Effects of food on bacterial community composition associated with the copepod *Acartia tonsa* Dana

Kam Tang[1,*]**, Claudia Dziallas**[2]**,
Kristine Hutalle-Schmelzer**[2,3]
and Hans-Peter Grossart[2]

[1]*Virginia Institute of Marine Science, Gloucester Point,
VA 23062-1346, USA*
[2]*Leibniz Institute of Freshwater Ecology and Inland Fisheries,
12587 Berlin, Germany*
[3]*Biological Sciences, University of Santo Tomas, Manila 1015,
The Philippines*
*Author for correspondence (kamtang@vims.edu).

The estuarine copepod *Acartia tonsa* naturally carried diverse strains of bacteria on its body. The bacterial community composition (BCC) remained very conservative even when the copepod was fed different axenic algal species, indicating that the food *per se* did not much affect BCC associated with the copepod. In xenic algal treatments, however, copepod-associated BCC differed with each alga fed, even though the same bacterial source was used to inoculate the algae. In addition, starved copepods taken at the same location but at different times significantly differed in their BCC. Algal species composition and copepod life history therefore serve to regulate BCC associated with copepods, and spatial and temporal variations in algal species composition and copepod origin would alter bacteria–copepod interactions.

1. INTRODUCTION

Conventional ecological research views mesozooplankton and bacteria as two weakly and indirectly connected functional groups (e.g. Azam & Malfatti 2007), but in reality they are closely linked in occurrence and ecological functions (Harris 1993). For example, the exoskeleton and gut lining of a copepod provide favourable surfaces for bacterial attachment (Carman & Dobbs 1997). The equivalent bacterial abundance associated with copepods can be orders of magnitude higher than that in ambient water, indicating active bacterial colonization and growth in these microenvironments (Tang 2005). The observations that the copepod's body and the surrounding water share similar bacterial groups, but in different proportions, suggest an active exchange of bacteria between the compartments, but the different environments tend to favour different bacterial groups

Electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2009.0076 or via http://rsbl.royalsocietypublishing.org.

(Sochard *et al.* 1979; Delille & Razouls 1994). These earlier studies relied on culturing techniques or biochemical assays, and may have missed many bacterial phylotypes that were present. Modern molecular techniques allow for a more detailed phylogenetic investigation of these bacterial communities (e.g. Møller *et al.* 2007; Peter & Sommaruga 2008). In the natural environment, food particles are already colonized by bacteria (Simon *et al.* 2002); copepod feeding will therefore bring new bacteria to its gut. The food environment as exploited by the copepod, and subsequent interactions between the ingested bacteria and the gut environment, would determine the resultant bacterial community composition (BCC) associated with the copepod. Here we used the molecular fingerprint technique denaturing gradient gel electrophoresis (DGGE) followed by the sequencing of individual DGGE bands to study how different algal species, under both axenic and xenic conditions, affect the BCC associated with the copepod *Acartia tonsa*.

2. MATERIAL AND METHODS

Axenic algal cultures (table 1) were maintained in *f*/2 medium at 20 psu; axenic status was confirmed by DAPI staining (Porter & Feig 1980) and DGGE using eubacterial primers. Xenic algal cultures were prepared by inoculating the algae with 5 μm filtered water from the York River estuary (VA, USA). Cell carbon content was estimated from cell size (Strathmann 1967), and the experimental food concentration was adjusted to approximately 350 ng C ml^{-1} to ensure maximum ingestion rate (Tang *et al.* 2001).

The calanoid copepod *A. tonsa* was collected from the York River estuary. Female copepods were first incubated in 0.2 μm filtered Instant Ocean artificial sea water (20 psu) for 24 hours to empty their gut contents. After starvation, a subsample of copepods were rinsed with sterile sea water and transferred to sterile Eppendorf vials as initial samples (5–7 copepods per vial; 3–4 replicates per treatment). The samples were preserved with 20 μl of 95 per cent ethanol (molecular biology grade) and stored at −20°C until DGGE analysis. Remaining starved copepods were transferred to incubation bottles with either axenic (15 copepods per 130 ml in triplicate) or xenic (13–15 animals per 130 ml in triplicate) algae. Because the axenic and xenic algae treatments were conducted at different times, the copepods were taken from different field populations for the experiments. The bottles were fastened onto a rotating wheel in an environmental room (19±1°C, 12 L : 12 D cycle) for 48 hours. The algae in the incubation bottles were renewed after 1 day; after 2 days of incubation, remaining live copepods were rinsed and preserved for DGGE analysis.

