



# Linkage between copepods and bacteria in the North Atlantic Ocean

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**ABSTRACT:** Copepods and bacteria are fundamental components of the pelagic food web and play a major role in biogeochemical cycles. Marine bacteria have a free-living or particle-attached lifestyle, but as members of the microbial food web, the only biotic interaction of bacteria is commonly assumed to be with their predators (protists and/or viruses). However, a copepod's body is highly enriched in organic matter and harbors a large and complex bacterial community. The aim of this study was to compare the composition of the free-living bacterial community of the open Atlantic to that associated with copepods. We used 454 high-throughput sequencing of the 16S rRNA gene to decipher the bacterial community composition associated with this zooplankton group and the ambient water. Significant differences were found between the bacterial communities associated with the dominant copepod families (Calanoida: Centropagidae and Clausocalanidae; Cyclopoida: Corycaidae, Oncaeidae, and Lubbockiidae) and the ambient water. *Bacilli* and *Actinobacteria* dominated the copepod-associated community and *Alphaproteobacteria*, *Deltaproteobacteria*, and *Synechococcus* dominated the free-living community. However, the presence of shared bacterial operational taxonomic units (OTUs) between these 2 distinct habitats suggests a dynamic exchange of bacteria between seawater and copepods. Taken together, our results support the hypothesis that the interior and exterior surfaces of copepods provide a specific niche with a strong selective pressure for bacteria.

**KEY WORDS:** Microbes · Zooplankton · Open ocean · 454 pyrosequencing

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## INTRODUCTION

Prokaryotes constitute the largest fraction of living biomass in marine ecosystems and are the main force driving biogeochemical cycles (Azam et al. 1983). In the oceanic water column, the majority of the prokaryotes are free-living, with commonly less than 5% of total prokaryotic cells associated with aggregates (Bell & Albright 1982, Unanue et al. 1992). However, several studies have reported

bacterial abundances associated with various microhabitats such as particles, aggregates, fecal pellets, and zooplankton exceeding those of free-living bacteria (Simon et al. 2002, Tang et al. 2010). Also, attached bacteria commonly exhibit high growth rates and enzymatic activities (Karner & Herndl 1992, Grossart et al. 2006). The microhabitats of these attached bacteria are frequently characterized by concentrations of organic matter and inorganic nutrients that are orders of magni-

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tude higher than in the surrounding water (Bochdansky & Herndl 1992, Alldredge 2000, Grossart & Tang 2010, Tang et al. 2010). These distinctly different microhabitats may favor biogeochemical reactions that otherwise would not occur in the oceanic water column (Grossart & Tang 2010). The anoxic and hypoxic conditions found in some pelagic aggregates, animal guts, and fecal pellets favor anaerobic reactions not occurring in the surrounding oxygenated water (Alldredge & Cohen 1987, Deangelis & Lee 1994, Grossart & Tang 2010, Tang et al. 2011). Furthermore, several studies have shown that the interactions between prokaryotes and predators such as protists and viruses are substantially different in these microhabitats as compared to the ambient seawater (Caron 1987, Riemann & Grossart 2008, Grossart & Tang 2010).

Marine copepods provide a complex microhabitat in marine ecosystems, with their complex body structure and extensive surface potentially available for microbial colonization (Tang et al. 2010). Furthermore, copepods contribute to the microbial food web through the release of biologically available dissolved organic matter and nutrients during the digestive processes (Azam et al. 1983, Møller et al. 2003, Tang 2005, Møller 2007, Tang et al. 2010). In contrast to detrital particles, copepods can collect organic compounds and cells through the ingestion of food, thereby allowing a continuous production and release of prokaryotes through their fecal pellets (Tang 2005). Moreover, many zooplankton species perform vertical migration (Kobari & Ikeda 2001). This migration in stratified waters favors the dispersal and acquisition of microbes from different water layers and allows them to cross physical barriers, such as pycnoclines (Grossart et al. 2010). Several abundant open-ocean copepods exhibit diel vertical migration potentially favoring dispersal of copepod-associated bacteria over the euphotic to mid-mesopelagic layers (Steinberg et al. 2000, Grossart et al. 2010, Tang et al. 2010).

