Chapter 7

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

ABSTRACT

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

INTRODUCTION

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

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

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

METHODS

Fecal pellet sample preparation

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

Atomic force microscopy

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

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

Bacterial cell volume estimation by AFM

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

Laser scanning confocal microscopy

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

RESULTS

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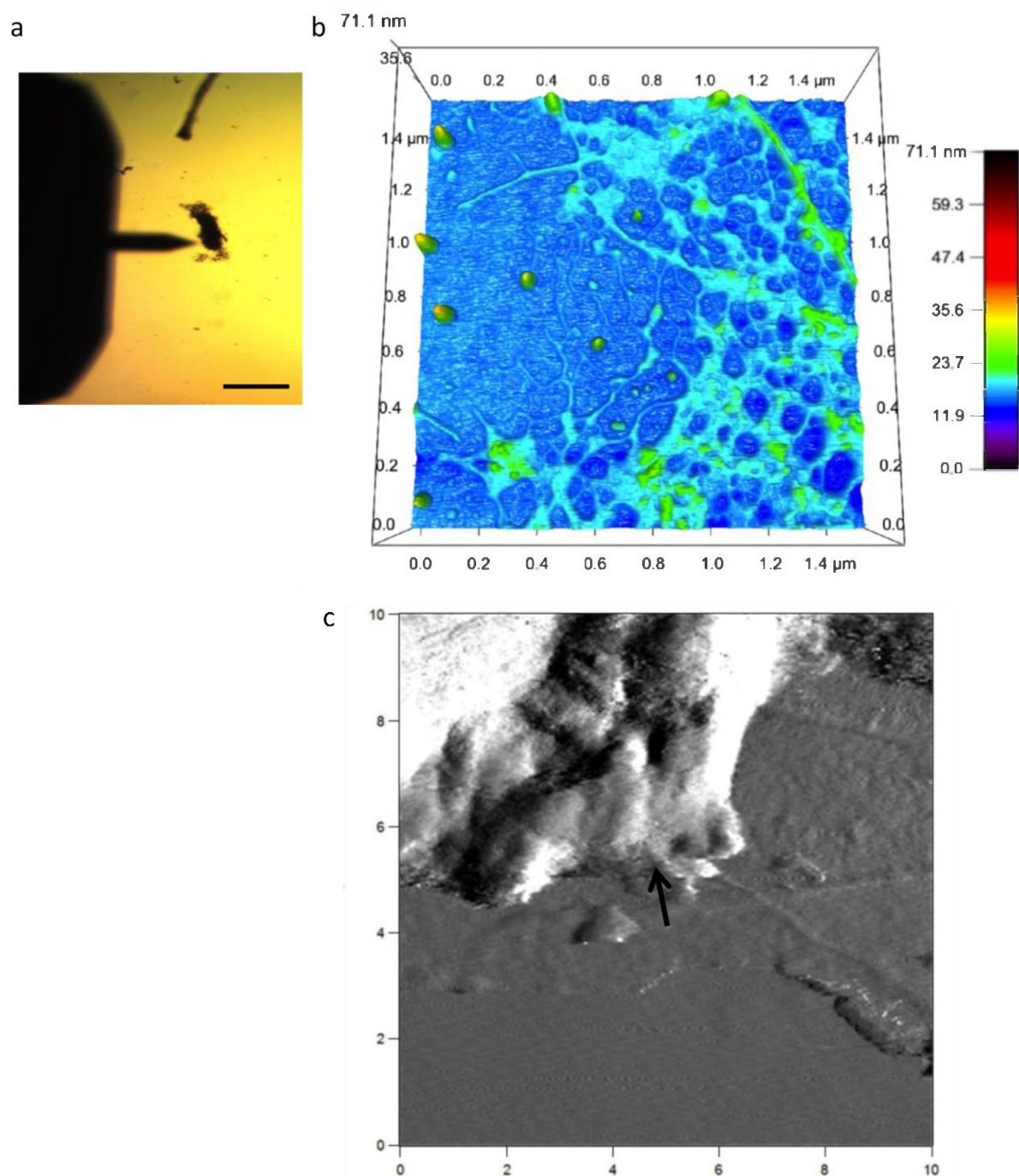
a