Bacterial DNA was extracted using phenol–chloroform–isoamylalcohol and zirconium beads (Zhou *et al.* 1996). Fragments of bacterial *16S rRNA* genes were amplified using universal primers 341f-GC and 907r (Muyzer & Ramsing 1995). Approximately 500 ng of amplification product were loaded in each lane of a 7 per cent polyacrylamide gel with a denaturing gradient ranging from 40 to 70 per cent (urea/formamide). The gels were run for 20 hours, stained with SYBRGold (Molecular Probes) for 30 min, then destained with Milli-Q water for 10 min and illuminated on a UV table (Biometra). Cluster analysis of the banding patterns was done by GELCOMPARE II, v. 3.5 (Applied Maths) using unweighted pair group method with arithmetic averages. A pairwise similarity matrix based on Dice correlation index was calculated. DNA from excised individual DGGE bands was re-amplified for sequencing using the primers 341f without GC-clamp and 907r (Muyzer & Ramsing 1995) and the PCR protocol of Grossart *et al.* (2005). Partial *16S rRNA* gene sequences were deposited in GenBank with accession numbers EU675687–EU675713 and EU680798–EU680807. Phylogenetic trees were constructed using the ARB software package (www.arb-home.de) and a database of approximately 52 100 aligned sequences. Only sequences of more than 1400 nucleotides were used. Phylogenetic analyses were performed by the maximum-likelihood algorithm. The resulting tree was compared with trees calculated with the neighbour-joining or maximum-parsimony algorithms to test for stability. Partial sequences from DGGE bands (approx. 560 nucleotides) were added to the tree according to maximum-parsimony criteria and with the 50 per cent base frequency filter.

Table 1. Axenic algal strains used in the present study. (Cell carbon content was estimated from cell size based on Strathmann (1967). All algal strains were obtained from Bigelow CCMP collection except for *Dunaliella tertiolecta* DE, which was obtained from NOAA-NMFS in Milford, CT.)

axenic algal strain	taxonomic group	CCMP no.	carbon content (pg C cell^{-1})
Phaeodactylum tricornutum	Bacillariophyceae	1327	11.6
Thalassiosira weissflogii[a]	Bacillariophyceae	1336	54
Rhodomonas salina[a]	Cryptophyceae	1319	29.9
Dunaliella tertiolecta[a]	Chlorophyceae	1320	31.1
Dunaliella tertiolecta DE	Chlorophyceae	n.a.	31.1

[a]Xenic algal food was prepared by inoculating the cultures with natural bacteria.

3. RESULTS

There was a large dissimilarity in the initial copepod-associated BCC between experiments (approx. 55%), reflecting differences in the experimental copepod populations (figure 1). In the axenic food experiment, the final copepod-associated BCC were very different from the initial samples (less than 40% similarity; figure 1), but were very similar among treatments: more than 80 per cent similarity except for one replicate of *Phaeodactylum tricornutum* (72%) and one replicate of *Thalassiosira weissflogii* (68%). This indicates that although the addition of food caused a change in copepod-associated BCC, the type of food *per se* had little effect on what bacterial community would establish on the copepods. Three bacterial classes were most common among the DGGE bands: α-proteobacteria; γ-proteobacteria; and Bacteroidetes (figure 2); all have members that commonly exist as symbionts or pathogens in marine organisms.

