**Bacteria associated with a marine planktonic copepod in culture. I. Bacterial genera in seawater, body surface, intestines and fecal pellets and succession during fecal pellet degradation**

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**Abstract.** In laboratory experiments, the bacterial flora of the zooplankton microbial environments seawater, fecal pellets and associated with the external and internal surfaces of the copepod *Acartia tonsa* (Dana) were examined. The bacteria associated with fecal pellets were dominated by *Bacillus* spp., *Cytophaga/Flavobacterium* spp., *Vibrio* spp. and *Pseudomonas* spp. The same genera were found in the seawater (0.22 μm filtered) in which the pellets were incubated. The bacteria showed a characteristic growth succession, and the abundance increased several orders of magnitude in the seawater during incubation of the pellets, indicating growth and proliferation based on the disintegrating/degrading fecal pellets. A carbon budget calculation revealed that organic matter from degrading fecal pellets could cover the carbon demand for the growing bacterioplankton. The composition of the bacterial community in the seawater and the fecal pellets also indicated a colonization of the pellets from bacterioplankton. The composition of the bacteria associated with the copepods showed that bacterial genera characterized as surface associated were preferentially associated with fecal pellets, animal surfaces and intestines. This suggests a specific intestinal flora in the cultivated copepods composed of 10^3 culturable bacteria per intestine (colony-forming units, c.f.u.) or 10^5 bacteria per intestine (acridine orange direct counts, AODC), possibly colonizing the intestine passively during filtration of algae. The activity of the bacterial communities was examined by the numerical ratio c.f.u.:AODC, where 1–19% of the bacteria were found active, with no significant difference between microbial environments.

**Introduction**

Generally, one-third of the suspended material ingested by filtrating zooplankton ends up as waste material, i.e. feces, constituting a considerable amount of organic matter (Smetacek, 1980; Jumars *et al.*, 1989; Noji, 1991).

Several studies report a massive colonization by bacteria on the surface of fecal pellets in the laboratory as well as *in situ* (Jacobsen and Azam, 1984; Lampitt *et al.*, 1990; Turner, 1991). After 2–3 days, the bacterial numbers reached a saturation level (Jacobsen and Azam, 1984).

Body surfaces of crustacean zooplankton are covered by bacteria, especially the oral region and the appendages (Huq *et al.*, 1983; Nagasawa, 1988), indicating bacterial exploitation of labile dissolved organic carbon (DOC_l) released from sloppy feeding and defecation. Generally, these bacteria are considered active (Carman, 1994) and represent genera known to attach to surfaces (e.g. Huq *et al.*, 1983).

There are different opinions about whether the source of bacteria associated with pellets is inside the fecal pellet matrix, arising from the intestine (Gowing and Silver, 1983), or associated with fecal pellet surfaces, indicating colonization by pelagic bacteria (Jacobsen and Azam, 1984). The bacteria degrading the feces can simply reflect the actual bacterioplankton probably colonizing the fecal pellets.
during sedimentation (Jacobsen and Azam, 1984). However, the feces can already be inoculated in the intestine and microbial degradation initiated before egestion by either (i) the ingested bacteria or (ii) a distinct intestinal flora (Olafsen, 1984; King et al., 1991; Nagasawa, 1992). Bacterial grazing is a significant energetic source for cladocerans, but not for copepods, hence excluding a significant passive inoculation of the pellets by intestinal bacteria (Hessen, 1985; Berggreen et al., 1988; Nagasawa and Nemoto, 1988). Photographic evidence for a specific intestinal flora in copepods (Nagasawa and Nemoto, 1988; Gowing and Wishner, 1992; Nagasawa, 1992), in prawns and crabs (Harris, 1993), in juvenile fish (Seki, 1969; Horsley, 1977; Hansen et al., 1992), and microbiological evidence for intestinal bacteria in cladocerans and copepods (Sochard et al., 1979; King et al., 1991) has been put forward, but Gowing and Wishner (1986) found no attached bacteria in benthopelagic calanoids, and Nagasawa (unpublished data referred to in Nagasawa and Nemoto, 1988) and Boyle and Mitchell (1978) claimed that there were no intestinal bacteria in marine crustaceans at all. A recent paper by Nagasawa (1992) suggests that some pelagic copepod species do not have an enteric microflora and their fecal material is even colonized slowly due to an inhibitory process.

The vast majority of studies have dealt with bacteria from a quantitative aspect as only a small fraction will grow on traditional microbial media (Jannash and Jones, 1959; Buch, 1979; Austin, 1988). Bacterioplankton species or genera degrading the fecal pellets and associated with zooplankton and their feces have not been investigated thoroughly (King et al., 1991). This is imperative to understand the source of the intestinal microflora. In the present study, we describe the bacterial succession associated with fecal pellets, and evaluate the quantitative as well as the qualitative significance of a specific intestinal microflora.

