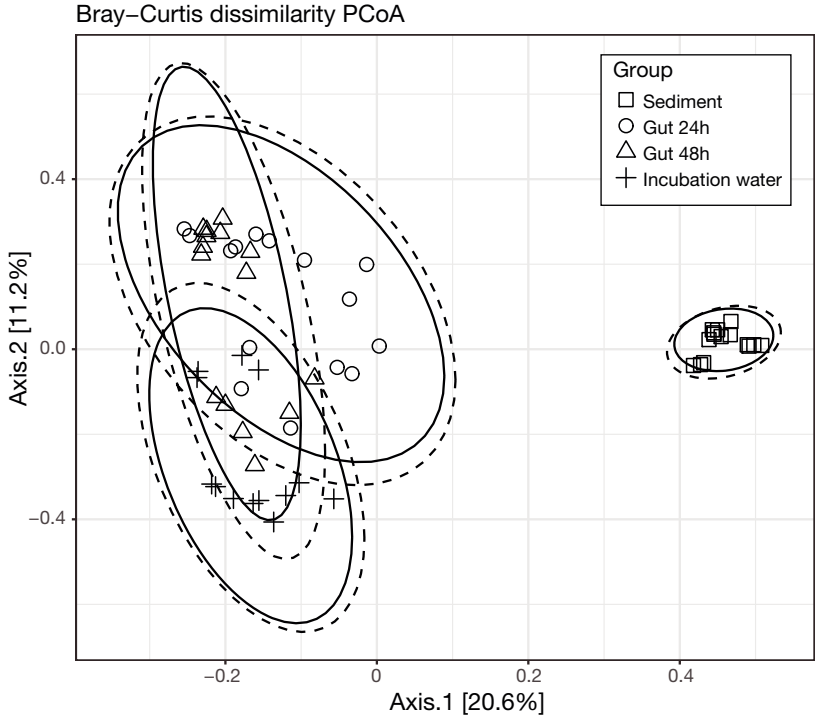

Fig. 4. Bray-Curtis dissimilarity principal coordinates analysis (PCoA) of the sediment microbiome, the 24 and 48 h starved copepod gut microbiome and the incubation water. Dashed line: normal distribution; solid line: *t*-distribution. PERMANOVA showed a significant difference between sediment and the 24 h gut microbiome ($p < 0.001$, $R^2 = 0.29$)

vation, bacterial cell growth in the incubation water reached densities of $10^4 - 10^6$ cells ml^{-1}.

3.3. Fatty acid content

Total FA content showed a seasonal trend throughout the sampling period, related to the expected seasonality in water temperature at the field location (Fig. 6). Total FA content reached a maximum at the end of winter (329 ± 15 µg ind.$^{-1}$, March 2023), containing 2.2 times more FAs compared to summer (150 ± 10 µg ind.$^{-1}$, July 2023). Biomarkers for diatoms accounted for the largest share of FAs, with 16 ± 6% of total FAs throughout the year for the specific marker 16:1 n-7, and 41 ± 5% when including other potential diatom FAs. Input from bacteria (4 ± 2%) and terrestrial green algae (0.8 ± 0.3%) were low. Flagellate input was low for the specific marker

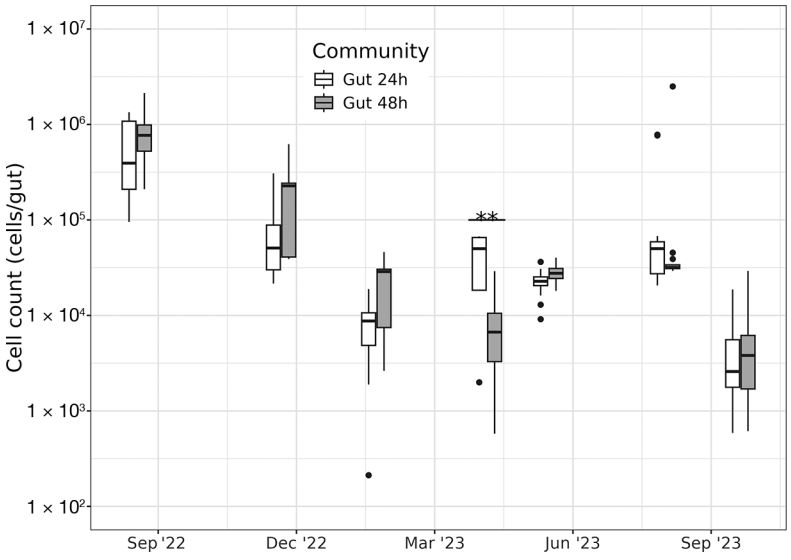

Fig. 5. Bacterial cell counts of the 24 and 48 h starved copepod gut microbiome throughout the sampling period of 1 yr. Boxplots indicate first quartile, median and third quartile; whiskers indicate minima and maxima, with dots as outliers; asterisks indicate significant differences ($p < 0.001$) between the 24 and 48 h conditions