Xenic food contributed new bacterial species to the copepod (seven bands that were not present in the initial samples; figure 1). To compare the two experiments, we normalized the number of bands in each sample to the total number of different bands in each experiment and then performed Welch's *t*-test. The result showed that the two experiments were significantly different from each other ($p < 0.001$, $t = -5.794$, d.f. $= 13$). In xenic treatments, the copepod-associated BCC clustered according to food type (except for one *Rhodomonas salina* sample), suggesting that different algal species delivered different bacterial assemblages to the grazers. The bacterial community mainly consisted of γ-proteobacteria, of which *Yersinia* of the Enterobacteriaceae group was frequently present (figure 2). In addition, the excised bands revealed several bacterial groups that are those commonly found on surfaces and in close association with marine organisms (Grossart *et al.* 2005): *Pseudoalteromonas* of the Alteromonadaceae group, *Sulfitobacter* and *Roseobacter* of the Rhodobacteraceae group and *Halomonas* species.

4. DISCUSSION

Our experiments showed that an external source of bacteria (e.g. via food intake) was required to maintain a diverse bacterial community associated with *A. tonsa*. The dissimilarity between the two sets of initial samples suggests that the copepods were initially exposed to and retained very different BCC even after gut clearance, similar to the observations by Grossart *et al.* (2009). This implies that the life history of copepods has an influence on the occurrence of specific bacterial communities associated with them.

Members of Vibrionaceae frequently appeared among our *16S rRNA* gene sequences. This group, including the pathogen *Vibrio cholerae*, is often found in close association with copepods (Heidelberg *et al.* 2002; Belkin & Colwell 2005). *Vibrio* species possess enzymes for chitin catabolism (Park *et al.* 2002) that make them well adapted to growing on the exoskeleton, gut lining and faecal pellets of copepods. Rhodobacteraceae are also commonly associated with copepods (Maran *et al.* 2007; Møller *et al.* 2007; this study), and form the second most abundant SSU rRNA gene cluster in marine plankton clone libraries (Giovannoni & Rappé 2000). The genus *Yersinia* is commonly found as gut flora (Brenner *et al.* 2005). Its frequent occurrence in our xenic treatments but near absence in the axenic treatments suggests that it relied on delivery via food intake to maintain a constant presence associated with the copepod. The absence of *Pseudoalteromonas*, *Sulfitobacter* and *Roseobacter* in the initial samples, but their frequent presence after xenic algal addition indicates that these species were also delivered via food intake, and were able to quickly establish a prominent presence associated with the copepod.

