Bacterial Colonization on Fecal Pellets of Harpacticoid Copepods and on Their Diatom Food
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Bacterial Colonization on Fecal Pellets of Harpacticoid Copepods and on Their Diatom Food

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Abstract Fecal pellets make up a significant fraction of the global flux of organic matter in oceans, and the associated bacterial communities in particular are a potential food source for marine organisms. However, these communities remain largely unknown. In the present study, the bacterial communities on fecal pellets of the benthic copepod *Paramphiascella fulvofasciata* feeding on the diatoms *Navicula phyllepta* and *Seminavis robusta* were analyzed. The aim of this study was to characterize the bacterial communities associated with the diatoms and the fecal pellets by means of DGGE profiling. Furthermore, isolated bacteria were characterized by means of partial 16S rRNA gene sequencing. The composition of the bacterial microflora on fecal pellets was studied in terms of the effect of the original food source, the age of the fecal pellets and the copepod's identity. Alphaproteobacteria, Flavobacteria, and Bacilli were found on the fecal pellets; whereas on diatoms, exclusively Gammaproteobacteria were identified. Especially after eating *N. phyllepta*, there was an important increase in bacterial diversity, although the diatom *N. phyllepta* harbored a less diverse bacterial community than *S. robusta*. Our data suggest that the additional bacteria originate from the copepod's digestive tract and largely depends on the initial food source.

Introduction

The traditional view of the marine food web depicts bacteria and zooplankton as separate units, indirectly connected via nutrient recycling (the 'microbial loop') and trophic cascade processes [1]. In addition, several recent studies have demonstrated that zooplankton and bacteria are directly connected [2–4]. This direct connection is mainly physical, e.g. bacteria are attached to zooplankton bodies with an active exchange of bacteria between copepods and their environment [3]. Indirect connections are mainly biological, e.g. zooplankton feeding supports bacterial growth through excretions [5]. Planktonic copepods (order Calanoida), in particular, produce dissolved organic matter by sloppy feeding, excretion, and leakage from fecal pellets [3]. Copepod fecal pellets in the water column rapidly become colonized by free-living bacteria [6].

Fecal pellets mostly contribute to the vertical flux of particulate material [7–9], representing often more than 85% of the average total sedimenting carbon [10] and represent a substantial substrate for bacteria. These bacteria in turn form a potential food source for a variety of organisms. Fecal material and associated bacteria reaching the seafloor may also provide food to benthic species [11]. Even during sinking, active reworking of fecal pellets by copepods in the water column has been observed, resulting in an increase in the pelagic residence time of particles, in the substrate area available for aerobic microbes, and presumably also in the remineralization rate of particulate organic matter [2]. In 2005, a conceptual model of a balance between
bacterial growth stimulated by the host’s feeding and bacterial loss through the host’s defecation was proposed [12]. To date, however, little is known about the structure of the bacterial communities that colonize copepod fecal pellets.

Most studies have hitherto focused on planktonic copepods (order Calanoida), their fecal pellets, and interactions with bacteria. Benthic copepods (mainly belonging to the order Harpacticoida) live in close contact with their (fresh) fecal material on the seafloor. *Paramphiascella fulvofasciata* frequently handles its own fecal pellets and seems to graze upon them (De Troch, personal observation). Moreover, we recently found that this harpacticoid species accelerates its fecal pellet production when fecal pellets are removed regularly [13]. These results suggest that this harpacticoid copepod needs fecal pellets in its vicinity and probably uses the bacterial community associated with them as an additional food source (see also [14]).

Therefore, the characterisation of the bacteria growing on fecal pellets is important not only for determining their functional roles in organic matter mineralization from fecal pellets, but also for our understanding of intricate trophic pathways at the base of marine benthic food webs.

In this study, we have characterized the bacterial communities associated with the food sources (diatoms) of harpacticoid copepods, and have compared them to the communities growing on copepod fecal pellets in terms of diversity and community composition. For planktonic copepods, it was previously documented that the bacterial communities on fecal pellets originate from the environment [6]. In addition, we tested whether the composition of the fecal pellets (as determined by the original food source), their age, and the species identity of the producers of the fecal pellets affected the associated bacterial communities.