Method

Cultivation of algal food and copepods

A continuous culture of the marine planktonic copepod Acartia tonsa was kept in a walk-in cold room at 18°C in 100 l tanks with 30 p.p.t. seawater. The culture tanks were provided with a bubble device to ensure water circulation and suspension of food particles [the haptophyte Rhodomonas baltica, diameter 7 μm, cultured in logarithmic growth phase in B1 medium (Hansen, 1989)]. Cultivation of copepods and food algae are described in detail in Støttrup et al. (1986).

Experiment I: Bacterial succession during fecal pellet degradation

To describe the bacterial succession associated with fecal pellets, ~400 pellets produced within 20 min by well-fed copepods (algal concentration >> intestinal saturation) were isolated. The copepods were observed in a dissecting microscope (20× magnification) equipped with cold light device. Each fecal pellet was collected immediately after egestion, pipetted onto a small dish and washed in sterile seawater. The fecal pellets were quickly video recorded through an inverted microscope (40× magnification) for later measurements of volumes (Sony CCD camera, Sony Trinitron monitor, Panasonic 6200 recorder). Batches of 20 fecal pellets were pipetted into 20 ml acid-cleaned and muffled glass vials with 0.22 μm
Bacteria associated with a marine planktonic copepod in culture. I

filtered seawater. In addition, a series of controls consisting of seawater without fecal pellets were run. The vials were topped up with water to avoid air bubbles and mounted on a plankton wheel (2 r.p.m.) to keep the fecal pellets suspended. During 90 h of incubation, the vials were regularly inspected for bacterial number, cell size and species succession (four times during the first day and two times per day during the rest of the period).

To isolate bacteria associated with fecal material, batches of fecal pellets were gently transferred to test tubes containing 3 ml of 0.22 μm filtered seawater. The suspensions were instantly sonicated (Branson Sonifier B12) for 4 s at 45 W in an ice bath in order to disrupt the fecal pellets without damaging the bacteria (King et al., 1991). Prior to the experiment, the significance of the procedure was tested for Vibrio-like bacteria in seawater on TCBS-agar (thiosulfate-sucrose-citrate-bile-sucrose-agar, Difco) (Figure 1).

For bacterial identification and enumeration, 1 ml from the sonicated suspensions and 1 ml from the seawater were diluted 10-fold by 0.22 μm filtered seawater, and 0.1 ml was spread plated on marine agar (MA) (Difco). The plates were incubated for 1 week at 18°C before colony-forming units (c.f.u.; number of culturable bacteria) were registered on five replicate plates (30–300 colonies on the plates). The c.f.u. were calculated as mean ± SD (Nimela, 1983). For every sample, c.f.u. were separated into groups by colony morphology criteria, and isolates were picked randomly and transferred to marine broth (75% seawater and 25% tap water; Schneider and Rheinheimer, 1988). All isolates were incubated for 48 h and tested biochemically following the criteria from Austin (1982) unless otherwise stated. All isolates were incubated at 20°C with daily inspection for 3 weeks, except where otherwise stated. Gram stain was performed after Gregersen (1978). The presence of catalase was tested with 3% H₂O₂. The presence of cytochrome C oxidase was tested by Kovacs method (see Gerhardt, 1994). Hemolysis and the purity of the cultures were tested by growth on blood agar base (BA) (Difco) containing 5% defibrinated calf blood. Some strains could not grow on BA, and were replated on marine agar (Difco). Morphology and motility were tested by phase-contrast microscopy on fresh broth culture. H₂S production was tested in triple sugar iron agar (TSI) (Merck). Arginine degradation was tested in bacto decarboxylase base (Difco) with the addition of L-arginine. Tolerance to 0 and 4% NaCl and growth at 37°C were tested in marine broth cultures, while the oxidative ability was tested in marine O/F medium (MOF) (Leifson, 1963). Growth on TCBS was observed on TCBS-agar (Difco) every day during a week. Strains able to grow on TCBS were tested for sensitivity to O/129 vibriostatic agent with diagnostic tablets (Rosco, Denmark) on BA.

To enumerate the total number of bacteria associated with the fecal pellets and suspended in the seawater, a volume of 3 and 10 ml was isolated from the sonicated fecal pellet suspension and from the seawater, respectively. The suspensions were fixed in 1% formalin final concentration and stored in a refrigerator. Before counting, the samples were filtered onto 0.2 μm black Nuclepore filters and stained with acridine orange (Hobbie et al., 1977). The bacteria were counted under an epifluorescence microscope (1250× magnification), ensuring 300 cells or 30 fields per slide. Bacterial biovolume was calculated from size measurements of enlarged
micrographs and calibrated with an object micrometer. Three size classes of bacteria were distinguished: small, 0.2–0.5 μm diameter; intermediate, 0.5–1 μm diameter; large, >1 μm diameter. Bacterial biomass was calculated assuming a variable carbon content per cubic micrometer for bacteria > 0.07 μm$^3$ and 20 fg C cell$^{-1}$ for the small bacteria (Lee and Fuhrmann, 1987; Simon and Azam, 1989).