Although we made no distinction between bacteria attached to the exterior of the copepod and bacteria inside the copepod's gut, there is no *a priori* reason to expect that food intake would affect the composition of externally attached bacteria. By contrast, food intake is expected to affect bacterial dynamics inside the gut (Harris 1993). For example, when the copepod *Calanus pacificus* switched its diet from diatoms colonized by ^{3}H-labelled bacteria to axenic diatoms, the copepod retained 31 per cent or less of the ^{3}H signal after 43 hours (Lawrence *et al.* 1993). The authors interpreted it as the amount of ingested bacterial biomass assimilated into the copepod tissues. We, however, suggest that some of the residual ^{3}H might represent ingested bacteria that became attached to the gut. Applying transmission electron microscopy (TEM) to paraffin sections, Peter & Sommaruga (2008) observed bacteria in the gut of starved *Daphnia pulex* (freshwater cladoceran), suggesting that some bacteria remained attached even after the zooplankton had cleared its gut content.
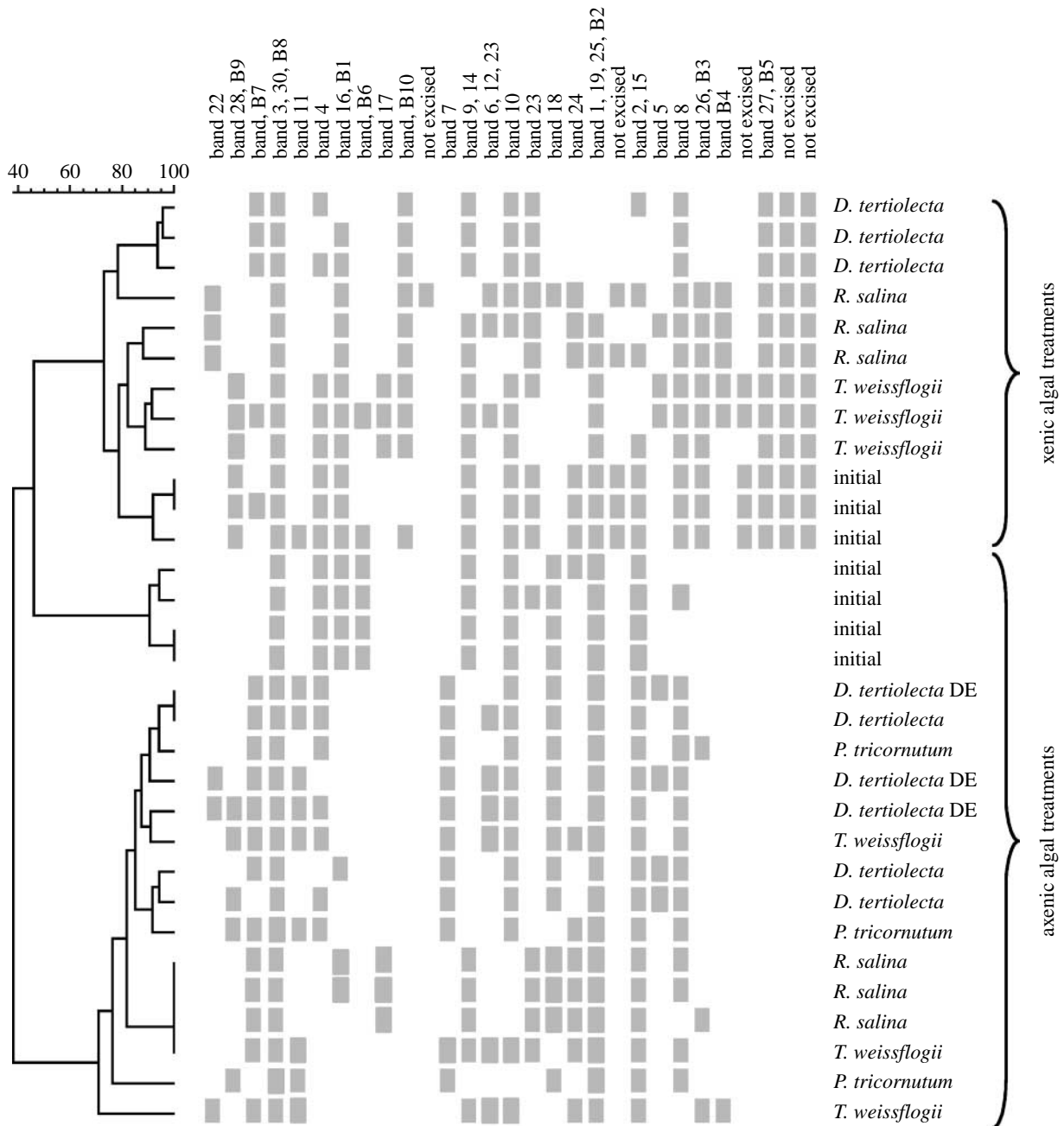
Figure 1. Combined cluster diagram of DGGE banding patterns and the presence/absence table for specific bacteria associated with *A. tonsa* in axenic and xenic algal treatments. Full species name are given in table 1. Initial samples after starvation and prior to algal treatments are included for comparison. The dendrogram was calculated by Dice correlation index and UPGMA. Scale bar at the top indicates per cent similarity.

They also reported the *absence* of bacteria in starved copepod *Acanthodiaptomus denticornis*. However, owing to the intrusive procedures of paraffin sectioning and TEM, even attached bacteria could have been lost, and negative results should be interpreted carefully. Using scanning electron microscopy, Nagasawa & Nemoto (1988) found gut bacteria in starved copepod *Eucalanus bungii*. Likewise, Hansen & Bech (1996) reported the presence of bacteria in the intestines of *A. tonsa* after the copepod had been starved and stripped of externally attached bacteria. In another study, when *A. tonsa* fed on axenic diatom, the abundance of attached bacteria exhibited a dome-shaped response to ingestion rate, which is consistent with the idea that bacterial abundance inside the gut was controlled by a balance between growth

