Dynamics of bacterial community composition during degradation of copepod fecal pellets

HONGMEI JING, LOKLUN SHEK, WAIYI YUNG, XIWAN JIN AND HONGBIN LIU*
DIVISION OF LIFE SCIENCE, THE HONG KONG UNIVERSITY OF SCIENCE AND TECHNOLOGY, CLEAR WATER BAY, KOWLOON, HONG KONG SAR, CHINA

*CORRESPONDING AUTHOR: liuhb@ust.hk

Received December 28, 2011; accepted in principle May 8, 2012; accepted for publication May 13, 2012

Corresponding editor: Roger Harris

The community of bacteria associated with the fecal pellets of planktonic copepods and those free living in surrounding seawater were investigated by denaturing gradient gel electrophoresis (DGGE) during a 10-day laboratory fecal pellet degradation experiment. Generally, fecal pellets containing bacteria were composed of different phylogenetic groups compared with those living in seawater. Bacteria in fecal pellets were dominated by γ-*Proteobacteria* and *Sulfitobacter* (α-*Proteobacteria*), whereas bacteria in seawater had higher species richness and mainly consisted of α-*Proteobacteria*. Remarkable bacterial community shifts occurred in the first 2 days of the experiment along with the apparent increase in dissolved organic carbon and decrease of dissolved oxygen in the incubation bottles. Throughout the incubation, bacteria that were initially unique to fecal pellets were never found in surrounding seawater, while the communities of bacteria in fecal pellets and seawater became more similar, indicating colonization of bacteria from seawater to fecal pellets during the degradation process. These results suggest that the colonization of free-living bacteria took place rapidly and that they might contribute significantly to the degradation of planktonic copepod fecal pellets.

KEYWORDS: Bacteria; population dynamics; fecal pellet degradation; DGGE

INTRODUCTION

Zooplankton fecal pellets are one of the primary contributors to the vertical flux of particulate organic carbon (POC) from surface waters to the deep ocean due to their high abundance and rapid sinking rates (Turner, 2002). Copepods, as the most abundant mesozooplankton, play a particular important role in this process (Honjo and Roman, 1978). Some of the fecal pellets are degraded in the upper part of water column by bacteria, protists and copepods (Smetacek, 1980; reviewed by Turner, 2002). Thus they serve as an important source of inorganic and organic carbon, nitrogen and silica for the microbial communities.

Fecal pellets have a high organic content that accounts for about one-third of copepod-ingested organic carbon with an elevated C:N ratio relative to ingested prey (Smetacek, 1980). Freshly produced copepod fecal pellets are rapidly colonized by bacteria, and the degradation process supplies nutrients for free-living bacteria (bacteria not attached to any particles) and other microorganisms (Cho and Azam, 1988). Bacteria associated with copepod fecal pellets are involved in the transformation process of POC to dissolved organic carbon (DOC), and affect the amount and types of DOC leached from fecal pellets (Urban-Rich, 1999). The transformation of POC to

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doi:10.1093/plankt/fbs043, available online at www.plankt.oxfordjournals.org. Advance Access publication June 1, 2012
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DOC and the diffusion of DOC from fecal pellets are important for oceanic carbon flux.

Copepods fecal pellets are often heavily colonized by bacteria, at population densities much higher than free-living bacteria (Turner, 1979; Delille and Razouls, 1994; Tang, 2005). However, there are uncertainties surrounding the decomposition mechanism of fecal pellets whether they are primarily from interior bacteria decomposing "inside out" or exterior bacteria working "outside in". Gowing and Silver (Gowing and Silver, 1983) concluded that internal bacteria of fresh fecal pellets initiated the decomposition process, which is in contrast to Honjo and Roman (Honjo and Roman, 1978) who found few internal bacteria, but bacteria attached to the fecal pellet surface were the major decomposers. The difference could be the result of different zooplankton species and environmental conditions as the bacterial composition on fecal pellets varies with the copepod species, the original food source and the age of the fecal pellets (De Troch *et al.*, 2010). For example, fecal pellets of the copepod *Pseudocalanus newmani* and the amphipod *Themisto japonica* lack intestinal bacteria (Nagasawa and Nemoto, 1988; Nagasawa, 1992), while bacteria are present in the fecal pellets of other planktonic calanoid copepods such as *Calanus pacificus* (Jacobsen and Azam, 1984), *Eucalanus bungii* and *Acatia omorii* (Nagasawa and Nemoto, 1988). Bacterial colonization on feces lacking intestinal bacteria is reported to be delayed compared with feces that possess enteric bacteria (Nagasawa, 1992).

Bacterial community composition associated with copepod fecal pellets has been assessed since the 1990s (Hansen and Bech, 1996; De Troch *et al.*, 2010). Bacterial communities inside copepod fecal pellets have been reported to be different from those in surrounding seawater (Delille and Razouls, 1994). However, bacterial community structures in that study were characterized by agar plating, which only reveals the composition of cultivable species and does not provide a detailed succession scenario for the bacterial community during fecal pellet degradation. In a recent study, De Troch *et al*. (De Troch *et al.*, 2010) investigated the composition of bacterial microflora on fecal pellets of a harpacticoid copepod using denaturing gradient gel electrophoresis (DGGE) profiling. Their results suggest that the high diversity of bacteria associated with fecal pellets mainly originates from the copepod's digestive tract and largely depend on the initial food sources.

