

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 *Platyhelipus littoralis* (family Laophontidae) and *Paramphiascella fulvofasciata* (family Miraciidae) (henceforth referred to by their genus names) were used for fp production. *Platyhelipus* 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- μ m 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 *Platyhelipus* 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 *Platyhelipus* 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 μ m 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* ($> 3 \times 10^3$ 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- μ m 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 V₃ 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 MgCl₂ 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 V₃ 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 °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 ($1-\lambda'$). 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 $100 - \text{similarity \%}$ 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 EcoPlates™

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.

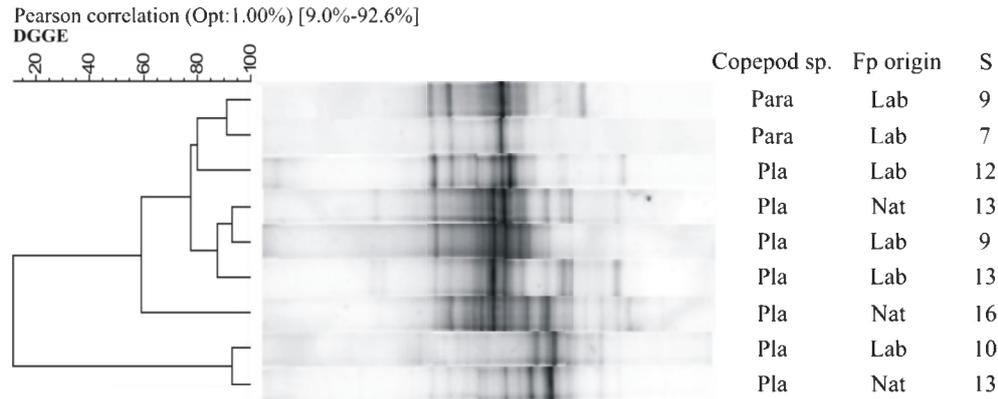


Fig. 1 Similarity between RNA-based DGGE profiles of fresh fecal pellets (fp), including natural (nat) and laboratory (lab) fp of copepods *Platychelipus* (Pla) and *Paramphiascella* (Para). S: number of DGGE bands.

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					
<i>Platyhelipus</i>					
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)