### Material and Methods

#### Stock Culture of Diatoms and Harpacticoid Copepods

Laboratory stock monocultures of the copepod species *P. fulvofasciata* (Harpacticoida, family Miraciidae) were maintained in 1 L glass beakers, in artificial seawater (c. 32 psu, Instant Ocean® salt, Aquarium Systems, France; see also [15]). They were regularly provided with a mixture of non-axenic benthic diatoms from lab cultures (see below). Mixed cultures of various copepod species were maintained under the same conditions.

Monoclonal cultures of the epipelic pennate diatoms *Seminavis robusta* and *Navicula phylepta*, hereafter referred to as *Seminavis* and *Navicula*, respectively, were used as primary food sources for the harpacticoid copepods. The *Seminavis* clones were originally isolated in November 2000 from the ‘Veerse Meer’, a brackish-water lake in Zeeland, The Netherlands [16]. The cells measured 55.89±0.94 µm in length (strain 84A, diatom culture collection of the Laboratory of Protozoology and Aquatic Ecology, Ghent University, Belgium). Monoclonal *Navicula* cultures were isolated from sediment from intertidal mudflats in the IJzer estuary (Nieuwpoort, Belgium) and the cells were on average 16.33±0.26 µm long.

Both diatom species were grown non-axenically in f2 culture medium [17] based on filtered (Schleicher & Schuell No. 597) and autoclaved artificial seawater (32 psu). Copepod and diatom cultures were kept at 17±1°C under a 12:12 h light-dark regime and with an irradiance of 25-50 µmol photons m$^{-2}$s$^{-1}$. Duplicate samples of the original diatom cultures were collected in order to investigate the bacteria associated with the food sources offered to the grazers.

In addition to the stock cultures, a grazing experiment was set up (see [13]) by picking up single copepod specimens with a micropipette from the stock cultures and placing them into Petri dishes containing artificial seawater where they were starved of food overnight. After starving, the copepods were transferred into small Petri dishes (polystyrene, surface area=21.2 cm$^2$) filled with 20 ml of artificial seawater (30-32 psu) that were placed at random on a shelf under the same controlled conditions as outlined above for stock cultures.

Each experimental unit received 20 adult copepods (*P. fulvofasciata*), which were allowed to graze on either *Seminavis* or *Navicula* as described previously [13].

### Fecal Pellets

Fecal pellets were picked up one-by-one from the experimental units or the stock cultures depending on the fecal pellet type (see further), rinsed in autoclaved artificial seawater (30-32 psu) and gathered in duplicate batches of 100 pellets each in Eppendorf tubes. One series served immediately for the isolation of bacterial strains and a second series (stored at −20°C) for culture-independent bacterial community analysis using denaturing gradient gel electrophoresis (DGGE).

The following types of copepod fecal pellets were collected in order to test the effect of content (original food source; fecal pellet types 1-3), age (fecal pellet type 4), content × age (fecal pellet type 5) and copepod species (fecal pellet type 6):

1. Fecal pellets (*Seminavis*): fresh fecal pellet (1-2 days old) from *P. fulvofasciata* having grazed on the diatom *Seminavis*;
2. Fecal pellets (*Navicula*): fresh fecal pellet (1-2 days old) from *P. fulvofasciata* having grazed on the diatom *Navicula*;

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(3) fecal pellets (*Navicula*+fecal pellets other copepod species): fresh fecal pellet (1-2 days old) from *P. fulvofasciata* having grazed on the diatom *Navicula* and additional fecal pellets produced by various harpacticoid copepod species from the Paulina salt marsh, present in mixed-species cultures;

(4) old fecal pellets (*Seminavis*): old fecal pellets (>3 days old) from *P. fulvofasciata* having grazed on *Seminavis*;

(5) old fecal pellets (*Seminavis*+*Escherichia coli*): old fecal pellets (>3 days old) from *P. fulvofasciata* having grazed on *Seminavis* and frozen-and-thawed *E. coli* (strain K12, BCCM/LMG collection, UGent), collected from the stock cultures;

(6) fecal pellets other copepod species: fresh fecal pellets (1-2 days old) produced by various harpacticoid copepod species from the Paulina salt marsh, collected from mixed-species stock cultures.