Nevertheless, no detailed studies on the bacterial community shifts during copepod fecal pellet degradation have been conducted, and fecal pellet degradation processes in the sea are still poorly understood (Poulsen and Iversen, 2008). Therefore, the present study mainly focuses on the phylogenetic composition and population dynamics of bacteria associated with fecal pellets and free-living bacteria in seawater during copepod fecal pellet degradation in a controlled laboratory experiment by using molecular techniques. The objective is to investigate the bacterial community composition and dynamics during copepod fecal pellet degradation and to identify the major decomposers.

METHOD

Collecting copepod fecal pellets

Live pelagic copepods were collected in July 2009 using a plankton net (open diameter 0.5 m, mesh size 200 μm) from coastal waters off Hong Kong. Copepod abundances reached their highest abundance in July (Chen *et al.*, 2011) and were mainly composed of *Acartia* spp. (47.36%), *Paracalanus parvus* (38.66%) and *Acartia spinicauda* (9.94%). The mixture of copepods was firstly incubated in GF/F filtered seawater for 3 h to empty their gut contents and then transferred again to GF/F filtered seawater and fed with a diatom *Thalassiosira pseudonana* at a saturating food concentration (~54 000 cells mL^{-1}) for fecal pellet collection. *Thalassiosira pseudonana* was grown in exponential phase in f/2 medium at 23°C under a 12 h:12 h light:dark cycle.

Fecal pellet degradation experiment

After 18 h incubation at 23°C in the dark for fecal pellet production, copepods were removed with a 200-μm sieve, fecal pellets were collected on a 20-μm sieve and rinsed twice with autoclaved 0.2-μm filtered seawater and then added into autoclaved 0.2-μm filtered seawater to form a fecal pellet sludge. An aliquot of the fecal pellet sludge (10 mL, ~ 2266 fecal pellets on average) was added to each 1 L polycarbonate bottle containing either 0.2 μm (to remove bacteria and grazers) or 1 μm (to remove grazers) filtered seawater. The fecal pellets used in our experiment were 109.3 ± 22.0-μm long and 33.1 ± 4.0-μm wide on average based on the measurement of 40 fecal pellets.

The treatment with fecal pellets incubated in 0.2-μm filtered seawater was named FP + BFSW (fecal pellets in bacteria-free seawater) and those incubated in 1-μm filtered seawater, FP + BCSW (fecal pellets in bacteria-containing seawater). A total of six bottles were prepared for each treatment and the control that contained 1-μm filtered seawater without fecal pellets (BCSW). All bottles were incubated at 28°C in the dark in a water-bath equipped with a slow rolling shaker. On Day

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2, 5 and 10, one set of bottles (two from each treatment) were terminated and filtered sequentially through 20 and 0.2-μm polycarbonate membranes to collect fecal pellets and bacteria in seawater, respectively. Initial (Day 0) samples were also taken for bacteria in fecal pellets and in 1-μm filtered seawater. Filters were stored at −80°C until DNA extraction.

Dissolved oxygen (DO) samples were collected in triplicate using a 60 mL plastic syringe and tubing transferring the samples from 1-L polycarbonate bottles to glass-stoppered bottles and analyzed by Winkler titration (Strickland and Parsons, 1972). Samples for inorganic nutrients, DOC and total nitrogen (TN) analyses were collected by filtering seawater through 550°C precombusted GF/F filters using a precombusted glass filtering unit. The filtrates for DOC/TN analysis were stored in amber glass vials in triplicate at 4°C, while those for nutrients were stored in clean plastic bottles in duplicate at −20°C until analysis. Nutrient analysis followed standard methods (Strickland and Parsons, 1972) adapted to a SKALAR autoanalyzer (SanPlus). DOC and TN were measured by a TOC/TN analyzer (TOC-V, TNM-1, Shimadzu Corporation, Japan). In addition, bacterial abundance in the water samples (free-living bacteria) collected on different sampling dates was counted using a Becton-Dickinson FACSCalibur flow cytometer using SYBR Green I (Molecular Probes) as a nucleic acid stain (Marie *et al.*, 1997).

DNA isolation and amplification

Total genomic DNA was recovered from both 20- and 0.2-μm PC membranes by phenol: chloroform extraction at 60°C after lysis with CTAB buffer containing RNase A (20 mg mL^{-1}) and Lyzome (20 mg mL^{-1}). Extracted DNAs were stored at −80°C after precipitation with isopropanol and then amplified with primer sets specific to bacterial 16S rRNA genes: 341F (5′-C CTACGGGAGGCAGCAG-3′) and 926R (5′-CCGTC AATTCMTTTRAGTTT-3′) (Muyzer *et al.*, 1996). A $(GC)_{40}$ clamp was attached to the 5′-end of the forward primer: 5′-CGCCCGCCGCGCCCCG CGCCCGT CCCGCCGCCCCCGCCCG-3′). The PCR reaction was carried out with a 50 μL master mix including 5 μL of 10 × Buffer, 2 μL of $MgCl_2$ (25 mM), 4 μL of dNTPs (2.5 mM), 0.2 μL of Taq polymerase (5 U, Invitrogen), 1 μL of each primer (10 μM) and 1 μL of DNA template (∼10 ng) with the following program: 95°C for 3 min; 30 cycles of 95°C for 1 min, 55°C for 50 s, 72°C for 1 min; final extension at 72°C for 10 min. PCR products were stained with ethidium bromide and visualized on 1% agarose gel with a UV illuminator.