as stimulated by the copepod's feeding and loss due to defecation (Tang 2005). Conceptually, some researchers distinguish between 'transient' and 'resident' gut bacteria (Harris 1993). The former are the ones that are ingested but are either digested or released through defecation; the latter are the ones that permanently reside inside the gut. Such a distinction, however, may be difficult in practice. A copepod's gut must initially acquire bacteria from an external source, most probably from pre-colonized food particles. As shown in this study, the rather stable copepod-associated BCC among the axenic algal treatments suggests that certain bacterial groups may be preferentially retained by the copepod; on the other hand, ingestion of different algae that were pre-colonized by bacteria resulted in varying bacterial
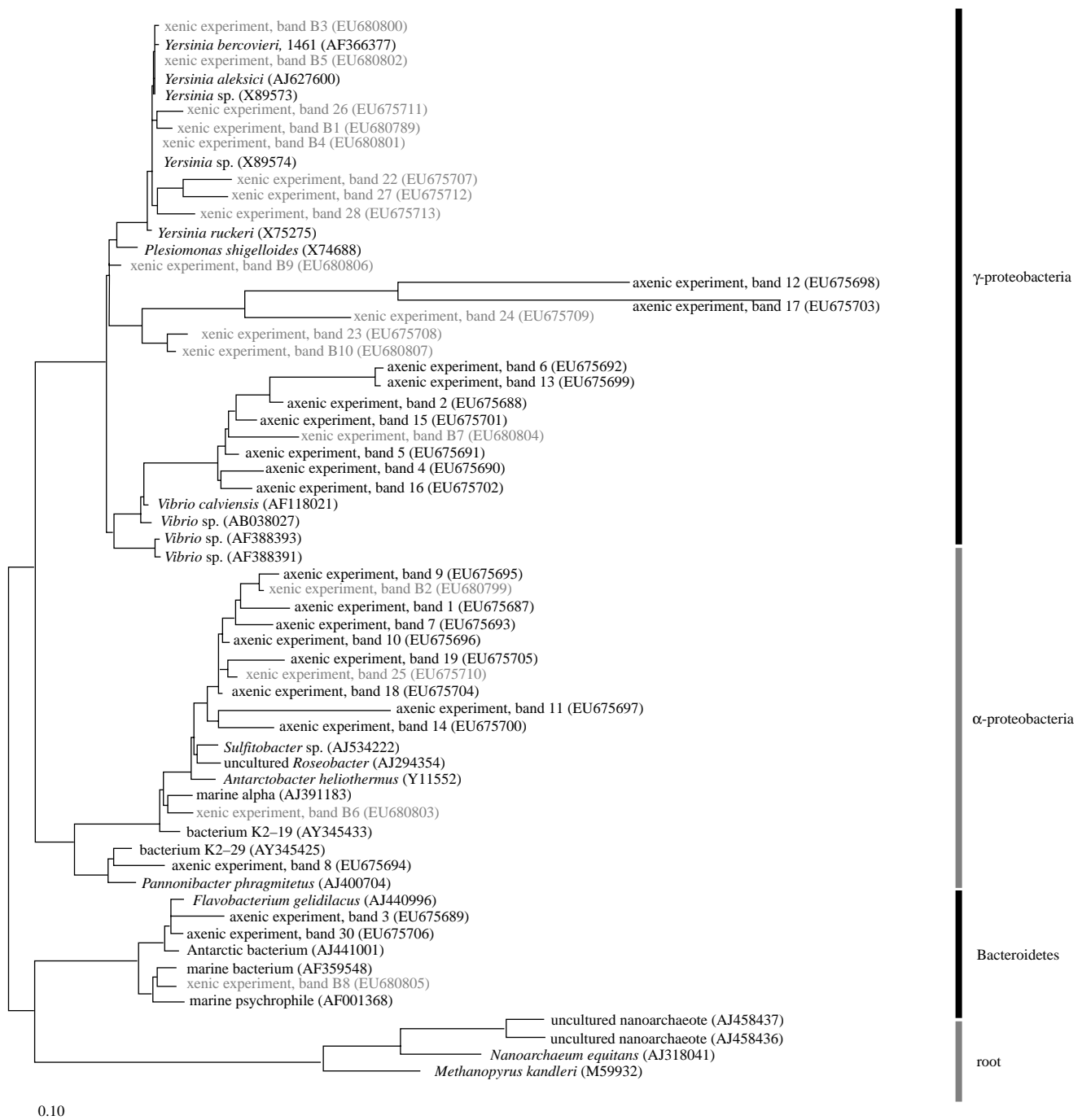
Figure 2. Phylogenetic tree based on maximum likelihood including *16S rRNA* sequences of all DGGE bands from feeding experiments with axenic (bold) and xenic (grey) algal food (see text for details). GenBank accession numbers are given in parentheses. Scale bar at the bottom represents relative phylogenetic distance.