b

c

Fig. 1. Fecal pellet images by AFM. a) Transmitted light image of a fecal pellet at the AFM. The cantilever (arrow shape) is visible as a shadow on the left-side of the pellet. Scale bar 160 µm length. b) Topography image of the fine structure of peritrophic membrane in air. Color scale indicates Z (height), scan size is 1.5 x 1.5 µm². c) Peritrophic membrane in liquid (black arrow is point at the perimeter of the membrane). Amplitude channel, scan size is 10 x 10 µm².
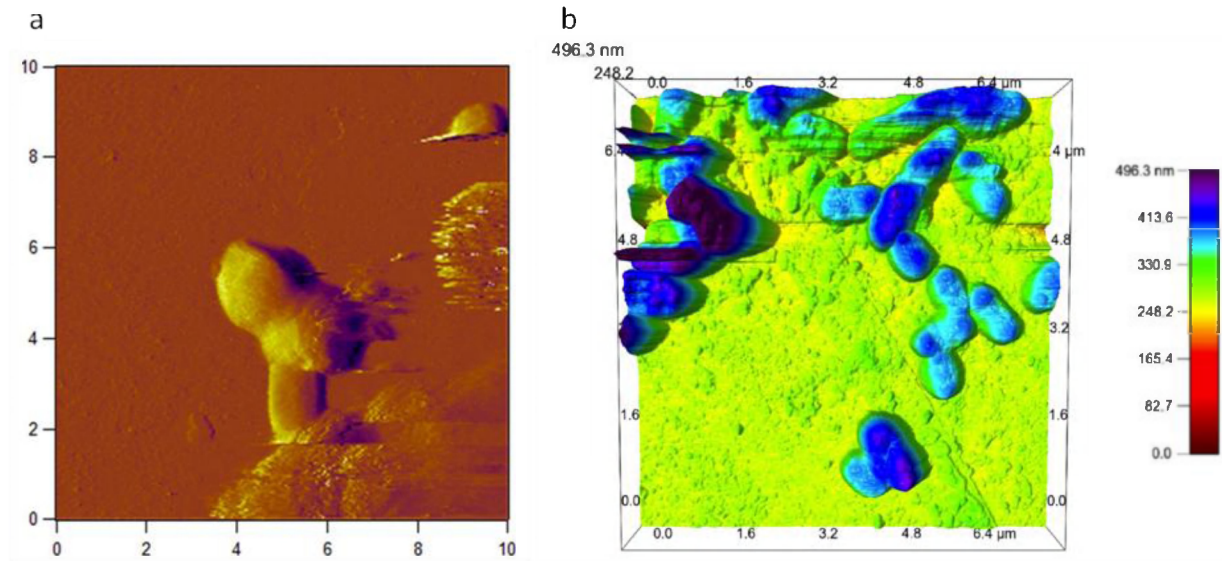
Fig. 2. Fecal pellets associated bacteria, images by AFM. a) Two bacteria imaged in liquid; a dividing cell adjacent to a rod shape cell (blue arrow is pointing to dividing cell). Amplitude channel, scan size 10 x 10 μm². b) Topographic image of bacteria in air. On the left side, some strips are visible indicating organic matter attaching to the cantilever tip thus influencing the scan quality. Color scale indicates Z (height), scan size 7 x 7 μm².

Peritrophic membrane

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

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

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

Fecal pellet bacteria

By AFM, we visualized bacteria associated with the peritrophic membrane (Fig. 2 a,b). We measured width (W), length (L) and height (Z) of 20 cells in liquid and 33 cells in air and then we have computed their cell volume (Table 1). Cell volume estimates, with either W≠Z or W=Z (see Methods), showed similar results when comparing liquid versus air measurements. When using the assumption W≠Z the average values (0.07± 0.084 μm³ in liquid and 0.04±0.342 μm³ in air) were statistically significantly different than those computed with W=Z (0.30±0.266 μm³ in liquid and 0.031±0.257 μm³ in air).