**Experiment II: Bacteria associated with the copepod**

A batch of 45 females (mean ± SD cephalothorax length = 859 ± 42 μm, $n = 27$) was isolated using a dissecting microscope (20× magnification) by pipette into Petri dishes with sterile seawater for defecation. After emptying their guts for ~2 h, 24 females were video recorded for body volume measurements and thereafter chlorinated to ensure that bacteria associated with external surfaces of the animals were killed (King et al., 1991). After sonication of the whole animals, the intestinal microflora was examined. The remaining 21 females were only sonicated, and surface-associated bacteria examined by assuming that the difference between chlorinated animals and non-chlorinated animals represented the surface-associated bacterial community (King et al., 1988, 1991). Fecal pellets ($n = 40$) were isolated and treated as in the previous experiment. A sample of 3 ml seawater was taken from the culture tank and sonicated for examination of c.f.u. in the seawater.

To compare the density of bacteria associated with the different microbial environments of the copepods, the biovolumes of animals were calculated from the linear dimensions measured on the videomonitor (Chojnasky, 1983). Volumes of intestines and fecal pellets were estimated following geometrical formulae of tubes and cylinders with half spheres at the ends, respectively. Counting and identification of the bacteria by c.f.u. and acridine orange direct counts (AODC) followed the same procedure as in experiment I.
Bacteria associated with a marine planktonic copepod in culture. I

Results

Experiment I

The numbers of bacteria associated with the fecal pellets are plotted as mean values without error bars. Generally, the SDs were within 10–20% of the mean c.f.u. and due to the counting procedure no SD could be calculated for AODC. In general, the development of bacterial numbers from plate countings and direct countings showed similar curve forms (Figure 2). The number of bacteria associated with fecal pellets increased slowly. After a short lag phase, the total number (AODC) and the culturable number of bacteria (c.f.u.) in the seawater increased two orders of magnitude and reached a maximum plateau after ~40 h. The mean c.f.u.:AODC ratio associated with pellets was 0.05 ± 0.04 and in the seawater 0.19 ± 0.19 (Figure 2). In control vials without fecal material, the bacterial abundances were initially two orders of magnitude lower than in the experimental vials (AODC) and remained constant during incubation (data not shown).

Bacteria associated with fecal pellets were small (50–75%) at delivery of fecal pellets, and the larger sized bacteria and in particular the intermediate sized bacteria increased relatively in number during incubation (data not shown). The bacterial size distribution in the seawater was 35% small, 25% intermediate and 40% large bacteria, and it did not change during incubation.

It was possible to characterize all the bacteria associated with fecal pellets and seawater during incubation by criteria listed in Austin (1982) and Bergey’s Manual Volumes I–III (1984–89) (Table I). Fifty-six isolates were characterized to 11 different genera with 90% similarity; three genera were numerically insignificant (Methylococcus, Flexibacter, Alcaligenes). The relative distributions of the other eight genera are shown in Figure 2. Colonies and cell morphology of the Cytophaga/Flavobacterium isolates were rather identical, and the biochemical testing was not sufficient to separate the isolates into two genera. Furthermore, it was very difficult to distinguish the gliding motility of Cytophaga spp. from the non-motile Flavobacterium spp. in broth cultures with fluid movements. Finally, the two genera are poorly characterized taxonomically (Holmes et al., 1984; Reichenbach, 1989) so we found it appropriate to group them together, as done by other authors (e.g. Austin, 1988). During the incubations, a bacterial succession was observed associated with the fecal pellets (Figure 2). Vibrio spp. as well as Pseudomonas spp. increased, Corynebacterium spp. and Bacillus spp. decreased, and Cytophaga/Flavobacterium spp. fluctuated. Bacillus spp. and Cytophaga/Flavobacterium spp. were the predominating bacteria. The succession in the filtered seawater showed that Vibrio spp. was the predominant bacteria with a rather constant ratio of relative number (Figure 2). Alteromonas spp. increased, while Pseudomonas spp. predominated after 89 h. Cytophaga/Flavobacterium spp. only appeared sporadically in the water, and after 26 h they disappeared totally from the water phase, while at the same time this group was dominating among the bacteria associated with the fecal pellets. The level of Aeromonas-like bacteria was generally constant in the first 48 h, thereafter it declined.