communities associated with the copepod. Other studies have shown that the bacterial communities attached to particles are more diverse than ambient free-living bacteria (Riemann & Winding 2001), and that different algal species harbour different bacterial communities (Grossart *et al.* 2006). Therefore, copepods exposed to different food environments will probably establish different resident bacterial communities inside their bodies. Some ingested bacteria will probably survive digestion and be incorporated into faecal matter (Lawrence *et al.* 1993). Although we did not examine the faecal matter, the dissimilarity in copepod-associated BCC among the xenic treatments in this study leads us to speculate that copepod

feeding on different xenic food particles would produce faecal pellets that contain different bacteria, which may lead to different dissolution rate of the faecal materials, with important ramification for material fluxes in the ocean.

In this study, we showed that BCC associated with *A. tonsa* depended on several factors: life history of the copepods; the source of bacteria; and the food that delivered them. Extrapolating these laboratory results to the field, we hypothesize that the same copepod species exposed to different environments and food would establish different bacterial communities associated with its body. Conversely, copepod species of different feeding habits would acquire different

bacterial communities via food intake, even if they share the same environment. Spatial and temporal variations in the food environment would therefore mediate changes in copepod–bacteria interactions.

Azam, F. & Malfatti, F. 2007 Microbial structuring of marine ecosystems. *Nat. Rev. Microbiol.* **5**, 782–791. (doi:10.1038/nrmicro1747)

Belkin, S. & Colwell, R. R. 2005 *Ocean and health: pathogens in the marine environment.* New York, NY: Springer.

Brenner, D. J., Krieg, N. R. & Staley, J. T. 2005. *Bergeys' manual of systematic bacteriology: part C*, vol. 2. New York, NY: Springer.

Carman, K. R. & Dobbs, F. C. 1997 Epibiotic microorganisms ion copepods and other marine crustaceans. *Microsc. Res. Tech.* **37**, 116–135. (doi:10.1002/(SICI)1097-0029(19970415)37:2<116::AID-JEMT2>3.0.CO;2-M)

Delille, D. & Razouls, S. 1994 Community structures of heterotrohpic bacteria of copepod fecal pellets. *J. Plankton Res.* **16**, 603–615. (doi:10.1093/plankt/16.6.603)

Giovannoni, S. & Rappé, M. 2000 Evolution, diversity, and molecular ecology of marine prokaryotes. In *Microbial ecology of the oceans* (ed. D. L. Kirchman), pp. 47–84. Chichester, UK: Wiley.

Grossart, H. P., Levold, F., Allgaier, M., Simon, M. & Brinkhoff, T. 2005 Marine diatom species harbour distinct bacterial communities. *Environ. Microbiol.* **7**, 860–873. (doi:10.1111/j.1462-2920.2005.00759.x)

Grossart, H. P., Kiørboe, T., Tang, K. W., Allgaier, M., Yam, E. M. & Ploug, H. 2006 Interactions between marine snow and heterotrophic bacteria: aggregate formation and microbial dynamics. *Aquat. Microb. Ecol.* **42**, 19–26. (doi:10.3354/ame042019)