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

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

Fecal pellets organization and bacterial distribution

By LSCM, we were able to 'see' the pellet 3D-organization (Fig 3 a, b). The pellet is a highly heterogeneous microenvironment. The fecal pellet interior was composed by food residues (algae), uncharacterized fluorescent debris and bacteria (as single cells and colonies). A very dim yellow fluorescent signal was visible at the pellet perimeter, i.e. the location of the peritrophic membrane (as reported by (Yoshikoshi & Ko 1988)) due to the N-acetyl-glucosamine residue fluorescence.
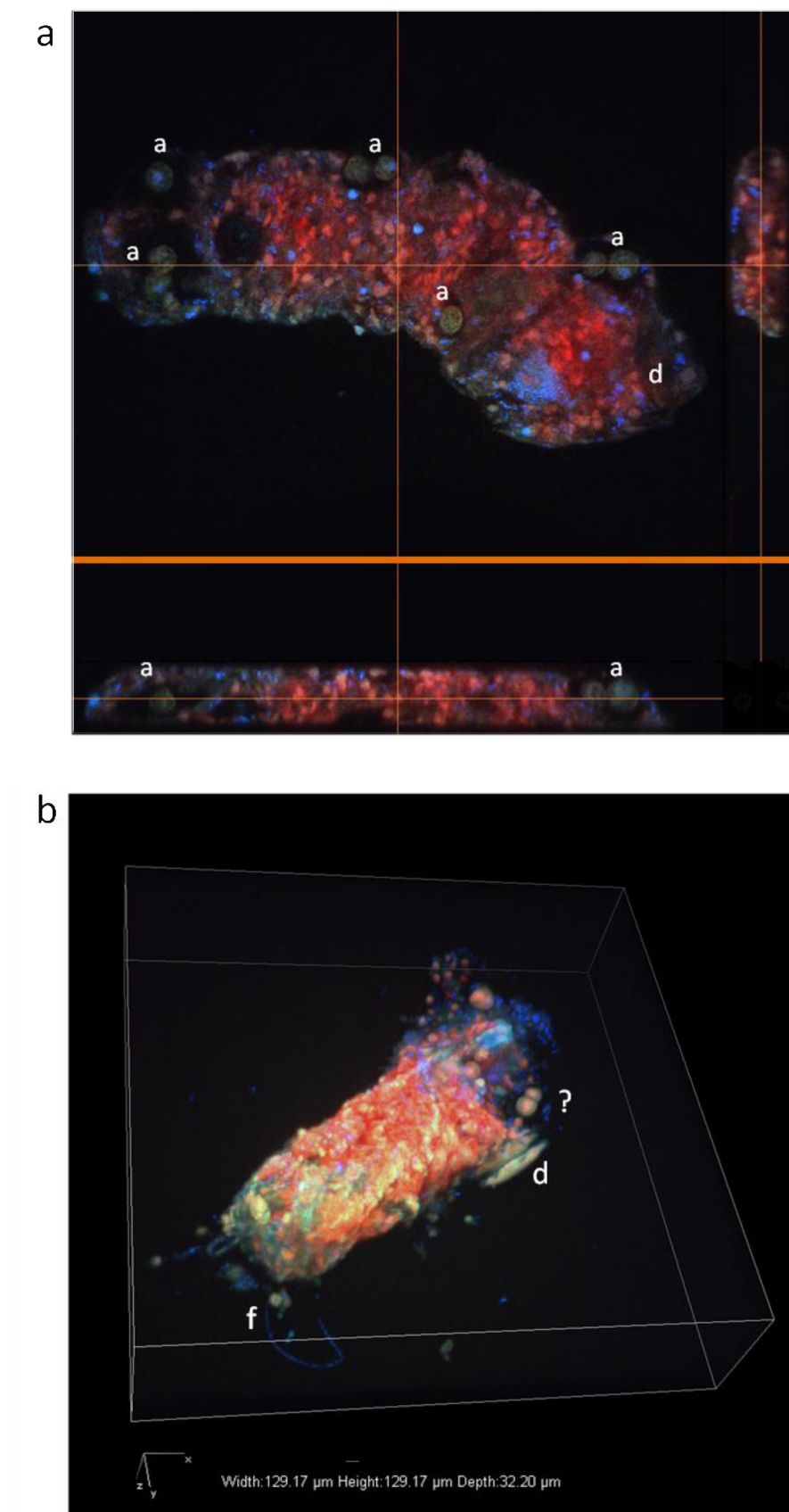
Fig. 3. Fecal pellets images by LSCM. a) 3D-sectioning of the pellet with top and side view details. Bacteria are stained with DAPI are blue, chlorophyll signal is red, microalgae (a) and diatom frustules (d) are visible; scale bar 5 μm. b) Volume rendering of a fecal pellet. Diatom frustule (d), microalgae (a) and filamentous bacteria (f) are visible.

DISCUSSION

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

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

According to the fecal pellet age, bacterial colonization can be very high. In our experiment, the fecal pellets were freshly egested in a sterile medium and we did not see an intense degree of bacterial colonization. The assumption W=Z (Bratbak 1985) gives larger volume than W≠Z, (Table 1). On the other hand, the average volume values in liquid and in air were similar i.e. 0.04 μm³ and 0.07 μm³ (assuming W≠Z), since the cells were dead. It has been shown that upon fixation and drying cell dimensions are deformed (Z collapses) but living cell volume has been found to be on average 3 time larger than dead cells (computed with W≠Z, Malfatti et al. 2010).

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

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

ACKNOWLEDGMENTS