The aim of this study was to compare the phylogenetic composition of the bacterial community associated with copepods collected in the North Atlantic Ocean to that of the ambient water using 454 pyrosequencing. Specifically, we hypothesized that a variable transient bacterial community is present in the copepods in addition to a stable resident community. The transient community reflects the composition of the ambient water, while the resident community is specifically adapted to the microenvironmental conditions in the copepods.

## MATERIALS AND METHODS

### Sampling of ambient water

Water samples were collected during the MEDEA-I cruise (October 2011) on board RV 'Pelagia' at 4 stations along a latitudinal transect in the North Atlantic from 24° 40' N, 34° 56' W to 30° 27' N, 24° 32' W (See Fig. S1 in the Supplement at [www.int-res.com/articles/suppl/a072p215\\_supp.pdf](http://www.int-res.com/articles/suppl/a072p215_supp.pdf)).

Seawater samples were collected with a Seabird conductivity-temperature-depth (CTD) rosette sampler equipped with 18 Niskin bottles (25 l each). To determine the bacterial community composition of the ambient seawater, 10 l of water were sampled from the lower euphotic zone (about 100 m depth) and from ~750 m depth.

The seawater was filtered through a 0.2 µm GTTP membrane filter (Millipore) and subsequently, the filters were stored at –80°C until further processing in the laboratory. Although these samples include some particle-attached bacteria, the free-living community is dominant (Bochdansky et al. 2010). Thus, we refer to the ambient seawater bacterial community as the free-living community.

### Sampling of zooplankton

Zooplankton were collected at the same stations as the ambient water using vertical plankton tows (200 µm mesh size, hoisted at 30 m min<sup>–1</sup>) from 750 m to the surface. Water samples were collected at the 2 depth layers (~100, ~750 m) within which the copepods migrate during the diel cycles (Steinberg et al. 2000, Tang et al. 2010), to compare the composition of the free-living bacterial community with the zooplankton-associated bacterial community obtained from the integrated net tows.

The content of the cod end of the plankton net was transferred into a plankton splitter and then concentrated over a 70 µm mesh Nitex screen. The zooplankton samples were then transferred into 2 ml Eppendorf tubes and stored at –80°C until sorting. In the laboratory, the zooplankton were thawed to room temperature, transferred to a Petri dish filled with 0.2 µm filtered seawater for sorting of the dominant copepod taxa (Calanoida: Centropagidae and Clausocalanidae; Cyclopoida: Corycaidae, Oncaeidae, and Lubbockiidae). To evaluate the gut-associated bacteria of different copepods, 10 individuals of each taxon were collected and washed 3 times with Milli-Q water to remove bacterial cells associated with the

external surface of the copepods. Subsequently, the copepods were transferred into sterile Eppendorf tubes for nucleic acid extraction.

### DNA extraction

The DNA of the ambient-water samples was extracted using an Ultraclean Soil DNA isolation Kit (MoBio Laboratories). The DNA from the copepod samples was extracted using a phenol-chloroform extraction protocol (Weinbauer et al. 2002), preceded by a bead-beating step to facilitate lysis of the copepods. To check the quality of the DNA following extraction, a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) was used.

### Pyrosequencing of the 16S rDNA bacterial community

The 16S rRNA genes (16S rDNA) of the zooplankton and ambient-water samples were PCR amplified with the bacterial primers 341f (5'-CCT ACG GGA GGC AGC AG-3') and 907r (5'-CCG TCA ATT CMT TTG AGT TT-3') (Muyzer et al. 1998, Grossart et al. 2009). The PCR amplification of the 16S rRNA gene of the samples was carried out in a 50 µl reaction volume using Fermentas *Taq* polymerase (Thermo Scientific) in a Mastercycler (Eppendorf) with the following parameters: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products were additionally purified with a PCR purification kit (5-Prime). The quality of the PCR product was checked on 2% agarose gel. The 16S rDNA amplicons were subsequently sequenced in a Roche 454 GS Junior next generation sequencing platform based on the Titanium chemistry by IMGM Laboratories GmbH (Martinsried, Germany). All samples were barcoded using multiplex identifiers and sequenced together in 1 run. The resulting sequences were divided into 4 groups: 2 orders of copepods (Calanoida and Cyclopoida) and 2 water layers (Deep, corresponding to ~750 m; and Surface, corresponding to 100 m; see Table S1 in the Supplement).