Grossart, H. P., Dziallas, C. & Tang, K. W. 2009 Bacterial diversity associated with freshwater zooplankton. *Environ. Microbiol. Rep.* **1**, 50–55. (doi:10.1111/j.1758-2229.2008.00003.x)

Hansen, B. & Bech, G. 1996 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. *J. Plankton Res.* **18**, 257–273. (doi:10.1093/plankt/18.2.257)

Harris, J. M. 1993 The presence, nature, and role of gut microflora in aquatic invertebrates: a synthesis. *Microb. Ecol.* **25**, 195–231. (doi:10.1007/BF00171889)

Heidelberg, J. F., Heidelberg, K. B. & Colwell, R. R. 2002 Bacteria of the gamma-subclass *Proteobacteria* associated with zooplankton in Chesapeake Bay. *Appl. Environ. Microb.* **68**, 5498–5507.

Lawrence, S. G., Ahmad, A. & Azam, F. 1993 Fate of particle-bound bacteria ingested by *Calanus pacificus*. *Mar. Ecol. Prog. Ser.* **97**, 299–307. (doi:10.3354/meps097299)

Maran, B. *et al.* 2007 Isolation and characterization of bacteria from the copepod *Pseudocaligus fugu* ectoparasitic on the panther puffer *Takifugu pardalis* with the emphasis on TTX. *Toxicon* **50**, 779–790. (doi:10.1016/j.toxicon.2007.06.021)

Møller, E. F., Riemann, L. & Søndergaard, M. 2007 Bacteria associated with copepods: abundance, activity and community composition. *Aquat. Microb. Ecol.* **47**, 99–106. (doi:10.3354/ame047099)

Muyzer, G. & Ramsing, N. B. 1995 Molecular methods to study the organization of microbial communities. *Water Sci. Technol.* **32**, 1–9. (doi:10.1016/0273-1223(96)00001-7)

Nagasawa, S. & Nemoto, T. 1988 Presence of bacteria in guts of marine crustaceans and on their fecal pellets. *J. Plankton Res.* **10**, 559–564. (doi:10.1093/plankt/10.3.559)

Park, J. K., Wang, L.-X. & Roseman, S. 2002 Isolation of a glucosamine-specific kinase, a unique enzyme of *Vibrio cholerae*. *J. Biol. Chem.* **227**, 15 573–15 578. (doi:10.1074/jbc.M107953200)

Peter, H. & Sommaruga, R. 2008 An evaluation of methods to study the gut bacterial community composition of freshwater zooplankton. *J. Plankton Res.* **30**, 997–1006. (doi:10.1093/plankt/fbn061)

Porter, K. G. & Feig, Y. S. 1980 DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* **25**, 943–948.

Riemann, L. & Winding, A. 2001 Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. *Microb. Ecol.* **42**, 274–282. (doi:10.1007/s00248-001-0018-8)

Simon, M., Grossart, H.-P., Schweitzer, B. & Ploug, H. 2002 Microbial ecology of organic aggregates in aquatic ecosystems. *Aquat. Microb. Ecol.* **28**, 175–211. (doi:10.3354/ame028175)

Sochard, M. R., Wilson, D. F., Austin, B. & Colwell, R. R. 1979 Bacteria associated with the surface and gut of marine copepods. *Appl. Environ. Microbiol.* **37**, 750–759.

Strathmann, R. R. 1967 Estimating the organic carbon content of phytoplankton from cell volume or plasma volume. *Limnol. Oceanogr.* **12**, 411–418.

Tang, K. W. 2005 Copepods as microbial hotspots in the ocean: effects of host feeding activities on attached bacteria. *Aquat. Microb. Ecol.* **38**, 31–40. (doi:10.3354/ame038031)

Tang, K. W., Jakobsen, H. H. & Visser, A. W. 2001 *Phaeocystis globosa* (Prymnesiophyceae) and the planktonic food web: feeding, growth and trophic interactions among grazers. *Limnol. Oceanogr.* **46**, 1860–1870.

Zhou, J., Bruns, M. A. & Tiedje, J. M. 1996 DNA recovery from soils of diverse composition. *Appl. Environ. Microbiol.* **62**, 695–724.