DGGE electrophoresis and sequence analysis

About 2 μg of GC-PCR products amplified from each sample were loaded into a 7% polyacrylamide DGGE gel, containing a linear denaturant gradient of 30−70%. Electrophoresis was conducted using the Bio-Rad DGGE system (Bio-Rad, USA) in 1 × TAE buffer (pH 8) at 60°C and 100 V for 15 h. After completion, the gels were stained with SYBR Green I (10 000 × dilution, Invitrogen) and photographed by Fluor-S MultiImager (Bio-Rad). Selected DGGE bands were excised and soaked overnight in TE buffer (pH 8) at 4°C before being re-amplified with bacteria primer sets. The resulting amplicons were electrophoresed again to verify that they aligned with their respective bands in the original sample and successful amplicons were purified with the PureLink™ Quick Gel Extraction Kit (Invitrogen) prior to automatic sequencing with the BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems).

All sequences obtained from the excised bands were submitted to the online RDP-II CHECK_CHIMERA program to check the sequence integrity and chimeras. For each non-chimera sequence, primer sequences were excluded and the NCBI GenBank BLAST program (www.ncbi.nlm.nih.gov) was used to determine their approximate phylogenetic affiliations.

Statistical analysis

DGGE profiles were analyzed with the GelCompar II software (Applied Maths) after being normalized against the 1 kb Plus DNA Ladder (Invitrogen), which was loaded and migrated along with samples in the gel. Gel images were converted to densitometric profiles and species richness (numbers of band position revealed in each lane) and inferred abundance (band intensity) was determined. The relative abundance of each phylotype (band position) was estimated based on the ratio of the specific peak height to that of the total bands in the profile.

In order to reveal the bacterial population dynamics in different treatments during the 10-day incubation, correspondence analysis (CA) was carried out using CANOCO V4.5 (Biometrics-Plant Research International). In addition, redundancy analysis (RDA) was performed for each treatment to reveal the relationships between the community structures and environmental variables, because the pre-run detrended correspondence analysis (DCA) demonstrated that the length of the first DCA axis is <4 for all treatments. Abiotic factors indicated in Figure 1 were all included

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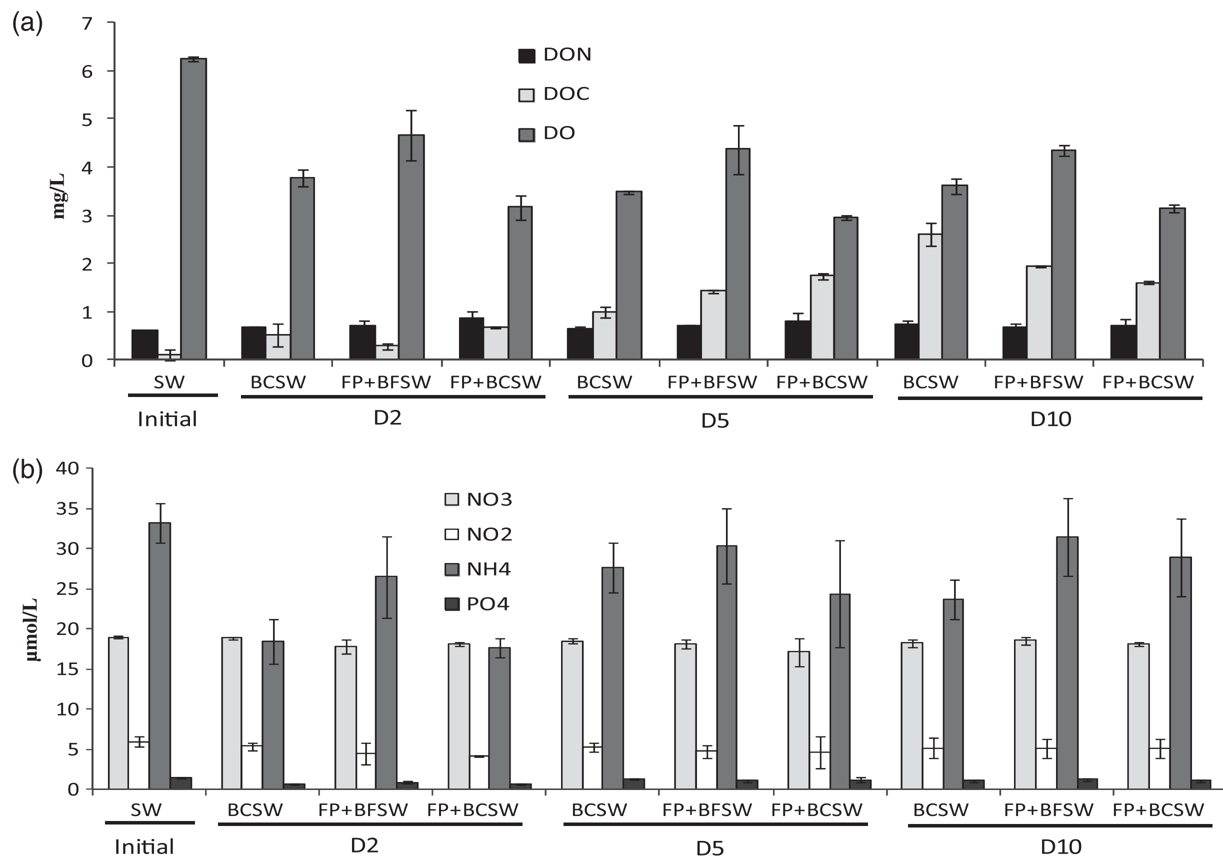