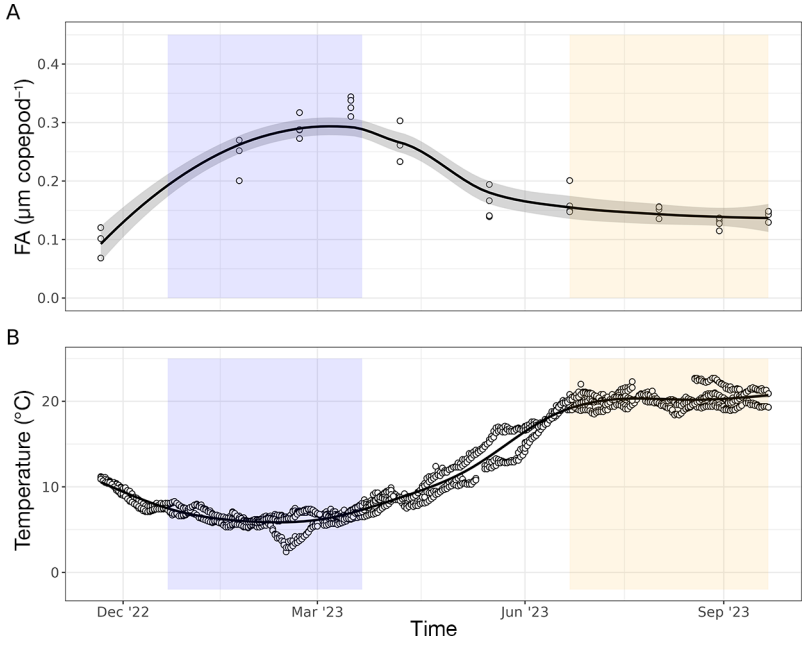

Fig. 6. Absolute fatty acid (FA) content in copepods (shadow: 95% CI for the modelled fit) and water temperature in the field over the sampling period. Winter (blue) and summer season (orange) are indicated. Water temperature is shown for the past 3 yr (21 November 2020–20 September 2023, see Section 2.1)

18:4 n-3 (0.6 ± 0.7%) and considerably lower (9 ± 4%) than diatom input when including other potential flagellate biomarkers.

4. DISCUSSION

4.1. Gut microbiome

The copepod species *Platychelipus littoralis* hosted on average (±SD) $2 \pm 4 \times 10^5$ microbial cells per gut. This translates to $4 \pm 7 \times 10^5$ cells mm^{-1} gut surface or $1 \pm 3 \times 10^{11}$ cells ml^{-1} gut volume. This is similar to cell densities reported for other copepod species; e.g. in the gut of the pelagic copepod *Pleuromamma* sp., with $1.1 \pm 0.7 \times 10^5$ cells mm^{-1} gut surface (Shoemaker & Moisander 2017) and bacterial densities of 10^7–10^{11} cells ml^{-1} body volume (Tang et al. 2010). *P. littoralis* inhabits intertidal mudflats where anoxia is quickly reached, and sulfate reduction is an important process. Sulfate reduction is determined by organic matter content and temperature (Stal 2016). However, in the sediment microbiomes, *Desulfobacterota* had a constant presence (11 ± 2%) throughout the year. The benthic copepod gut is a selective microhabitat with lower species richness and evenness compared to sediment. This includes the presence of *Desulfobacterota* at a maximum of 2% in guts. *Archaea* were also present in the sediment but they were not detected in gut samples. Although the sediment had higher bacterial diversity than copepod guts, community composition and richness in the gut were more variable with sampling time. However, no difference was found between 24 and 48 h starved guts in overall cell counts and composition. Our original hypothesis was rejected and prolonged egestion was excluded as a stressor for the bacterial community. The egestion of gut microorganisms is likely accounted for by regrowth. Reingestion of faecal pellets might introduce bacteria, and it would be ideal to have observation data for the ingestion of individuals as complementary information to our experiments. However, that was not feasible in the framework of the present study. A multi-year seasonal study on the guts of the pelagic copepod *Pleuro-*

mamma sp. showed quite stable cell counts, whereas there was seasonal variability in the composition of grouped gut microbiomes (Shoemaker & Moisander 2017). Copepod feeding behaviour and gut characteristics can be generally selective towards a specific gut microbiome, independent of the environment in which the copepod occurs, whether it is sediment or the water column.