The number of c.f.u. and the total number of bacteria (AODC) in the microbial environments associated with the copepods are listed in Table II. The c.f.u. was
Fig. 2. Abundance of bacteria (upper left) and qualitative composition (lower left) associated with the incubation water, and abundance of bacteria (upper right) and qualitative composition (lower right) associated with fecal pellets during fecal pellet degradation in 0.22 μm seawater. Solid symbols = AODC; open symbols = c.f.u.
Table I. Diagnostic table for the identification of bacteria associated with fecal pellets produced by the copepod *A. tonsa*

<table>
<thead>
<tr>
<th>Taxon</th>
<th>No. of isolates</th>
<th>Gram stain</th>
<th>Motility</th>
<th>Fermentative (F)</th>
<th>Oxidative (O)</th>
<th>Catalase production</th>
<th>Hemolysis on BA</th>
<th>Sensitivity to O1/2</th>
<th>Arginine</th>
<th>Growth at 37°C</th>
<th>Growth on TCBS-agar</th>
<th>Growth on 0% (w/w) NaCl</th>
<th>Growth on 4% (w/w) NaCl</th>
<th>Glucose fermentation</th>
<th>H₂S production</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio</em> spp.</td>
<td>8</td>
<td>-</td>
<td>+</td>
<td>F +</td>
<td>+</td>
<td>d +</td>
<td>+ +</td>
<td>- +</td>
<td>d +</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Aeromonas</em> spp.</td>
<td>2</td>
<td>-</td>
<td>d</td>
<td>F +</td>
<td>+</td>
<td>d +</td>
<td>n +</td>
<td>d -</td>
<td>d -</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Alteromonas</em> spp.</td>
<td>8</td>
<td>-</td>
<td>+</td>
<td>O +</td>
<td>+</td>
<td>n -</td>
<td>+ +</td>
<td>- +</td>
<td>d -</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.</td>
<td>10</td>
<td>-</td>
<td>+</td>
<td>O d</td>
<td>d</td>
<td>- n</td>
<td>+ +</td>
<td>- d</td>
<td>d -</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Alcaligenes</em> spp.</td>
<td>1</td>
<td>-</td>
<td>+</td>
<td>O +</td>
<td>+</td>
<td>+ n</td>
<td>- +</td>
<td>- +</td>
<td>+ -</td>
<td>-</td>
<td>d +</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Corynebacterium</em> spp.</td>
<td>5</td>
<td>+</td>
<td>-</td>
<td>O +</td>
<td>+</td>
<td>- n</td>
<td>+ d</td>
<td>- -</td>
<td>- +</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. 1</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>F +</td>
<td>+</td>
<td>+ n</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. 2</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>O +</td>
<td>+</td>
<td>+ n</td>
<td>+ +</td>
<td>- -</td>
<td>+ +</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Flexibacter</em> spp.</td>
<td>2</td>
<td>-</td>
<td>+</td>
<td>O +</td>
<td>+</td>
<td>n -</td>
<td>+ +</td>
<td>- -</td>
<td>+ +</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Methyllobacterium</em> spp.</td>
<td>2</td>
<td>-</td>
<td>?</td>
<td>+ +</td>
<td>?</td>
<td>n -</td>
<td>+ +</td>
<td>- +</td>
<td>+ +</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Acinetobacter</em> spp.</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>O +</td>
<td>-</td>
<td>n +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Cytophaga/Flavobacterium</em> spp.</td>
<td>8</td>
<td>-</td>
<td>d</td>
<td>d +</td>
<td>d</td>
<td>- n</td>
<td>- d</td>
<td>- -</td>
<td>- -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+, ≥90% of strains are positive.
-, ≤90% of strains are negative.
d, 11–89% strains are positive.
?, very weak signal.
n, not determined.

Table II. Number of bacteria in the microbial environments associated with the copepod *A. tonsa* registered by culturable bacteria (c.f.u.) and direct counts (AODC). The ratio c.f.u.:AODC indicates the bacterial culturability (Albrechtsen and Winding, 1992). For comparison between the milieus, the bacterial numbers are given in terms of bacteria per biovolume μm⁻³

<table>
<thead>
<tr>
<th>Milieu</th>
<th>CFU Numerical</th>
<th>Volume μm⁻³</th>
<th>AODC Numerical</th>
<th>Volume μm⁻³</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seawater</td>
<td>0.32 ± 0.06 x 10⁴</td>
<td>3 x 10⁻³</td>
<td>1.04 x 10⁴</td>
<td>1 x 10⁻⁴</td>
<td>3</td>
</tr>
<tr>
<td>Animal surface</td>
<td>0.79 ± 0.06 x 10⁴</td>
<td>2 x 10⁻³</td>
<td>7.87 x 10⁴</td>
<td>2 x 10⁻⁴</td>
<td>10</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.11 ± 0.04 x 10⁴</td>
<td>1.2 x 10⁻³</td>
<td>12.1 x 10⁴</td>
<td>1.4 x 10⁻⁴</td>
<td>1</td>
</tr>
<tr>
<td>Fecal pellets</td>
<td>0.61 ± 0.08 x 10⁴</td>
<td>2.1 x 10⁻³</td>
<td>5.26 x 10⁴</td>
<td>1.8 x 10⁻⁴</td>
<td>12</td>
</tr>
</tbody>
</table>