Fig. 1. Concentrations (mean ± SD, $n = 2$) of dissolved organic carbon (DOC) and nitrogen (DON) and dissolved oxygen (DO) (**a**) and inorganic nutrients (**b**), in control and treatments over time. Initial DO concentration in the 1-μm filtered seawater was 6.25 ± 0.06 mg/L and in the 0.2-μm filtered seawater was 5.91 ± 0.08 mg/L. SW, seawater; FP, fecal pellets; FP + BFSW, fecal pellets in 0.2-μm filtered seawater; FP + BCSW, fecal pellets in 1-μm filtered seawater; BCSW, 1-μm filtered seawater serving as control.

as explanatory variables. Variables with variance inflation factors >20 indicating co-linearity between variables were excluded from analysis. Variables that best described the most influential gradients were identified by forward selection and their significance was assessed by the Monte Carlo permutation test (999 permutation, $P \leq 0.05$).

Nucleotide sequence accession numbers

All partial16S rRNA gene sequences obtained from this study were submitted and deposited in GenBank under accession numbers GU576912 to GU576968.

RESULTS

Chemical parameters

DO in the incubation bottles dropped remarkably in the first 2 days, and became rather stable after (Fig. 1a). DOC concentration increased during the incubation, while dissolved organic nitrogen (DON) increased remarkably on Day 2 and then decreased on Day 5 and Day 10. In addition, ammonium concentrations were also notably lower on Day 2 than on Day 0 and then increased through the rest of the incubation (Fig. 1b). Concentrations of nitrate and nitrite were rather constant. Phosphate concentrations in all bottles showed an even more dramatic drop than ammonium in the first 2 days of incubation and recovered on Day 5 and remained constant until Day 10.

Changes in bacterial abundance

Abundance of free-living bacteria in control and treatments reached a peak on Day 2, declined notably on Day 5 and then increased again on Day 10 (Fig. 2). Particularly, bacterial abundance in the seawater control (BCSW) increased by about four times in the first 2 days of incubation and was higher than that in the fecal

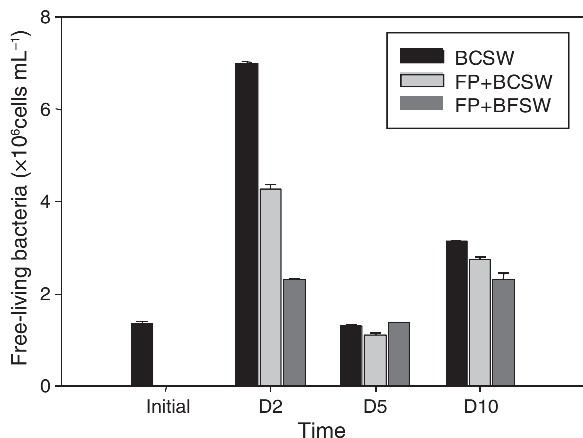

Fig. 2. Abundance of free-living bacteria in different treatments over time. *Note*: Cyanobacteria *Synechococcus* were found in samples of initial seawater (630 cells mL^{-1}) and on Day 2 of BCSW (494 cells mL^{-1}) and FP + BCSW (296 cells mL^{-1}). BCSW, 1-μm filtered seawater serving as control; FP + BCSW, fecal pellets in 1-μm filtered seawater; FP + BFSW, fecal pellets in 0.2-μm filtered seawater.

pellet containing bottles. Fecal pellet suspension in 1-μm filtered seawater (FP+BCSW) had lower levels of free-living bacteria, while the treatment of fecal pellets in 0.2-μm filtered seawater had the lowest abundance.

Changes in bacterial community composition

A total of 90 DGGE bands were excised and successfully sequenced and those bands not cut and sequenced were treated as unidentified (Fig. 3). Bacteria in seawater displayed much higher species richness than those in fresh fecal pellets, as indicated by the remarkably greater number of DGGE bands in the former (Fig. 3a). A remarkable change of band pattern in the seawater control can be seen on Day 5 (Fig. 3a). When fecal pellets were added into 1-μm filtered seawater (FP+BCSW), the free-living bacterial assemblage mostly resembled to that of the seawater rather than the fecal pellets, and a remarkable change in bacterial composition was also observed on Day 5 as indicated by the emergence of many new bands (Fig. 3c). In FP + BFSW, Day 2 DGGE showed a few light bands similar to the initial seawater (maybe due to incomplete removal of free-living bacteria) and the intensity of the bands was overall faint compared with that of FP + BCSW (Fig. 3b and c). Bacteria that were associated with fecal pellets appeared less variable though species richness increased with incubation time in both treatments, especially in those incubated with seawater with free-living bacteria (FP + BCSW) (Fig. 3d).

Initial phylogenetic composition in seawater and fecal pellets

The bacterial community in the natural seawater was dominated by *α-Proteobacteria*, especially *Rhodobacterales*. *γ-Proteobacteria* was the second major group accounting for ~28% of the whole community and *Actinobacteria* and *Cyanobacteria* each accounted for ~10% of the assemblage (Fig. 4). On the other hand, freshly produced fecal pellets after feeding on *T. pseudonana*, contained mainly *r-Proteobacteria* with approximately the same amounts of *Cyanobacteria* and *Sulfitobacter* (*α-Proteobacteria*).