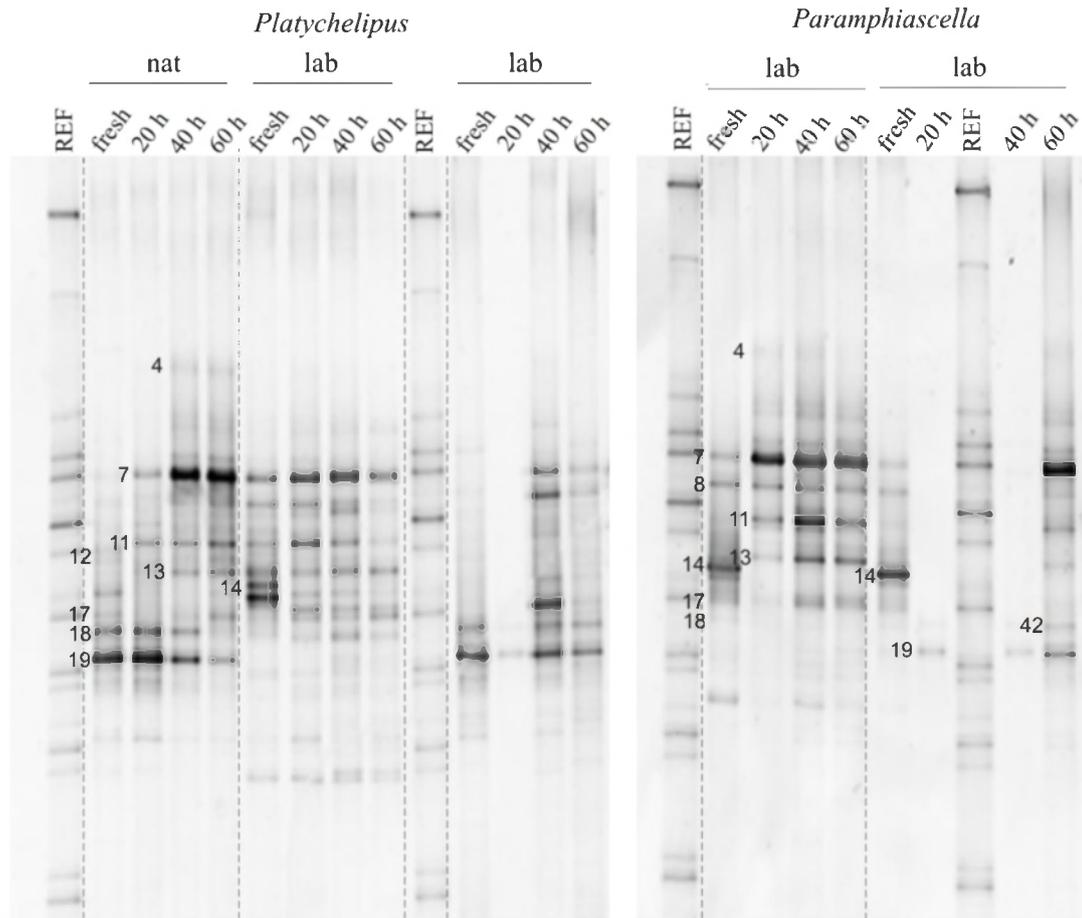
b					
<i>Paramphiascella</i>					
Band	OTU	Fp (n = 4)	Food (n = 4)	Cop - fed (n = 2)	Cop - empty (n = 2)
39.3 %	7	7.7 (2.3)	26.9 (17.1)	11.0 (6.7)	6.0 (0.4)
43.0 %	8	10.2 (2.1)	12.1 (5.3)		
51.0 %	12	8.0 (10.7)	0.8 (1.5)	5.2 (4.1)	1.0 (0.5)
54.0 %	14	51.4 (13.4)	11.7 (7.9)	33.0 (13.5)	
57.8 %	17	9.9 (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 *Platyhelipus* 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 *Platyhelipus* 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.



	<i>Platychelipus</i>												<i>Paramphiascella</i>							
	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				
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 (phylogeny 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 phylogeny 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.