On the one hand, fecal pellet types 1, 2, 3, and 4 from *P. fulvofasciata* were collected from small Petri dishes, while the fecal pellet types 5 and 6 were collected from stock cultures (jars of 1 L). While treatments 1 and 2 tested the effect of food types, treatment 3 assessed whether the presence of fecal pellets of other copepod species grown together in one stock culture (jar of 1 L; fecal pellet type 6) were analyzed. Unfortunately, the fact that they were fed a variety of diatoms for several weeks implied a potential additional factor of variance.

Culturing and Isolation of Bacteria

For the isolation of bacteria, samples were emulsified using a vortex mixer and serially diluted (10⁻¹ to 10⁻³) in a sterile (0.2 µm filtered) saline solution. From each dilution, an aliquot was spread onto ‘Marine Agar’ plates, containing 37.4 g/l Marine Broth (DIFCO) and 20 g/l Bacto agar (DIFCO), and incubated at 16-18°C for 5 days. In total, 150 pure isolates originating from fecal pellets and diatoms were preserved at −80°C in Marine Broth (DIFCO) with addition of 15% glycerol.

Template DNA Preparation of Bacteria

DNA was extracted through alkaline lysis [18]. For the analysis of total bacterial communities on fecal pellets and diatoms, a fourfold concentrated DNA extract was made by reducing the amounts of lysis buffer and Milli-Q water. DNA extracts were stored at −20°C.

Culture-independent Bacterial Community Analysis

Polymerase chain reaction (PCR)-DGGE was carried out on the 16S rDNA V3 region with the universal bacterial primer set 357f and 518r as described by [19]. A GC-clamp (5’-GCACGCGCCGCAGCGGACGCGCCCGCCCGCCGCCCGCCGCCGCCCGCCGCCGCCGCCGCCGAGCCGCCGCCGCCGCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGC⁴-3) [20] was coupled to the primer 357f. Each mixture contained primers at a concentration of 5 µM, each dNTP at a concentration of 2 mM, 250 ng of bovine serum albumin, 6 µl of 10× PCR buffer and 1.25 U Taq DNA polymerase (AmpliTaq, Applied Biosystems) and was adjusted to a final volume of 46 µl with sterile MilliQ water. Large amounts of template DNA (4 µl) were added. A touchdown PCR was performed with a Bio-Rad DNA thermal cycler as described by [21]. Subsequently, a second identical PCR reaction was conducted on the obtained products to reinforce band intensity.

DGGE analysis of the PCR amplicons of the fecal pellet and diatom samples was performed as described by [22]. Samples of 30 µl PCR product and 10 µl loading dye were loaded on a denaturing gradient gel of 35-70%. The gels were stained with SYBR gold (100 µL L⁻¹ 1× TAE buffer; Invitrogen, California, USA) followed by digital capturing of the profiles under UV light with a CCD Camera (Fotodyne Inc., Hartland) and Iris Video Digitize software (Inside Technology, Amersfoort).

Characterization and Identification of Bacterial Strains

For fast genomic fingerprinting of the isolated bacteria, repetitive extragenic palindromic PCR (rep-PCR) was carried out using the (GTG)₅-primer with its optimal PCR program [23, 24]. PCR master mix was prepared as described by [25] and amplifications were performed as described by [24] in a DNA thermal cycler Perkin-Elmer 9600 (Applied Biosystems, USA) using Goldstar DNA polymerase (Eurogentec, Belgium). Electrophoresis was performed as described previously [23]. Profiles were captured digitally as described above for DGGE.