~0.6 x 10⁴ bacteria per fecal pellet for freshly egested fecal pellets. The copepod surface was colonized by 0.8 x 10⁴ bacteria ind.⁻¹, and the intestines were host to 0.1 x 10⁴ bacteria. The biovolume specific bacterial abundance showed that 5% (c.f.u.) and 65% (AODC) of the bacteria were associated with the intestines as.
Table III. Diagnostic table for the identification of bacteria from the different microbial environments associated with the copepod *A. tonsa*

<table>
<thead>
<tr>
<th>Taxon</th>
<th>No. of isolates</th>
<th>Gram stain</th>
<th>Motility</th>
<th>Fermentative (F)oxidative (O)</th>
<th>Oxidase production</th>
<th>Catalase production</th>
<th>Hemolysis to O/129</th>
<th>Arginine</th>
<th>Growth at 37°C</th>
<th>Growth on TCB agar</th>
<th>Growth on 0% NaCl</th>
<th>Growth on 4% NaCl</th>
<th>Glucose fermentation</th>
<th>H₂S production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pin-point colonies</td>
<td>3</td>
<td>d</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>n</td>
<td>-</td>
<td>-</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio-like</td>
<td>8</td>
<td>-</td>
<td>+</td>
<td>F</td>
<td>+</td>
<td>d</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio spp.</td>
<td>12</td>
<td>-</td>
<td>+</td>
<td>F</td>
<td>+</td>
<td>d</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aeromonas spp.</td>
<td>7</td>
<td>-</td>
<td>d</td>
<td>F</td>
<td>+</td>
<td>d</td>
<td>n</td>
<td>+</td>
<td>-</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Photobacterium spp.</td>
<td>7</td>
<td>-</td>
<td>+</td>
<td>F</td>
<td>+</td>
<td>-</td>
<td>n</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alteromonas spp.</td>
<td>5</td>
<td>-</td>
<td>+</td>
<td>O</td>
<td>+</td>
<td>d</td>
<td>n</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>9</td>
<td>-</td>
<td>+</td>
<td>O</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>n</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alcaligenes spp.</td>
<td>2</td>
<td>-</td>
<td>?</td>
<td>O</td>
<td>+</td>
<td>+</td>
<td>n</td>
<td>-</td>
<td>+</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Corynebacterium spp.</td>
<td>8</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus spp.</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>n</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytophaga/Flavobacterium spp.</td>
<td>16</td>
<td>-</td>
<td>d</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>n</td>
<td>+</td>
<td>-</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Symbols as in Table I.

compared to the fecal pellets. The bacterial culturability, registered as c.f.u.:AODC, suggests that only 1% of the intestinal microflora was active, implying that the medium could sustain bacterial growth.

**Experiment II**

A total of 74 isolates were taken from the four samples representing the bacteria associated with the seawater, the surface and the intestines of the copepods and the fecal pellets. The isolates were characterized by the same criteria as in the former experiments. Two isolates failed to grow in all media and were therefore omitted. The isolates were classified into 11 groups identified with 90% similarity as the genera shown in Table III. A large number of unidentified ‘pin-point’ colonies were found in the water sample wherein the copepods resided. Three isolates were tested, but they reacted weakly or not at all during the test procedures and were omitted.

The composition of the bacterial populations in the seawater showed that 63% of the bacteria were pin-points (the main part of ‘others’, of which the rest were *Bacillus* spp. and *Alcaligenes* spp.) (Figure 3). These bacteria were not associated with the copepods and the fecal pellets. Practically all the identified taxa were associated with all environments, except that no *Corynebacterium* spp. were found at the surface of the copepods. There was a qualitative overlap between the com-
Bacteria associated with a marine planktonic copepod in culture. I

Fig. 3. Bacterial composition in terms of percentage of the population in the different microbial environments (Water = seawater; Surface = whole animals + intestine; Intestine = chlorinated animals; Feces = fecal pellets) associated with the copepod *A. tonsa* in culture. Abbreviations are as in Figure 3, but with some genera missing and some new. Others = mainly unidentified pin-point colonies.

The size distribution of the bacteria showed that a majority of the cells were 0.2–0.5 µm in diameter (Figure 4). In the seawater, there were relatively fewer small bacteria than those associated with copepods. The relative cell size distributions in the seawater, at the animal surfaces and in the intestines were similar with a dominance of small cells and ~20% intermediate cells and 9% large cells. The bacteria associated with fecal pellets were significantly smaller, however, with no large rods or cocci present.