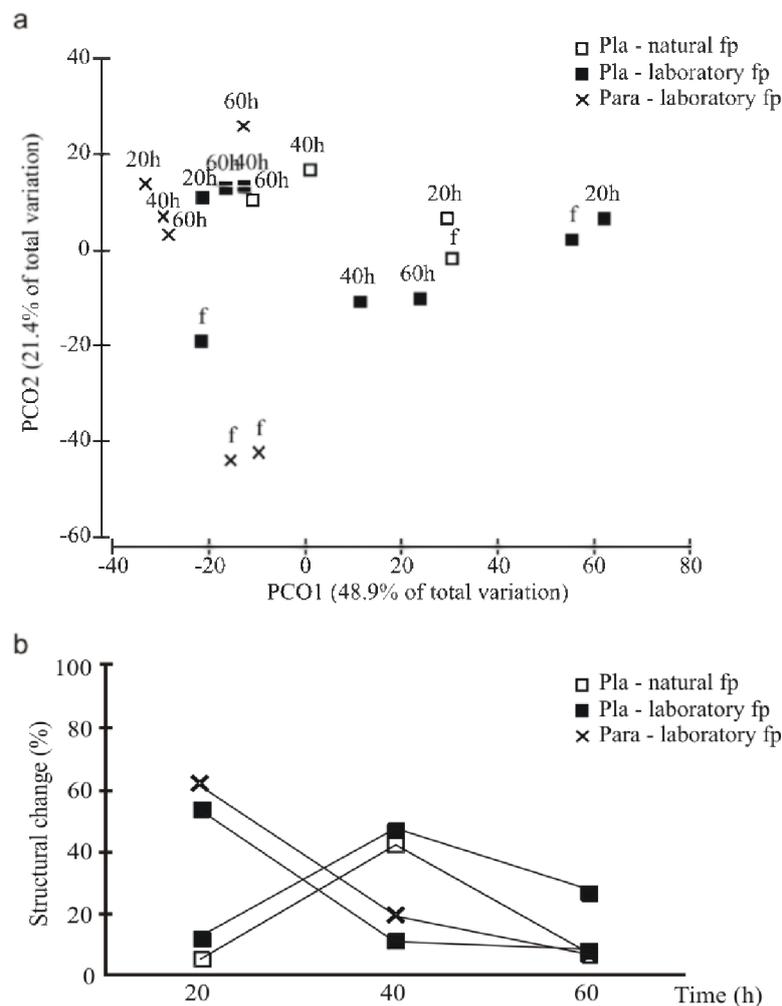


Fig. 3 Successive change in the DGGE profiles of active bacteria during fecal pellet (fp) degradation, from fresh fp to 20-h, 40-h and 60-h aged fp assessed by (a) multivariate PCO analysis and (b) by univariate moving window analysis. In the latter, data points indicate the % change of the bacterial 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 *Platyhelipus* (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.

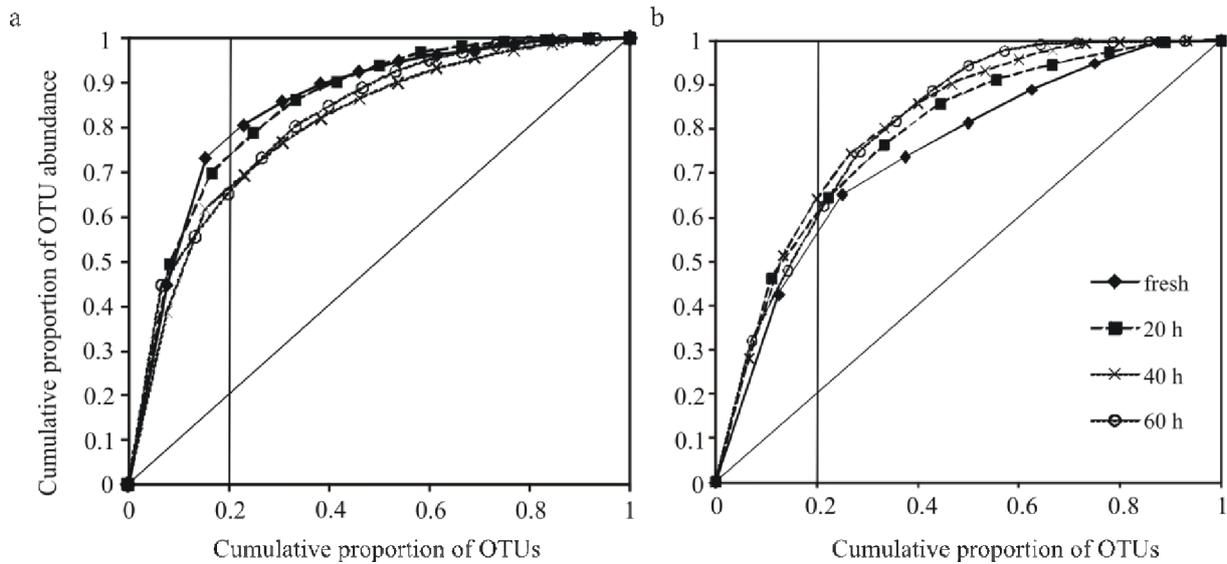


Fig. 4 Pareto-Lorenz curves of active bacterial communities associated with aging (a) natural *Platyhelipus* fecal pellets and (b) laboratory *Paramphiascella* fecal pellets. The diagonal line represents perfect evenness. Curves are compared at the 0.2 x-axis value.

For both *Platyhelipus* 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 *Platyhelipus* 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 *Platyhelipus* 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).