Furthermore, a selection of isolates representing the clusters in the rep-PCR dendrogram (see data treatment) was subjected to 16S rDNA sequencing. The 16S rRNA gene was amplified using the conserved primers 16F27 and 16R1522 [26] as described previously [27]. PCR-amplified 16S rRNA genes were purified using the NucleoFast® 96 PCR Cleanup Kit (Macherey-Nagel). Sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and purified using the Montage™...
SEQ96 Sequencing Reaction Cleanup Kit (Millipore, Bedford, MA). A smaller fragment of the 16S rRNA gene (the first 500 bp approximately), was sequenced using the primer 16R516/BKL1 [14]. Sequencing was performed using an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems).

Data Treatment

Digitized DGGE and (GTG)5-PCR images were processed and analyzed with BioNumerics software (version 4.61, Applied Maths, Sint-Martens-Latem, Belgium).

To visualize differences in microbial community composition on diatoms, on fecal pellets and in artificial seawater, similarity between DGGE patterns was calculated by comparing the densitometric curves using the Jeffrey’s X correlation coefficient. The unweighted pair group method with arithmetic mean (UPGMA) was applied to calculate the dendrogram. Bacterial diversity of DGGE samples was quantified by means of the number of operational taxonomic units (OTU) in each sample corresponding to the number of DGGE bands. The DGGE profiles were analyzed by means of a non-metric multidimensional scaling using the PRIMER 5.0 software package [28] and based on square root transformations of the relative band intensities in order to trace relative changes in community structure.

Similarities between the (GTG)5 profiles of isolates were calculated using the Pearson correlation coefficient and visualized in mean linkage (UPGMA) dendrograms. Furthermore, a dendrogram incorporating all isolates was constructed using BioNumerics. Based on a similarity level of >60% using the Pearson correlation coefficient, 18 (GTG)5-PCR clusters (A-R) were defined. Of each cluster, one isolate was identified through partial 16S rRNA gene sequencing. Partial 16S rRNA gene sequences were compared with the EMBL database using the BLAST algorithms.

Results

Bacteria Associated with Diatoms

The bacterial communities on the two diatom species differed in terms of diversity as indicated by the number and intensity of the bands on the DGGE gel (Fig. 1). Navicula harbored a less diverse bacterial community (4 OTUs), while Seminavis showed a more diverse community (15 OTUs). The large number of unclustered isolates (Fig. 2a) illustrates the richness and distinctiveness of the Seminavis bacterial assemblage.

Both diatom species were characterized by specific bacterial strains with ≤40% rep profile similarity (Fig. 2a). Only one strain of Seminavis showed a 90% similarity with Navicula strains and clustered together with 3 Navicula isolates (cluster C, see supplemental material, Tables 1 and 2). These strains were identified as Marinobacter sp. based on 16S rRNA gene sequencing (Tables 1 and 2). Other bacterial strains isolated from Navicula were identified as belonging to the genera Alteromonas, Pseudoalteromonas, and Roseobacter (Table 2).

Bacteria Associated with Fecal Pellets vs. Diatoms

The DGGE analysis of the total bacterial community on Seminavis and on the fecal pellets produced by P. fulvofasciata after grazing on Seminavis (Fig. 1, two lower lanes) showed more than 60% similarity in the dendrogram and a comparable bacterial diversity in terms of number of sources, with fecal pellets (fp) after consumption of Seminavis (fp (Seminavis)) or Navicula (fp (Navicula)) and with fp produced by other copepod species. Artificial seawater was used as a control.