Bacterial community succession

Clear bacterial community changes occurred in seawater of both control and treatments. In the 1-μm filtered control seawater *γ-Proteobacteria* and *Rhodobacterales* became undetectable on Day 2 and *Cyanobacteria* were not detected after Day 2, while at the same time *Bacteroidetes* and *Bacteriovorax were* detected from Day 2 and *Sphingobacteria* (*Bacteroidetes*) also emerged on Day 10 (Fig. 4). By the end of the incubation on Day 10, these three groups accounted for ~60% of the whole community, while the rest were *α-Proteobacteria* and *Actinobacteria*, which were little changed from Day 0.

In the fecal pellets incubated in 1-μm filtered seawater (FP + BCSW), the bacterial community composition in seawater changed dramatically when compared with the initial seawater. After the incubation, α-*Proteobacteria*, including *Rhodobacterales*, *Roseobacter* and *Sphingomonadales*, were the dominant group and its proportion increased throughout the incubation. *Cyanobacteria* and *Methylophaga* became undetectable after Day 2, while *Sphingobacteria*, which were not detected in both seawater and fecal pellets initially, were found during the whole degradation process.

The phylogenetic composition of free-living bacteria was also examined in the fecal pellets incubated in "bacteria-free" seawater (FP + BFSW). The possible sources of the free-living bacteria in the "bacteria-free" treatment included releasing from fecal pellets, introduction from fecal pellet sludge and incomplete removal of bacteria by 0.2 μm filtration. Free-living bacteria in seawater on Day 2 contained α-*Proteobacteria*, *Rhodobacterales*, *Roseobacter*, *Bacteroidetes*, *Sphingobacteria*, γ-*Proteobacteria* and *Methylophaga* (γ-*Proteobacteria*). The latter was not detected on Day 5 and Day 10, when *Cytophaga-Flavobacter* (*Bacteroidetes*) appeared. *Sulfitobacter* (α-*Proteobacteria*) and *Vibrio*, which occurred initially in fecal pellets, were not detected in seawater. The composition and relative abundances of bacterial populations associated with the fecal pellets also shifted over time in both treatments; most noticeably the disappearance of *Cyanobacteria*, γ-*Proteobacteria* and *Sulfitobacter*, and

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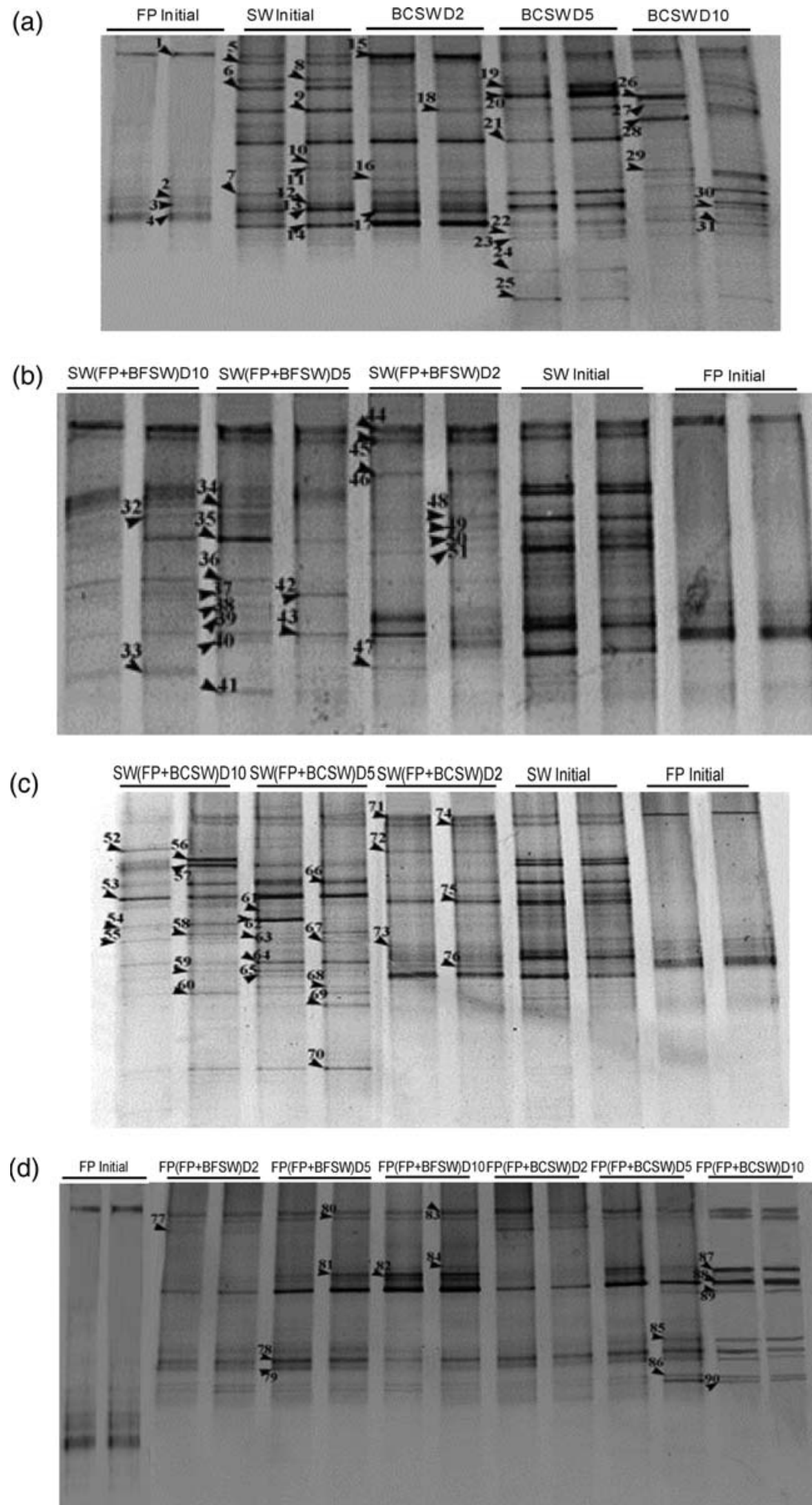