**Discussion**

*Fecal pellet degradation*

The disintegration/degradation of fecal pellets caused a 5–25 times increase in the number of associated bacteria registered by c.f.u. and AODC. When considering...
the number of bacteria per volume of habitat, a factor of 30 times the initial bacterial density was found by direct counts (Figure 2). This was also observed in a study by Jacobsen and Azam (1984), where bacteria enumerated by AODC reached $10^9$ per fecal pellet (volume $3 \times 10^6 \mu m^3$) after 24 h. We found $5 \times 10^8$ bacteria per fecal pellet in experiment I and $0.5 \times 10^8$ bacteria per fecal pellet in experiment II (mean fecal pellet volume $3 \times 10^6 \mu m^3$), revealing the same order of magnitude. Jacobsen and Azam argue that due to sedimentation the fecal pellets catch up bacteria passively at a significant rate. A simple calculation of fecal pellets incubated in seawater in the present study can ascribe for 30–65% of the bacterial increase on the surface of the pellets assuming a fecal pellet volume of $3 \times 10^6 \mu m^3$ with a sinking speed of 50 m day$^{-1}$ (Paffenhofer and Knowles, 1979) at the actual bacterial density, assuming all bacteria encountered by the fecal pellets to adsorb to the surface. This indicates that the bacterial increase in association with fecal pellets includes adsorption and growth in combination.

Whether fecal pellet-associated bacteria are just surface associated or embedded in the matrix is unclear. Honjo and Roman (1978) found no bacteria inside pellets and Jacobsen and Azam (1984) claim, based on 24 h incubations, that the bacteria are associated with the surface only (0.1–0.4 bacteria $\mu m^{-2}$ from $t_0$ to $t_2$, equal to 5–27% of the fecal pellet surface). However, bacteria were found inside the fecal pellets from *Eucalanus bungii* and *Calanus pacificus* (Nagasawa and Nemoto, 1988; Lawrence *et al.*, 1993), and recently anaerobic bacteria have been observed inside mesozooplankton pellets (Bianchi *et al.*, 1992). In the present study, we can calculate that 3–7 bacteria were present per $\mu m^2$ on newly egested fecal pellets, increasing to 172 bacteria $\mu m^{-2}$ during incubation, assuming they are only surface associated. If the bacteria are 0.2 $\mu m$ in diameter (much less than we
Bacteria associated with a marine planktonic copepod in culture. I

actually measured), one layer of bacteria accounts for 25 bacteria $\mu m^{-2}$, leading to a surface coverage of seven layers of bacteria in the biofilm after several days. On the other hand, if the bacteria are evenly distributed in the matrix of the pellets and not surface associated, there is only room for the observed 3 bacteria $\mu m^{-3}$ (Figure 2) if 45% of the fecal pellet matrix is bacterial biomass. These conservative calculations suggest that the bacterial community is mostly surface associated, without excluding the possibility of bacteria inside the fecal pellets (Gyllenberg and Lundquist, 1978; Johnson et al., 1982; Lawrence et al., 1993).

The numerical response of bacteria in seawater showed saturation developments. This is in agreement with what has been described elsewhere (Jacobsen and Azam, 1984; Lawrence et al., 1993). Virtually all of the bacterial biomass can be ascribed to carbon originating from the newly egested fecal pellets in the present study if (i) all the pellet carbon was mineralized, (ii) the fecal pellet and the bacterial carbon:volume was 0.5 and 0.22 pg C $\mu m^{-3}$, respectively, and (iii) with a bacterial growth yield of 0.4 (Middelboe and Søndergaard, 1993) (Table IV).

In association with fecal pellets, a succession with increasing significance of *Vibrio* spp. was observed (Figure 2). These bacteria are described as being very common in seawater and to attach to surfaces (Huq et al., 1983; Austin, 1988). *Alteromonas* spp. were slightly increasing, not found internally in zooplankton or fish, but common in coastal waters and in oysters (Austin, 1982; Olafsen et al., 1993). *Pseudomonas* spp. were also increasing in association with fecal material. Bacteria from this variable genus are, however, sometimes described as not adhering to surfaces (Huq et al., 1983) and sometimes to be the predominating bacteria on copepod fecal pellets (Sochard et al., 1979). *Pseudomonas* spp. were associated with internal organs of cladocerans, fish and oysters (Sanders and Fryer, 1988; King et al., 1991; Olafsen et al., 1993) and were non-viable in the intestines of zooplankton (King et al., 1991), suggesting that the bacteria colonizing the fecal pellets (in the intestine) arise from the seawater and not from the intestinal environment. *Aeromonas* spp. are negligible in association with pellets, but not in seawater (Figure 3), which is surprising as aeromonads often are related to freshwaters and coastal waters (Larsen and Willeberg, 1984). Nevertheless, *Aeromonas* spp. have been isolated from marine fish (Larsen and Jensen, 1977; Saunders and Fryer, 1988), and this genus may have a tolerance to higher salinity than earlier expected (6 p.p.t., J.L.Larsen, personal communication). *Cytophaga/Flavobacterium* spp. dominated the pellets, and were also found in seawater as well as in the gut of *A.tonsa* reared in other laboratories (5.7% of the total bacterial counts; Sochard et al., 1979). *Corynebacterium* spp. were present in the pellets, and are described as being resistant to the internal environment in zooplankton (King et al., 1991). The frequently observed *Bacillus* spp. are also from zooplankton fecal pellets commonly associated with fish intestines (Sanders and Fryer, 1988).