Figure 1 Dendrogram (UPGMA) based the Pearson correlation of DGGE fingerprints of PCR-amplified 16S rRNA gene (V3 region) of bacteria associated with the diatoms Seminavis en Navicula (food sources), with fecal pellets (fp) after consumption of Seminavis (fp (Seminavis)) or Navicula (fp (Navicula)) and with fp produced by other copepod species. Artificial seawater was used as a control.
Figure 2 Dendrograms (UPGMA) based on the Pearson correlation of the (GTG)5-PCR profiles of the pure cultures isolated from a the diatom species Seminavis and Navicula, b the diatom species Seminavis and the fecal pellets (fp) produced by *P. fulvofasciata* after grazing on this diatom (fp (Seminavis)), and c the diatom species Navicula and the fecal pellets (fp) produced by *P. fulvofasciata* after grazing on it (fp (Navicula)). The corresponding (GTG)5-PCR clusters are indicated between square brackets; NC not clustered. The asterisk in b represents the clusterpoint with maximum similarity between *Seminavis* and fp produced by *P. fulvofasciata* after grazing on it.
Table 1 Identification of the (GTG)₅-PCR clusters (see supplementary material) by means of partial 16S rRNA gene sequencing with indication of strain name, accession number, and the source of the isolates

<table>
<thead>
<tr>
<th>(GTG)₅-PCR cluster</th>
<th>Isolate (accession number)</th>
<th>Identification by partial 16S rRNA gene sequencing</th>
<th>Source of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CC58 (FN661957)</td>
<td>Ruegeria sp.</td>
<td>fp (Navicula), old fp (Seminavis)</td>
</tr>
<tr>
<td>B</td>
<td>CC74 (FN661962)</td>
<td>Alteromonas sp.</td>
<td>fp other copepod species, fp (Seminavis), fp (Navicula), old fp (Seminavis + E. coli), Navicula</td>
</tr>
<tr>
<td>C</td>
<td>CC85 (FN661965)</td>
<td>Marinobacter sp.</td>
<td>fp (Navicula), Navicula, Seminavis</td>
</tr>
<tr>
<td>D</td>
<td>CC71 (FN661960)</td>
<td>Alteromonas sp.</td>
<td>fp (Seminavis), old fp (Seminavis)</td>
</tr>
<tr>
<td>E</td>
<td>CC67 (FN661958)</td>
<td>Roseobacter sp.</td>
<td>fp (Navicula), fp (Seminavis)</td>
</tr>
<tr>
<td>F</td>
<td>CC73 (FN661961)</td>
<td>Pseudoalteromonas sp.</td>
<td>fp(Seminavis), fp (Navicula)</td>
</tr>
<tr>
<td>G</td>
<td>CC40 (FN661956)</td>
<td>Pseudoalteromonas sp.</td>
<td>fp (Navicula), old fp (Seminavis)</td>
</tr>
<tr>
<td>H</td>
<td>CC7 (FN661953)</td>
<td>Bacillus sp.</td>
<td>fp (Seminavis), fp other copepod species</td>
</tr>
<tr>
<td>I</td>
<td>CC87 (FN661966)</td>
<td>Sulfitobacter sp.</td>
<td>fp (Navicula), fp other copepod species, fp (Seminavis)</td>
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<td>Pseudomonas sp.</td>
<td>fp (Seminavis)</td>
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<td>CC26 (FN661954)</td>
<td>Joostella sp.</td>
<td>Old fp (Seminavis)</td>
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<td>CC84 (FN661964)</td>
<td>Pseudoalteromonas sp.</td>
<td>fp (Navicula), Navicula</td>
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<td>M</td>
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<td>Maribacter sp.</td>
<td>fp (Seminavis), fp (Sem.), old fp (Seminavis + E. coli), fp other copepod species</td>
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<td>N</td>
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<td>fp other copepod species</td>
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<tr>
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<td>Bacillus sp.</td>
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<td>Q</td>
<td>CC109 (FN661967)</td>
<td>Croceibacter sp.</td>
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<tr>
<td>NC</td>
<td></td>
<td></td>
<td>fp (Seminavis), fp (Navicula), old fp (Seminavis), old fp (Seminavis + E. coli), fp other copepod species, Seminavis, Navicula</td>
</tr>
</tbody>
</table>

fp fecal pellets, NC not clustered

strains (diatoms: 15 OTUs, fecal pellets: 17 OTUs) but not in terms of their identity. Strains on fecal pellets (Seminavis) included the genera Alteromonas, Roseobacter, Pseudoalteromonas, Bacillus, Sulfitobacter, Pseudomonas, Maribacter, and Croceibacter (Table 2). The strains isolated from Seminavis and the fecal pellets (Seminavis), were very different (max. 50% similarity) based on the (GTG)₅-fingerprinting (see asterisk in Fig. 2b). Moreover, no identified genera were shared between the bacterial strains recovered from the diatom Seminavis and the copepod’s fecal pellets after grazing on this diatom.