Fig. 3. Denaturing gradient gel electrophoresis fingerprints of 16S rRNA genes of bacterial population dynamics over time in different treatments: control BCSW (**a**), free-living bacteria in FP + BFSW (**b**) and FP + BCSW (**c**); associated bacteria in both FP + BFSW and FP + BCSW (**d**). SW, seawater; FP, fecal pellets; FP + BFSW, fecal pellets in 0.2-μm filtered seawater; FP + BCSW, fecal pellets in 1-μm filtered seawater; BCSW, 1-μm filtered seawater serving as a control. Arrows indicate bands that were cut and sequenced.

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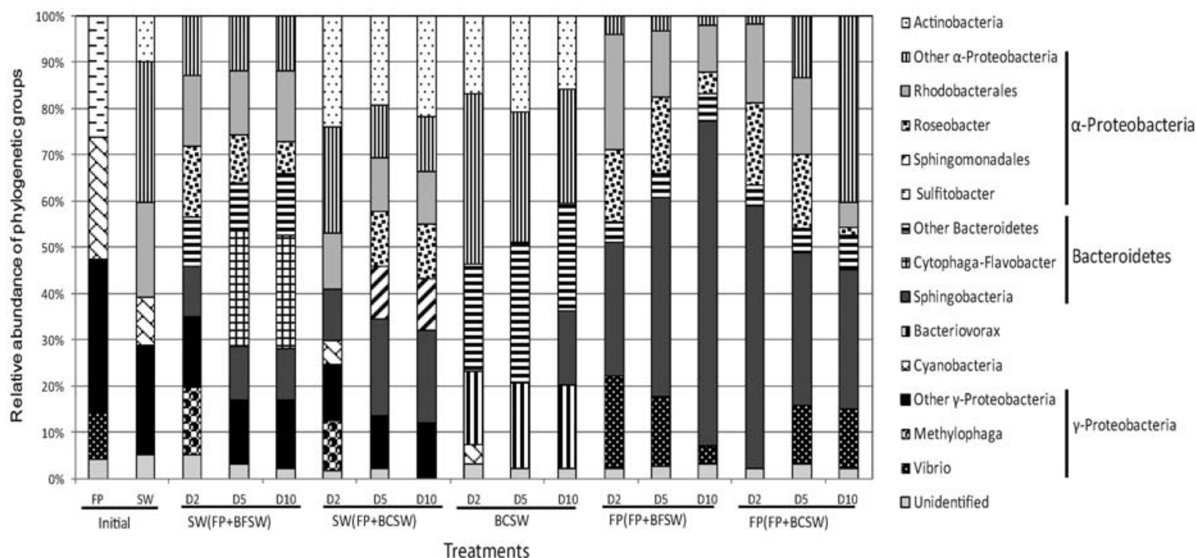

Fig. 4. Bacterial community shifts in different treatments over time based on the percentages of band intensities in the DGGE profile. SW, seawater; FP, fecal pellets; FP + BFSW, fecal pellets in 0.2-μm filtered seawater; FP + BCSW, fecal pellets in 1-μm filtered seawater; BCSW, 1-μm filtered seawater serving as control. Unidentified: DGGE bands that were not cut and sequenced.

Table I: Summary of RDA analysis for the free-living bacterial communities in different treatments over time

Treatments	Eigenvalue of axis 1	Eigenvalue of axis 2	Total variances explained by two main axes	Major parameter	λ_A	*F*-ratio
BCSW	0.850	0.115	96.5%	DO	0.85	11.3*
BCSW − I	0.711	0.289	100%	DOC	0.71	2.46
(FP + BFSW) − I	0.965	0.035	100%	DO	0.96	22.23
(FP + BCSW)	0.816	0.18	99.5%	DOC	0.79	7.72*
(FP + BCSW) − I	0.985	0.015	100%	DOC	0.98	55.85

− I, not including initial (Day 0).
$\boldsymbol{\lambda}_A$ (Lambda-A), represents the variance that the major parameter explains during forward selection and its statistical significance is assessed using Monte Carlo tests (*F*-statistics listed).
*Statistically significant, $P \leq 0.05$.

the emergence of many taxa originally found in seawater (Fig. 4). Both were dominated by *Sphingobacteria* and α-*Proteobacteria* (including *Rhodobacterales* and *Roseobacter*), though the succession patterns were different between the treatments. For example, the proportion of *α-Proteobacteria* decreased and the proportion of *Sphingobacteria* increased throughout the incubation in FP + BFSW, but the opposite was observed in FP + BCSW. The only bacterial group associated to fecal pellets of both the initial and in both of the treatments was *Vibrio* (*γ-Proteobacteria*).