The seawater only contained four genera, while the newly egested fecal pellets contained eight genera at the initiation of the incubation (Figure 2). The observed *Vibrio* spp., *Pseudomonas* spp., *Acinetobacter* spp. and *Aeromonas* spp. in the seawater are all known to be rapidly proliferating in eutrophic environments (Bau- man and Schubert, 1984; Juni, 1984; Palleroni, 1984). This could explain why they are found at $t_o$, while the bacteria more typically related with the fecal pellets and
Table IV. Carbon budget for bacterial growth based on DOC\textsubscript{c} leaked and mineralized from fecal pellets produced by *A. iona*. Data on fecal pellet number and mean volume, bacterial number and bacterial size are from the present study.

<table>
<thead>
<tr>
<th>Number</th>
<th>Fecal pellet carbon input\textsubscript{fp}</th>
<th>Bacterial output\textsubscript{bo}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 pellet ml\textsuperscript{-1} incubated</td>
<td>Fecal pellet\textsubscript{fp}: 3 \mu m\textsuperscript{3} x vol = 0.9 \times 10\textsuperscript{6} ml\textsuperscript{-1}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Incubation water\textsubscript{w}: 1 \times 10\textsuperscript{6} ml\textsuperscript{-1}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total\textsubscript{t}: 1.9 \times 10\textsuperscript{6} ml\textsuperscript{-1}</td>
</tr>
<tr>
<td>Mean volume (\mu m\textsuperscript{3})</td>
<td>0.297 \times 10\textsuperscript{6}</td>
<td>0.15 \times 1.9 \times 10\textsuperscript{6} ml\textsuperscript{-1}</td>
</tr>
<tr>
<td>Carbon:volume (pg C \mu m\textsuperscript{3})</td>
<td>0.5\textsuperscript{a} = 0.15 \mu g C ml\textsuperscript{-1}</td>
<td>0.35 \times 0.1 \mu g C ml\textsuperscript{-1}</td>
</tr>
<tr>
<td></td>
<td>0.39\textsuperscript{a} = 0.12 \mu g C ml\textsuperscript{-1}</td>
<td>0.1 \mu g C ml\textsuperscript{-1}</td>
</tr>
<tr>
<td></td>
<td>0.05\textsuperscript{a} = 0.015 \mu g C ml\textsuperscript{-1}</td>
<td>0.22 \times 0.063 \mu g C ml\textsuperscript{-1}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Total bacterial volume was calculated from the relative size distribution and corresponding mean volumes at 0.02 and 0.2 \mu m\textsuperscript{3} for small and intermediate sized bacteria, and 0.28 \mu m\textsuperscript{3} (the mean measured size) for the large bacteria.

\textsuperscript{b} Lampitt et al. (1990) *Centropages hamatus*; B. Hansen (unpublished) *Acartia longiremis*.

\textsuperscript{c} Hansen et al. (1996).

\textsuperscript{d} Gonzales and Smetacek (1994).

\textsuperscript{e} Bjørnsen et al. (1986).

\textsuperscript{f} Bratbak and Dundas (1984).

copepod intestines (*Corynebacterium*, *Bacillus* and *Cytophaga/Flavobacterium* spp.) appeared in the water phase only after 2.5 h (Figure 2). Other explanations for the initial four genera can be ascribed to either a rapidly proliferating inoculation from the fecal pellets or, more speculatively, ultramicrobacterial 'dwarf' cells passing the 0.22 \mu m filter and increasing in size and number (Kjelleberg et al., 1987). After 2.5 h, the number of genera in the seawater was already eight, which was constant during the rest of the incubation. This indicated that bacteria from the fecal pellets inoculated the filtered seawater, and either the fecal pellets delivered an inoculum of bacteria during degradation or the bacteria were budding off and the pellets acted as 'baby machines' (Jacobsen and Azam, 1984). We cannot conclude whether the bacteria arise from the surface of the fecal pellets or from the fecal pellet matrix, but they probably originate from both sources (Nagasawa, 1992).

All the genera growing in the 0.22 \mu m filtered seawater were also present in the copepod intestines (Figure 3) and possibly could inoculate the fecal pellets before defecation.

The detailed bacterial succession in the seawater was characterized by an increase in the relative number of *Alteromonas* spp., and a decrease in *Acinetobacter* spp. *Cytophaga/Flavobacterium* spp. were constant, they were simultaneously the most abundant on the fecal pellets. This fecal pellet association can possibly be ascribed to the ability to degrade cellulose by *Cytophaga* spp. (Reichenbach and Dworkin, 1981). In 0.22 \mu m filtered seawater, *Pseudomonas* spp. increased at the
Bacteria associated with a marine planktonic copepod in culture. I

end of incubation, indicating that these bacteria are not solely surface dependent, emphasizing the dynamics between fecal material and seawater.