4.2. Environmental drivers of the microbiome

In sediments, certain taxa had a year-round presence (*Ilumatobacteraceae* and *Flavobacteriaceae*) while others fluctuated throughout the year (O_B2M28, *Cyanobacteria*, *Rhodobacteraceae*). Intertidal mudflats are usually inhabited by benthic diatoms. These motile diatoms produce a biofilm with exopolysaccharides, which provides bacteria with a carbon and energy source (Stal 2016). However, as diatoms have a patchy distribution, the exopolysaccharides are also irregularly distributed at both the macro and micro scales. The observed within-species variability in copepod gut microbiomes at the sampling time points of July 2023 and September 2023 might have been caused by this patchiness in diatoms and their associated microbiome.

Diatom seasonality is largely controlled by temperature (Stal 2016). In spring, increased temperature and daylight allow for diatom population growth. Blooms can be observed as temperature and desiccation limit growth again in summer (Sahan et al. 2007, Schnurr et al. 2020). Diatom biomarkers continuously accounted for the highest percentage of total FAs of our copepod samples. This indicates that copepods were mainly and continuously feeding on diatoms throughout the year even though FA content was higher in winter than in summer. Continuous feeding on diatoms and their associated complex biofilms are likely to be a driver for gut microbiome composition. The presence of *Holosporaceae*, a known endosymbiont of protists (Santos & Massard 2014), indicated that protists were an unquantified portion of the copepod diet. Sequencing of the gut's eukaryotic community would be needed to clarify the copepods' full prokaryotic and eukaryotic dietary preferences.

4.3. Core taxa

Core taxa were slightly different for 24 and 48 h starved guts. We identified 3 core taxa that remained present in the gut microbiome throughout 48 h of egestion. These were also found among shared core taxa of marine copepods from previous studies: *Rhodobacteraceae* in *Acartia*, *Calanus*, *Sinocalanus* and *Pseudodiaptomus*; *Flavobacteriaceae* in *Calanus*, *Pleuromamma* and *Temora*; and *Saprospiraceae* in *Temora* (Moisander et al. 2015, Shoemaker & Moisander 2015, Cregeen 2016, Dorosz et al. 2016, Shoemaker & Moisander 2017, Wäge et al. 2019, Sadaiappan et al. 2021, Velasquez et al. 2023): (1) *Rhodobacteraceae* were reported as being associated with coral, sponges and microalgae (De Corte et al. 2018). This family was also identified as a transient core taxon for 3 brackish copepod species (Chae et al. 2021). (2) *Flavobacteriaceae* are known for their potential to degrade high molecular weight organic matter (Wäge et al. 2019, Velasquez et al. 2023). De Corte et al. (2018) characterized the microbial community associated with zooplankton in the North Atlantic and their metabolic potential. A high number of glycosyl hydrolase encoding genes were associated with the *Flavobacteria* clade and suggested the metabolization of polysaccharides and amino sugars (De Corte et al. 2018). Both *Rhodobacteraceae* and *Flavobacteriaceae* are consistently associated with diatoms (Helliwell et al. 2022). (3) *Saprospiraceae* are known to hydrolyse and use complex carbon sources (McIlroy & Nielsen 2014). Continuous feeding of the copepods on diatoms and the metabolic potential of the core taxa suggests a potential role in food-assisted degradation for gut bacteria. However, there is likely turnover on lower taxonomic levels, as specifically observed for *Rhodobacteraceae*. This suggests that species-specific bacterial interactions are hard to predict.

5. CONCLUSIONS

This study provided a characterization of the gut microbiome of *Platychelipus littoralis*, a key benthic copepod species in western European intertidal mudflats. The copepod gut was shown to be a selective microhabitat, with lower species richness and evenness compared to the surrounding sediment. Gut microbiome cell counts and composition varied with time, while starvation up to 48 h was eliminated as a stressor for the gut community.

The copepod species showed seasonal changes in food availability by varying FA content. However, diatoms were the main and consistent food source. The core taxa *Rhodobacteraceae*, *Flavobacteriaceae* and *Saprospiraceae* were identified and are known degraders of complex organics. They are likely intro-

duced through the diet and could play a role in the microbial-assisted degradation of food. This study further elucidated bacteria–copepod interactions, relevant for the food web ecology of benthic systems and potentially the optimization of copepod culturing.