Statistical analysis

The succession of bacterial populations in each incubation bottle was likely related to the changes of abiotic factors as indicated by the RDA analysis (Table I). RDA analyses for each treatment and control including initials (the whole experimental period) and excluding initials (Day 2 through Day 10) were both performed, because changes in DO and nutrients mainly occurred in the first 2 days of the incubation (Fig. 1). In the control (BCSW), DO ($\lambda_A = 0.85$) explained the greatest part of bacterial community changes during the whole experimental period; while DOC ($\lambda_A = 0.71$) appeared to be the main driver for the community shifts in dark incubation from Day 2 to Day 10. In the FP+BFSW treatment, bacterial community shifts in the seawater during the incubation were mostly affected by DO ($\lambda_A = 0.96$). On the other hand, in the FP+BCSW treatment, DOC was the key factor explaining the variance of bacterial communities in the seawater over the incubation period, no matter whether the initial samples were included ($\lambda_A = 0.79$) or excluded ($\lambda_A = 0.98$).

CA exhibited a clear distribution pattern between bacterial populations and associated treatments (Fig. 5). Bacteria associated with the initial FP samples were located at the far right of the plot, while those associated

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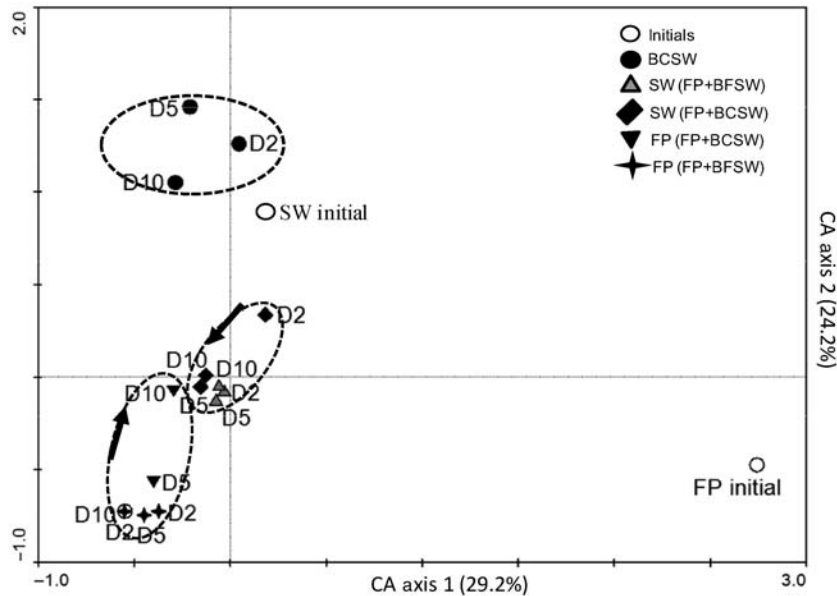

Fig. 5. Correspondence analysis biplot of bacterial communities in different treatments over time. Distances between sample dots give an indication of the bacterial community dynamics in different treatments and time. The most important (first) CA axis describes the population succession during the incubation and the second axis describes different treatments. SW, seawater; FP, fecal pellets; FP + BFSW, fecal pellets in 0.2-μm filtered seawater; FP + BCSW, fecal pellets in 1-μm filtered seawater; BCSW, 1-μm filtered seawater serving as control.

with seawater appeared at the top of the plot. Bacterial populations in different treatments were separated on the second CA axis: free-living bacteria in control bottles during the incubation period shared a high similarity with the seawater initial, and were located distantly from bacteria in other treatments. Free-living bacteria in the two treatments located around the cross-center of the two axes, and those associated with fecal pellets, located at the lower part of the plot. The succession of bacterial communities through the incubation can also be traced in the CA plot. In treatment bottles containing fecal pellets and 1-μm filtered seawater (FP + BCSW), bacterial composition in seawater was similar to the seawater initial on Day 2 and distinct from the bacterial community of fecal pellets, but they moved toward each other as the incubation proceeded and clustered together on Day 10. On the other hand, bacterial composition in fecal pellets and the surrounding seawater in the treatment bottles containing fecal pellets and 0.2-μm filtered seawater (FP + BFSW) were both relatively constant and remained distinct from each other throughout the incubation (Fig. 5).

DISCUSSION

Zooplankton fecal pellets make up a significant fraction of the oceanic flux of organic matter, but the processes governing their degradation are poorly known. A complete suite of organisms, from bacteria to protozoa and copepods contribute to the degradation (Poulsen and Iversen, 2008). Copepod fecal pellets in the water column are rapidly colonized by free-living bacteria (Jacobsen and Azam, 1984), but the succession of the bacterial community during copepod fecal pellet degradation has not been studied. Therefore, this study documents the population succession of bacteria associated with fecal pellets and in surrounding seawater during a 10-day copepod fecal pellet degradation experiment using molecular techniques.

Clear bacterial community dynamics during fecal pellet decomposition were demonstrated by DGGE profiles, which provide a semi-quantitative estimation of the relative abundance of various bacterial groups. However, it should be noted that the DGGE band intensity does not necessarily represent the abundance of each phylotype and some rare taxa in environmental samples could be undetectable (Muyzer *et al.*, 1993). In addition, different regions of the 16S rRNA gene under different DGGE conditions might result in differing separation resolution and only relatively short sequences (∼500 bp) were suitable for separation (Myers *et al.*, 1985). DNA fragments with a certain amount of sequencing variation may not always be separable (Vallaeys *et al.*, 1997). Due to the intrinsic limitations of DGGE, plus the possibility of bias introduced during DNA extraction and PCR, DGGE profiles could not be used for absolute quantitative estimation of specific

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phylogenetic groups. Consequently, relative abundance has been used throughout to refer to the proportions of DNA from each bacterial group contributing to the total DNA amplified. The fact that *Sphingobacteria* were undetected in both initial samples, but were present in the two treatments throughout the degradation process may reflect that their initial abundances were too low to be detected by DGGE.