### Bioinformatic analysis and phylogenetic classification

The bioinformatic analysis of the 16S rDNA sequences largely followed the standard operating pro-

cedure pipeline of Mothur software, version 1.31 (Schloss 2009). The 16S rDNA pyrotags were sorted according to their respective barcode into the different samples. The raw sequence reads were filtered, trimmed, and quality checked, and sequences smaller than 200 bp were discarded. Subsequently, the sequences were aligned with the SILVA database, and the pairwise distance matrix was calculated. The 16S rDNA sequences with a 97% sequence similarity were clustered into operational taxonomic units (OTUs). Taxonomic assignment was performed using QIIME (Caporaso et al. 2010), and all unclassified bacteria at the phylum level were discarded. Additionally, MEGAN (Huson et al. 2007) was used to build the hierarchical phylogenetic tree of the bacterial community as an alternative to QIIME for taxonomic identification. The MEGAN analysis was based on BLAST results (Altschul et al. 1997) against SILVA and Greengenes databases (data not shown) following the NCBI taxonomy (Sayers et al. 2012). Rarefaction curves, Chao1, ACE richness, and the Shannon index of diversity were calculated with Mothur (Schloss 2009).

Pairwise UniFrac distance and principal coordinate analysis (PCoA) (Lozupone & Knight 2005) were used to compare the bacterial community composition in the different samples (implemented in QIIME). The established phylogenetic tree was built with Mothur (Schloss 2009), and the Unifrac distance matrix was calculated with FastUnifrac (Lozupone & Knight 2005). The Unifrac distance matrix was calculated unweighted using only presence-absence information of bacterial OTUs, or weighted and thus taking the relative proportion of each bacterial OTU to the total bacterial community into account. A *t*-test (implemented in Sigma Plot v.11) was used to test for the statistical difference between samples.

## RESULTS

### Analysis of the pyrosequencing library

The 454 pyrosequencing analysis was performed to investigate the differences between the bacterial community composition associated with 2 orders of copepods (Cyclopoida and Calanoida) and the bacterioplankton community collected from the 2 boundary depth layers (~750 and 100 m depths) at the same location as the copepods were collected. In total, we obtained 65 855 reads for the entire set of samples with an average length of 450 bp. The trimming of low-quality reads resulted in 25 101 sequences with

an average length of 307 bp used for further analyses (Table S1 in the Supplement). From the total number of trimmed sequences, 3970 and 12978 reads were categorized as unique at the 97 % and 100 % similarity level, respectively.

### OTU richness in ambient water and copepods

The rarefaction analysis showed different trends for the 2 sets of samples (Fig. 1). While the rarefaction curves for the copepod samples approached a plateau, the rarefaction curves of the ambient water samples did not level off (Fig. 1). Hence, the sequencing effort was sufficient to sample most members of the bacterial community associated with the copepods, while it was not sufficient for the ambient water samples. Moreover, these results indicate a lower diversity in the copepod-associated bacterial community as compared to the ambient-water community (Table 1). The Chao richness index estimated, on average,  $231 \pm 57$  (average  $\pm$  SD) OTUs (ranging from 120–306,  $N = 9$ ) for the copepod-associated bacterial communities and  $1870 \pm 693$  OTUs (ranging from 791–3026,  $N = 7$ ) for the ambient water (Table 1). Similar results were obtained with the ACE richness index (Table 1). Shannon and Simpson diversity indices

Table 1. Total number of operational taxonomic units (OTUs; cutoff 97 % similarity), Chao and Ace species richness, and Shannon and Simpson diversity indices obtained from 16S rDNA sequence libraries from ambient-water and copepod-associated bacteria