The bacterial community on Navicula (4 OTUs) was clearly less diverse than the community on the fecal pellets of P. fulvofasciata having grazed on Navicula (9.7 OTU), resulting in a very low similarity in the dendrogram based on the DGGE outcome (Fig. 1). Some of the fecal pellets (Navicula) strains showed more similarity to strains on the original food source (Navicula) than in the case of Seminavis and fecal pellets (Seminavis; Fig. 2b vs. Fig. 2c). However, rep profile similarity levels between the isolates of Navicula vs. fecal pellets (Navicula) remained moderate with maximum values ranging between 65% and 75% (Fig. 2c).

The fecal pellets of P. fulvofasciata having eaten Navicula generally contained an enriched bacterial community, including species of the genera Ruegeria, Alteromonas, Bacillus, Pseudoalteromonas, and Roseobacter (Table 2). Although the genera Alteromonas and Pseudoalteromonas were found on both Navicula and on fecal pellets (Navicula), their profiles did not cluster together ((GTG)₅-PCR clusters B vs. P and L vs. G), suggesting that the species may differ.

Four bacterial genera (Alteromonas, Pseudoalteromonas, Marinobacter, Roseobacter) were found in common between diatoms and fecal pellets. The genera Bacillus, Joostella, Maribacter, Pseudomonas, and Sulfitobacter were only found on fecal pellets. No bacteria were identified as restricted to the diatoms.

The MDS (Fig. 3) illustrated also that the bacterial communities on the diatoms differed largely from the communities on the fecal pellets, especially in the case of Navicula. In addition, fecal pellets produced after eating...
Table 2  Overview of the bacterial communities associated with the diatoms *S. robusta* and *N. phyllepta* and fecal pellets (fp)

<table>
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<th>Cluster:</th>
<th>A</th>
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<td>fp (<em>Navicula + fp other copepod species</em>)</td>
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<td>fp Other copepod species</td>
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<td><em>Seminavis</em></td>
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<td><em>Navicula</em></td>
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<td>Fresh fp (<em>Seminavis</em>)</td>
<td>X</td>
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<tr>
<td>Fresh fp (<em>Navicula</em>)</td>
<td>X</td>
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<tr>
<td>fp (<em>Navicula + fp other copepod species</em>)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>5</td>
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<tr>
<td>Old fp (<em>Seminavis</em>)</td>
<td>X</td>
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<tr>
<td>Old fp (<em>Seminavis</em> + <em>E. coli</em>)</td>
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<td>10</td>
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</tr>
<tr>
<td>fp Other copepod species</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>8</td>
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different diatoms (*Seminavis* vs. *Navicula*) did not cluster together.

Effect of Food Source, Age, Producer on the Bacterial Community of Fecal Pellets

The effect of the copepod food source on the species richness of bacteria associated with fecal pellets was considerable, as evidenced by the substantial differences between the fecal pellet associated bacteria of copepods fed with *Navicula* and the bacterial communities present on *Navicula* diatoms, but not between the bacteria on *Seminavis* and the respective fecal pellets associated bacteria.

The MDS (Fig. 3) showed that, in addition to the original food source, the age of the fecal pellets (see old fecal pellets after consumption of *Seminavis* and *E. coli* (old fp (*Seminavis*+fp other copepod species)) and on fecal pellets produced by other copepod species (fp other copepod species) and with old fecal pellets (*Seminavis + E. coli*; upper part of dendrogram, Fig. 1). This was also illustrated by the fact that the majority of the strains on fecal pellets of different copepod species could not be classified into one of the rep-PCR clusters (requiring a 60 % similarity; see supplementary material), suggesting a specific bacterial community on these fecal pellets.