Fp sample		Persistence over time (%)									
		# OTUs	Highly contributing phylotypes*				# OTUs	Medium contributing phylotypes**			
			fresh	20 h	40 h	60 h		fresh	20 h	40 h	60 h
<i>Platyhelipus</i>	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)
<i>Paramphiascella</i>	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)

Fp sample		Contribution of total aged active community over time (%)									
		fresh	Highly contributing phylotypes*				fresh	Medium contributing phylotypes**			
			fresh	20 h	40 h	60 h		fresh	20 h	40 h	60 h
<i>Platyhelipus</i>	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)		
<i>Paramphiascella</i>	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 *Platyhelipus* (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 *Platyhelipus*, fresh fp bacteria used 7 ± 2 substrates ($N = 10$) while aged fp bacteria consistently used a lower number of substrates ($S = 2 \pm 2$, $N = 7$) (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 ($S = 7 \pm 2$, $N = 3$) (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.

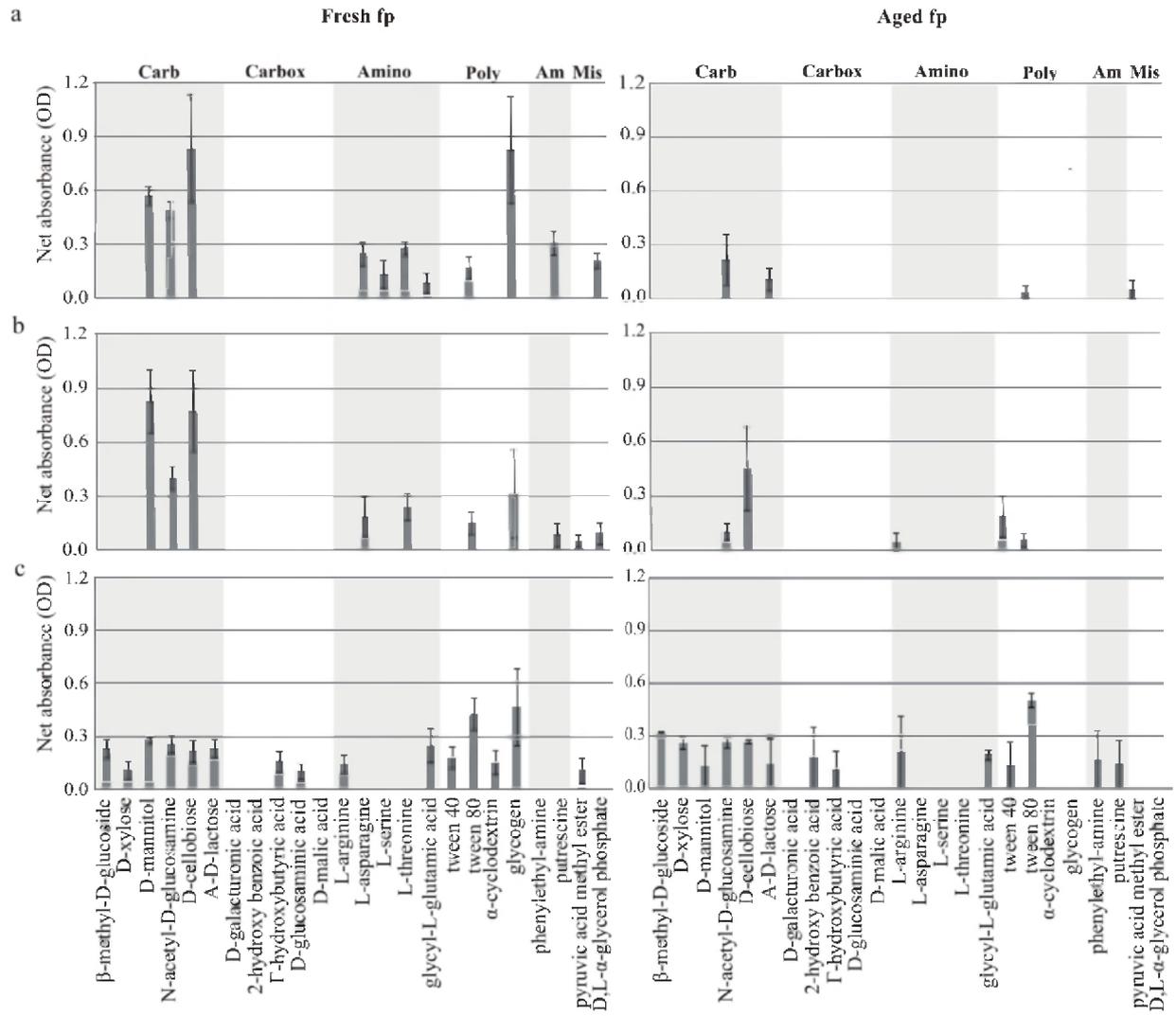


Fig. 5 Carbon substrate utilization patterns (CSUP) of bacteria associated with fresh (left) and 60-h aged (right) fecal pellets (fp): (a) natural *Platyhelipus* fp, (b) laboratory *Platyhelipus* fp and (c) laboratory *Paramphiascella* fp. When the substrate was positive in at least one replicate, OD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

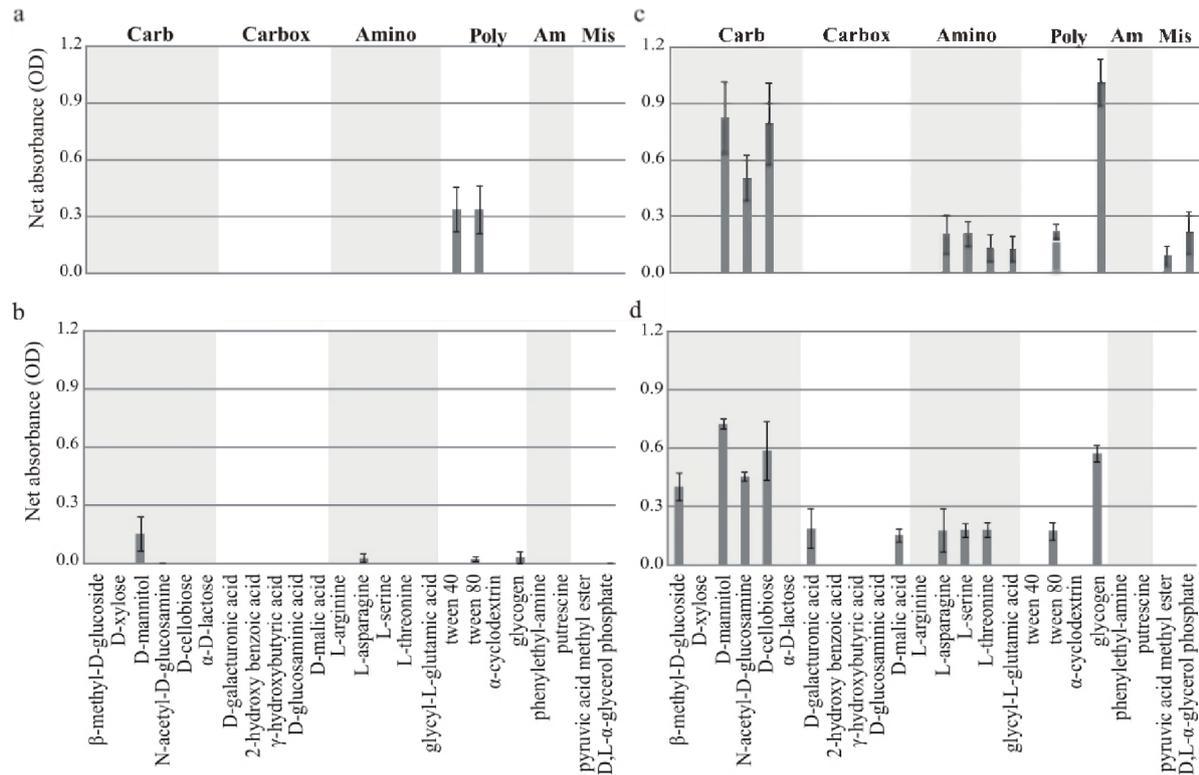


Fig. 6 Carbon substrate utilization patterns (CSUP) of bacteria associated with (a) *Seminavis*, (b) field-caught *Platycheilipus* copepods, (c) laboratory-fed *Platycheilipus* copepods and (d) natural seawater (NSW). When the substrate was positive in at least one replicate, OD values of all replicates were averaged and represented by bars (average \pm 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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