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LITERATURE CITED

Baguley JG, Coull BC, Chandler GT (2019) Meiobenthos. In: Cochran JK, Bokuniewicz HJ, Yager PL (eds) Encyclopedia of ocean sciences, 3rd edn, Vol 1: Marine biogeochemistry. Elsevier, Amsterdam, p 742–748

Bass D, Rueckert S, Stern R, Cleary AC, Taylor JD, Ward GM, Huys R (2021) Parasites, pathogens, and other symbionts of copepods. Trends Parasitol 37:875–889

Boxshall GA, Halsey SH (2004) An introduction to copepod diversity. Ray Society, Andover

Brucet S, Compte J, Boix D, López-Flores R, Quintana XD (2008) Feeding of nauplii, copepodites and adults of *Calanipeda aquaedulcis* (Calanoida) in Mediterranean salt marshes. Mar Ecol Prog Ser 355:183–191

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 13: 581–583

Carli A, Pane L, Casareto L, Bertone S, Pruzzo C (1993) Occurrence of *Vibrio alginolyticus* in Ligurian coast rock pools (Tyrrhenian Sea, Italy) and its association with the copepod *Tigriopus fulvus* (Fisher 1860). Appl Environ Microbiol 59:1960–1962

Chae YJ, Oh HJ, Chang KH, Kwak IS, Jo H (2021) Application of next-generation sequencing for the determination of the bacterial community in the gut contents of brackish copepod species (*Acartia hudsonica*, *Sinocalanus tenellus*, and *Pseudodiaptomus inopinus*). Animals 11:542

Coull BC (1999) Role of meiofauna in estuarine soft-bottom habitats. Austral Ecol 24:327–343

Couturier LIE, Michel LN, Amaro T, Budge SM and others (2020) State of art and best practices for fatty acid analysis in aquatic sciences. ICES J Mar Sci 77:2375–2395

Cregeen SJJ (2016) Microbiota of dominant Atlantic copepods: *Pleuromamma* sp. as a host to a betaproteobacterial symbiont. PhD dissertation, University of Southampton

Datta MS, Almada AA, Baumgartner MF, Mincer TJ, Tarrant AM, Polz MF (2018) Inter-individual variability in copepod microbiomes reveals bacterial networks linked to host physiology. ISME J 12:2103–2113

De Corte D, Srivastava A, Koski M, Garcia JAL and others (2018) Metagenomic insights into zooplankton-associated bacterial communities. Environ Microbiol 20:492–505

De Troch M, Cnudde C, Willems A, Moens T, Vanreusel A (2010) Bacterial colonization on fecal pellets of harpacticoid copepods and on their diatom food. Microb Ecol 60: 581–591

De Troch M, Boeckx P, Cnudde C, Van Gansbeke D, Vanreusel A, Vincx M, Caramujo MJ (2012) Bioconversion of fatty acids at the basis of marine food webs: insights from a compound-specific stable isotope analysis. Mar Ecol Prog Ser 465:53–67

Dorosz JNA, Castro-Mejia JL, Hansen LH, Nielsen DS, Skovgaard A (2016) Different microbiomes associated with the copepods *Acartia tonsa* and *Temora longicornis* from the same marine environment. Aquat Microb Ecol 78:1–9

El-Sabaawi R, Dower JF, Kainz M, Mazumder A (2009) Characterizing dietary variability and trophic positions of coastal calanoid copepods: insight from stable isotopes and fatty acids. Mar Biol 156:225–237

Fleeger JW (2007) The potential to mass-culture harpacticoid copepods for use as food for larval fish. In: Le CS, O'Bryen PJ, Marcus NH (eds) Copepods in aquaculture. Blackwell Publishing, Ames, IA, p 11–24

Gantner S, Andersson AF, Alonso-Sáez L, Bertilsson S (2011) Novel primers for 16S rRNA-based archaeal community analyses in environmental samples. J Microbiol Methods 84:12–18

Gentsch E, Kreibich T, Hagen W, Niehoff B (2009) Dietary shifts in the copepod *Temora longicornis* during spring: evidence from stable isotope signatures, fatty acid biomarkers and feeding experiments. J Plankton Res 31: 45–60

Helliwell KE, Shibl AA, Amin SA (2022) The diatom microbiome: new perspectives for diatom–bacteria symbioses. In: Falciatore A, Mock T (eds) The molecular life of diatoms. Springer International Publishing, Cham, p 679–712

Iversen MH, Poulsen LK (2007) Coprorhexy, coprophagy, and coprochaly in the copepods *Calanus helgolandicus*, *Pseudocalanus elongatus*, and *Oithona similis*. Mar Ecol Prog Ser 350:79–89