**Discussion**

Bacterial Diversity Determined by the Grazer

This study documents the bacterial communities on original food sources (diatoms) and on the fecal pellets produced by harpacticoid copepods after having fed on these diatoms. In total, 150 isolates were obtained and characterized. Our results show clear differences, which is in contrast with the findings of [29] that degradation of the peritrophic membrane of fecal pellets was not accompanied by significant changes in bacteria.

Our results stress the diversifying role of *P. fulvofasciata* for benthic bacterial communities. The extra substratum provided by the fecal pellets of this copepod appears a significant factor contributing to the bacterial diversity of the environment. Fecal pellets harbored Alphaproteobacteria (*Roseobacter*, *Sulfitobacter*), Flavobacteria (*Joostella*, *Maribacter*, *Croceibacter*), and Bacilli (*Bacillus*), whereas on diatoms exclusively Gammaproteobacteria were identified. *Sulfitobacter* and *Roseobacter* are commonly reported on surfaces and in close association with marine organisms [30, 31]. Recently, [31] reported that these bacterial groups associated with copepods originated from food intake while in the present study, *Sulfitobacter* was not identified on the
food source (diatoms). In the present study, also the genera *Bacillus*, *Joostella*, *Maribacter*, *Croceibacter*, and *Pseudomonas* were found to be restricted to fecal pellets. *Pseudomonas* species have previously been found attached to fecal pellets, and were suggested to degrade fecal pellets of pelagic copepods [32, 33].

Copepods stimulate bacterial growth by producing extra substrate (fecal pellets) and nutrients through excretions (e.g. [34]). Different types of bacterial gardening in marine meiofauna [35] have been observed before, e.g. on the mucus tracks of nematodes (e.g. [25, 36, 37]), and in macrofauna like e.g. *Arenicola marina* [38]. In the latter case, however, both bacterial abundance and biomass peaked at the input side (foregut) and decreased by 70% towards the egestive side (hindgut and fecal casts) [38]. In contrast, we observed that the initial food (diatoms) was (partly) consumed and the increased bacterial diversity was initiated during the gut passage towards the egestive side. Passage through the copepod gut is crucial for this diversification of the bacterial community and not only the copepod’s presence as illustrated for zooplankton (e.g. [39]). Grazing by protozoans has been shown to be one of the major determinants of bacterial community structure in lake water [32]. This consumer’s effect can even be species specific as shown for bacterivorous nematodes on bacteria on decaying cordgrass detritus [21].

Although in general, a more diverse bacterial community was found on fresh fecal pellets in comparison to the original food source, this phenomenon was mainly observed in copepods grazing on *Navicula* cells. The production of fecal pellets may upgrade the biochemical composition of food by adding more bacteria rather than merely repackaging the food and waste products (trophic upgrading, [40]). This ‘trophic upgrading’ might be a way to balance any nutritional shortfalls of herbivory [40]. Our findings suggest that the bacterial enrichment in terms of diversity is probably governed by the available (initial) food or the bacterial communities associated with this food since the increased diversity was clearer in the case of *Navicula* than *Seminavis*. Further biochemical screening of these diatoms remains, however, necessary to substantiate this statement. Different growth kinetics in enrichment cultures of bacteria were recovered from the calanoid copepod *Acartia tonsa* feeding on different diets [4] illustrating that diet types may be a selective force for different bacterial communities inside the host’s body.

Although this increased bacterial diversity has hitherto not been documented for harpacticoid copepod species other than *P. fulvofasciata*, this phenomenon appears to be copepod-species specific as we found only a limited number of bacterial genera in common between the fecal pellets produced by our test species and the fecal pellets collected from mixed copepod stock cultures. However, the latter were fed a mixture of diatoms. It is therefore unclear whether pellet enrichment with intestinal bacteria is a more general phenomenon in harpacticoid copepods, or is restricted to certain species, as in some other crustacean taxa [41, 42].