Bacteria associated with the copepods

The bacterial number in the seawater was \(1 \times 10^7\) ml\(^{-1}\) (AODC), a factor of two higher than in eutrophic coastal areas (Fenchel, 1988). We worked with a culture with algal food concentration above intestinal saturation (Kiørboe et al., 1985) and an unknown amount of detritus, so we expected high bacterial densities in the seawater and also in association with the copepods (Huq et al., 1983; Jumars et al., 1989). The bacterial number associated with animal surfaces was similar to c.f.u. counts on field-caught neritic copepods: \(<10^2-3 \times 10^4\) ind.\(^{-1}\) (Sochard et al., 1979).

The reported range of c.f.u. from individual copepod intestines are from zero to \(10^6\) bacteria per intestine, mostly \(>10^2\) per intestine (Sochard et al., 1979), and for freshwater cladocerans in the range of \(10^2-10^3\) per intestine (King et al., 1991). These numbers are, however, orders of magnitude lower than densities reported photographically (Nagasawa and Nemoto, 1988; Nagasawa, 1992). The discrepancy could be ascribed to the recent discovery of intestines harboring a population of anaerobic or autotrophic bacteria, not registered by the plate count technique (Bianchi et al., 1992).

The pin-point bacteria (Figure 3) were either very slow-growing bacteria belonging to the genera described, or different bacterial species not willing to grow on plates. It is noteworthy that they only occurred in the seawater in this particular part of the study. They could not be identified in the bacterial size distribution (Figure 4). The most significant distinction between the microbial environments associated with copepods is that \(Vibrio\) spp. and \(Pseudomonas\) spp. apparently were associated with surfaces. This has been described before for isopods and copepods (Huq et al., 1983; El-Shanshoury et al., 1994), and for skin and intestinal mucus from wild fish (Olsson et al., 1992). Some of the bacterial strains are chitin digesting and cause damage to crustacean exoskeletons (Nagasawa, 1988; Sanders and Fryer, 1988). The significance of \(Cytophaga/Flavobacterium\) spp. on the copepod surfaces, fecal pellets and intestines was also described on field-caught as well as on laboratory-reared copepods (Sochard et al., 1979). The association of \(Corynebacterium\) spp. with the intestine was similar in cladocerans and was described as being resistant to the internal micromilieu (King et al., 1991). \(Pseudomonas\) spp. were, however, suggested to be either non-viable or to be digested by cladocerans. These qualitative similarities between our study and other investigations suggest that the isolated and identified bacterial genera are either just those able to grow on plates or pretty universal in association with pelagic crustaceans. The fecal pellets were presumably inoculated by the intestinal microbial environment and bacteria from the copepod surface, reflecting the observations on \(Calanus plumchrus\) (Nagasawa, 1992). In the present laboratory study, a population of intestinal bacteria remained in the intestines of \(A. tonsa\) after all the fecal material was egested. This intestinal flora reflected the surrounding seawater flora and might have colonized the intestines passively (Bianchi et al., 1992), in association with detritus ingested by the copepods (Lawrence et al., 1993), by the filtration of the algal particles or by drinking the seawater. The planktonic pin-point bacteria might then be
physiologically activated and become cultivable when they are transferred to the intestinal eutrophic micromilieu.

Comparing the bacterial composition associated with fecal pellets in our two different experiments gave a reproducible overlap of *Vibrio* spp. and *Cytophaga/Flavobacterium* spp. in particular, with some of the other genera represented as minor groups. *Bacillus* spp. and *Acinetobacter* spp. were only represented in experiment I, while *Photobacterium* spp. were only represented with fecal pellets from experiment II. We used seawater from the same locality, copepods from the same laboratory strain and algal food from a continuous culture in all our experiments. In addition, all working procedures were similar. The qualitative differences in the bacterial populations must therefore be ascribed to biological fluctuations in the culture tanks, in which we have observed significantly daily numerical fluctuations of *Vibrio* spp. Thus, we could only describe the microbial activity as 'snapshot' views in such small tanks, which makes it difficult to reproduce same qualitative scenarios in experiments performed at different times.

The bacterial culturability during fecal pellet incubation (experiment I) was relatively high in the seawater compared to bacteria associated with the fecal pellets (the ratio c.f.u.:AODC was 0.05 and 0.019, respectively), which was in contrast to the observations from experiment II (Table II). The study indicates that intestinal bacteria are relatively less active than bacteria associated with fecal pellets. When the fecal pellets are egested they grow inside the pellets (Lawrence *et al*., 1993), they proliferate into the water (Jacobsen and Azam, 1984) and pelagic bacteria colonize the fecal pellets. These processes change the culturability and the bacterial size distribution. This is possibly due to differences in oxygen concentration and nutrient accessibility in the intestine versus the external environments (Gowing and Wishner, 1992).

Qualitative and quantitative insight into the microbial genera associated with zooplankton can be applicable to the field, thereby increasing the insight into the interactions between the substrate producers, phyto- and zooplankton (grazer-mediated DOC), and the consumers (heterotrophic bacteria).

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Bacteria associated with a marine planktonic copepod in culture. I


Bacteria associated with a marine planktonic copepod in culture. I


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