Jacobsen TR, Azam F (1984) Role of bacteria in copepod fecal pellet decomposition: colonization, growth rates and mineralization. Bull Mar Sci 35:495–502

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41:e1

McIlroy SJ, Nielsen PH (2014) The Family *Saprospiraceae*. In: Rosenberg E, DeLong EF, Lory S, Stackenbrandt E, Thompson F (eds) The prokaryotes: other major lineages of *Bacteria* and the *Archaea*. Springer, Berlin, p 863–889

Moisander PH, Sexton AD, Daley MC (2015) Stable associations masked by temporal variability in the marine copepod microbiome. PLOS ONE 10:e0138967

Møller EF, Riemann L, Søndergaard M (2007) Bacteria associated with copepods: Abundance, activity and community composition. Aquat Microb Ecol 47:99–106

Oh HN, Myeong NR, Kim T, Min GS, Kim S, Sul WJ (2022) Changes in fecal pellet microbiome of the cold-adapted Antarctic copepod *Tigriopus kingsejongensis* at different

temperatures and developmental stages. Microb Ecol 84: 1029–1041
Peter H, Sommaruga R (2008) An evaluation of methods to study the gut bacterial community composition of freshwater zooplankton. J Plankton Res 30:997–1006
Quast C, Pruesse E, Yilmaz P, Gerken J and others (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590–D596
Sadaiappan B, PrasannaKumar C, Nambiar VU, Subramanian M, Gauns MU (2021) Meta-analysis cum machine learning approaches address the structure and biogeochemical potential of marine copepod associated bacteriobiomes. Sci Rep 11:3312
Sahan E, Sabbe K, Creach V, Hernandez-Raquet G, Vyverman W, Stal LJ, Muyzer G (2007) Community structure and seasonal dynamics of diatom biofilms and associated grazers in intertidal mudflats. Aquat Microb Ecol 47:253–266
Sahandi J, Sorgeloos P, Xiao H, Mu F and others (2023) The use of microbes in culture of harpacticoid copepod (*Tigriopus japonicus* Mori) for suppression of *Vibrio* and enhancement of population growth, enzyme activity and microbial longevity. Aquaculture 563:739008
Santos HA, Massard CL (2014) The Family *Holosporaceae*. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (eds) The prokaryotes: *Alphaproteobacteria* and *Betaproteobacteria*. Springer, Berlin, p 237–246
Schnurr PJ, Drever MC, Elner RW, Harper J, Arts MT (2020) Peak abundance of fatty acids from intertidal biofilm in relation to the breeding migration of shorebirds. Front Mar Sci 7:63
Shoemaker KM, Moisander PH (2015) Microbial diversity associated with copepods in the North Atlantic subtropical gyre. FEMS Microbiol Ecol 91:fiv064
Shoemaker KM, Moisander PH (2017) Seasonal variation in the copepod gut microbiome in the subtropical North Atlantic Ocean. Environ Microbiol 19:3087–3097
Skovgaard A, Castro-Mejia JL, Hansen LH, Nielsen DS (2015) Host-specific and pH-dependent microbiomes of copepods in an extensive rearing system. PLOS ONE 10: e0132516
Stal LJ (2016) Coastal sediments: transition from land to sea. In: Stal LJ, Cretoiu MS (eds) The marine microbiome: an untapped source of biodiversity and biotechnological potential. Springer, Cham, p 283–304
Tang KW (2005) Copepods as microbial hotspots in the ocean: effects of host feeding activities on attached bacteria. Aquat Microb Ecol 38:31–40
Tang KW, Turk V, Grossart HP (2010) Linkage between crustacean zooplankton and aquatic bacteria. Aquat Microb Ecol 61:261–277
Tang KW, Glud RN, Glud A, Rysgaard S, Nielsen TG (2011) Copepod guts as biogeochemical hotspots in the sea: evidence from microelectrode profiling of *Calanus* spp. Limnol Oceanogr 56:666–672
Thor P, Koski M, Tang KW, Jónasdóttir SH (2007) Supplemental effects of diet mixing on absorption of ingested organic carbon in the marine copepod *Acartia tonsa*. Mar Ecol Prog Ser 331:131–138
Velasquez X, Morov AR, Belkin N, Kurt TT and others (2023) Feeding strategy and dietary preference shape the microbiome of epipelagic copepods in a warm nutrient-impoverished ecosystem. Environ DNA 5:38–55
Wäge J, Strassert JFH, Landsberger A, Loick-Wilde N and others (2019) Microcapillary sampling of Baltic Sea copepod gut microbiomes indicates high variability among individuals and the potential for methane production. FEMS Microbiol Ecol 95:fiz024

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