Origin of Bacterial Enrichment

The diatoms were used as main food source for the copepods in this study and form an important source of bacteria in addition to the original gut flora of the copepods. In general, food intake is expected to affect the bacterial dynamics inside the gut [42]. [4] reported that an external source of bacteria (e.g. via food intake) is required to maintain a diverse bacterial community associated with *A. tonsa*. However, they made no distinction between bacteria attached to the exterior of the copepod and bacteria inside the copepod’s gut. In the present study, we screened the bacterial communities on the initial food and on the fecal pellets produced.

Although all diatoms were grown in the same 2 culture medium and under the same conditions, the *Navicula* cells carried a significantly less diverse bacterial community. *Navicula* cells have a tendency to clump together and this may have biased the amount and diversity of the bacteria that were obtained at emulsification as some bacteria may remain attached to the diatom cells. It has been shown that diatoms harbor distinct bacterial communities, diatom-associated bacteria especially differing in relation to the algal growth phase and less so between diatom species [30]. In the present study, potential temporal changes in bacterial communities according to the growth phases of the diatoms were not taken into account; they cannot in any case explain the differences between the diatom species used here since the initial cultures were started in similar conditions with comparable cell densities and were harvested after the same period of growth, in a comparable growth phase. In the present study we analyzed diatom-bound and fecal pellets-bound bacteria and the reported differences between the bacterial communities on the closely related diatoms *Navicula* and *Seminavis* were mainly due to substrate-bound bacteria. The only bacterial strain that was found in common between *Seminavis* and *Navicula* was identified as *Marinobacter*, a bacterial genus that has previously been reported from marine environments, both in seawater [43, 44] and in sediments [11].

In the natural situation, seawater is a major source of bacteria and sinking particles like fecal pellets quickly become colonized by bacteria from the environment [6]. The sole potential source of additional bacteria in our setup, however, is the copepods themselves. The bacteria associated with the fecal pellets could originate from the copepod’s cuticle. This is, however, very unlikely since this does not explain the difference between different treatments (*Seminavis vs. Navicula* as food source) with the same copepod species. Bacteria may rather originate from the
copepods’ gut and intestine (enteric bacteria) [45]. Fecal pellets of copepods are surrounded by a peritrophic membrane elaborated by the epithelial cells of the gut [46]. The passage through the digestive tract is essential for the formation of this membrane and meanwhile the bacterial colonization takes place. It was also considered very unlikely that bacteria entered pelagic tunicate fecal pellets from the water [47]. On the other hand, of all commonly reported genera of gut bacteria [42], we only identified Pseudomonas in our samples.

The data reported here underpin our initial expectations when we found P. fulvofasciata producing a lot of small fecal pellets with many intact diatom cells when fecal pellets were removed on a daily basis [13]. This increased production of fecal pellets seems to be a fast way of substrate (fecal pellets) creation for bacterial growth by ‘pumping through’ the diatoms without assimilating them. Detailed observations on the surface structure of fecal pellets will be necessary to clarify the exact position of the bacteria and determine whether they are accessible to surface grazers.

Ecological Implications

P. fulvofasciata is known as a grazer on diatoms but the increased production of fecal pellets upon removal of fecal pellets [13] and the changes in bacterial communities on fecal pellets (present study) suggest a potential role of the fecal pellets in the feeding ecology of this species. So far, the importance of fecal pellets for harpacticoid copepods has not been documented. Here, we illustrate that fecal pellets are a substrate for bacterial colonization and that they can potentially be used to upgrade the initial food source (in this case, diatoms). The trophic upgrading of fecal pellets may be considered a potential mechanism to guarantee the copepod’s energy requirements and production, especially during periods of low algal abundance or low-quality primary production.

The close interaction between bacteria and diatoms on the one hand [30] and between grazers, their fecal pellets and bacteria on the other hand (this study) are crucial to organic matter processing and nutrient cycling in the sea. The role of bacteria in the degradation of fecal pellets can be particularly important.

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References

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