The difference of bacterial community structures between the initial samples and those during degradation was possibly caused by the apparent decrease in oxygen in the first 2 days of incubation. Fermentative *Vibrio* were found only in the fecal pellets during our incubations, consistent with the study of Delille and Razouls (Delille and Razouls, 1994), but were absent in the water samples. This bacterial group was found predominantly attached on the body surface of planktonic copepods and in their guts and involved in the microbial decomposition of zooplankton (Simidu *et al*., 1971; Heidelberg *et al*., 2002). The fermentative microhabitats are formed by the peritrophic membrane of the fecal pellets, which could be a substrate for the growth of *Mythylophaga* (Janvier and Grimont, 1995) that emerged on Day 2 in our study. In adddition, *Sulfitobacter* occurred only in the initial fecal pellet samples, which agrees with previous studies that have shown its close association with food particles, especially diatoms (Grossart *et al*., 2005; Tang *et al*., 2009), suggesting that this bacterial group is intrinsic to fecal pellets and that conditions were not favourable during the degradation process. Similarly, unicellular cyanobacteria, *Synechococcus*, which have been found intact in *Calanus* sp. fecal pellets (Johnson *et al*., 1982), were only detected at the beginning but dispeared during the dark incubation. The high proportion of *Synechococcus* in fecal pellets is probably due to the undigested cells that were introduced into the prey for fecal production through GF/F filtered seawater.

It has been reported that fecal pellets contain much more bacteria than the surrounding seawater (Delille and Razouls, 1994) and bacterial abundance decreased over time during the degradation of diatom-based fecal pellets of the copepod *Acartia tonsa* (Hansen *et al*., 1996). The fact that some major bacterial groups in fresh fecal pellets, such as *Sulfitobacter* and *Vibrio*, were not detected in seawater throughout our incubation experiments, and that bacterial groups abundant in seawater were also found in fecal pellets after incubation, indicates that colonization of bacteria from surrounding seawater instead of bacteria that are originally associated with fecal pellets might be more important in fecal pellet degradation.

Our RDA analysis demonstrated that DO and DOC played the most important role in the successions of bacterial community structures in both fecal pellets and seawater throughout the incubation period. Recent study has suggested that oxygen consumption due to degradation of copepod fecal pellets can contribute significantly to the formation of coastal hypoxia in river impacted Hong Kong coastal waters (Shek and Liu, 2010). Although the microbial adaptations to hypoxic marine environment are not well documented, it is suggested that a number of microbial processes that are uncommon for aerobic water columns may flourish in low oxygen conditions (Bertagnolli *et al*., 2010).

Zooplankton fecal pellets are an important source of DOC for the growth of free-living bacteria (Thor *et al*., 2003). The degradation of zooplankton fecal pellets is rather fast. It is reported that >90% of fecal pellets could be degraded within the first 24 h (Hansen *et al*., 1996), and ~45% of fecal pellet carbon could be remineralized within 20 h (Olsen *et al*., 2005). The colonization of bacteria onto particles also occurs rapidly (Jacobsen and Azam, 1984). In our study, noticeable shifts in bacterial community structure were detected on Day 2, corresponding to the apparent increase in DOC and DON concentrations (Fig. 1a). The increase in DOC and DON were likely a result of the leakage from fecal pellets (Møller *et al*., 2003), while the subsequent decline in the following days represented bacterial utilization.

Besides bacteria, zooplankton including copepods and protozooplankton also play a role in fecal pellet degradation through consumption and repackaging (Poulsen and Kiørboe, 2006; Poulsen and Iversen, 2008). Copepods as microbial hotspots in the ocean harbor a highly diverse bacterial community that is affected by host feeding activities and prey composition (Tang, 2005; Tang *et al*., 2009). We are aware that copepods were not present in our degradation experiments, and that could have had an effect on the composition of both free-living and fecal pellet-associated bacterial communities.

In conclusion, we have characterized the bacterial community and its succession during the decomposition process of copepod fecal pellets. In subtropical estuarine and coastal shallow seas, such as those surrounding Hong Kong, the water column is fully mixed during the most time of the year (Harrison *et al*., 2008; Chen *et al*., 2009), and fecal pellets will be degraded rapidly and continuously during the sinking and resuspension processes. Moreover, our results suggest that colonization of bacteria from seawater onto fecal pellets occurred rapidly, and that they might play an important role in the degradation process. It needs to be pointed out, however, that since 90% of fecal pellets can be degraded within the first 24 h (Thor *et al*., 2003; Olsen

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et al., 2005), it is possible that a significant portion of the decomposition may be accomplished by bacteria already present in the fecal pellet, for instance fermentative *Vibrio*, perhaps before free-living bacteria even colonize on the pellet. Therefore, further study of the metabolic pathways of bacteria involved in different stages of fecal pellet degradation is needed to elucidate the complexity of the processes governing the degradation.

ACKNOWLEDGEMENTS

The authors thank Tracy Guo for analysis of the bacteria abundance in the seawater samples and Emma Rocke for manuscript polishing.

FUNDING

This research is supported by the University Grants Committee of Hong Kong AoE project (AoE/P-04/04-4-II), Hong Kong Research Grants Council grants (661407 and 661809), and TUYF Charitable Trust (TUYF10SC08) to H.L.

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