Sample	OTUs observed	Chao	Ace	Shannon	Simpson (1-D)
Calanoida					
St.1	196	258	276	4.18	0.97
St.1	151	231	215	3.88	0.95
Cyclopoida					
St.2	162	276	275	4.18	0.97
St.2	153	206	206	4.12	0.97
St.3	132	181	170	4.14	0.97
St.3	189	306	346	4.36	0.97
St.4	193	279	341	4.40	0.98
St.4	65	120	210	3.25	0.91
St.4	116	226	386	3.86	0.95
Water samples					
Stn 1_750 m	486	2268	3707	5.33	0.98
Stn 1_100 m	358	1740	3755	4.65	0.96
Stn 2_900 m	470	3026	7600	5.30	0.98
Stn 2_100 m	292	1507	3115	4.30	0.94
Stn 3_100 m	258	791	1303	4.15	0.94
Stn 4_750 m	493	2081	4830	5.28	0.98
Stn 4_100 m	346	1683	2716	4.50	0.95

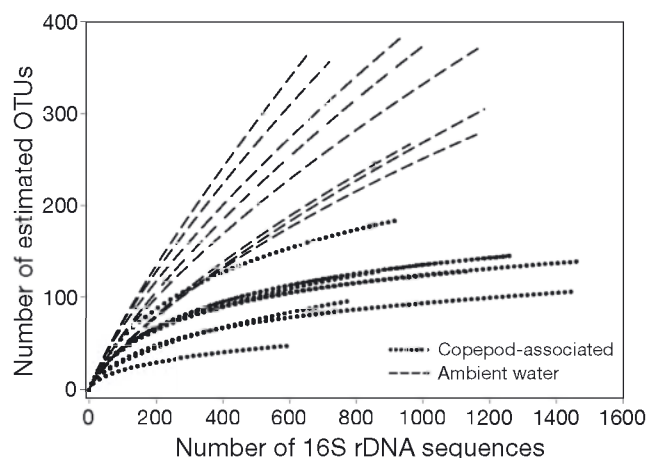


Fig. 1. Rarefaction curves obtained for the 16S rDNA sequences of calanoid and cyclopoid copepod-associated and ambient-water bacterial communities. Operational taxonomic units (OTUs) were defined at 97 % sequence identity

were computed for each sample using the 97 % similarity threshold. These indices also indicated a higher diversity in the bacterial community of the ambient water as compared to the copepod-associated community (Table 1). Two of the 3 samples of the Cyclopoida-associated bacterial community collected at Stn 4 exhibited significantly lower (*t*-test,  $p < 0.01$ ) diversity than the other copepod samples. These 2 samples of Cyclopoida had the lowest number of OTUs (*t*-test,  $p < 0.01$ ) and the lowest Shannon (*t*-test,  $p = 0.01$ ) and Simpson indices (*t*-test,  $p < 0.01$ ) of the entire dataset (Table 1).

In the ambient water, the bacterial community at 100 m depth exhibited a lower diversity (*t*-test,  $p = 0.02$ ), a lower number of OTUs (*t*-test,  $p = 0.02$ ) and lower Shannon (*t*-test,  $p < 0.01$ ) and Chao (*t*-test,  $p = 0.03$ ) indices than that of the 750 m layer, although the ACE index was not significantly different between these depth layers (*t*-test,  $p = 0.86$ ; Table 1).

The most abundant bacterial OTU of the ambient water contributed on average 25 % to the total number of ambient-water OTUs, while the most abundant copepod-associated bacterial OTU contributed only 18 % to the total copepod-associated bacterial OTUs (see Fig. S2 in the Supplement). Singletons (OTUs appearing only once in the entire pyrosequencing library) accounted for 27 % of the total number of bacterial OTUs of the ambient water, but only 8 % of the copepod-associated OTUs (Fig. S2).



The bacterial communities of the ambient water were dominated by a few very abundant OTUs and a large number of rare OTUs (Fig. S2). In contrast, members of the copepod-associated bacterial community were more evenly distributed than those of the ambient water with a comparatively low number of rare OTUs (Fig. S2).

### Composition of the bacterial community in the ambient water and in copepods

The phylogenetic analysis of the 25 101 sequences performed in QIIME using the Greengenes database revealed a clear clustering into 4 groups of bacterial communities (ambient water at 100 m and 750 m and calanoid and cyclopoid copepods; Table 2). *Firmicutes* contributed 23 % and 27 %, *Actinobacteria* 22 % and 19 %, and *Alphaproteobacteria* 20 % and 11 % to the Calanoida- and Cyclopoida-associated bacteria, respectively. *Betaproteobacteria* contributed 1.5 % and 16 % to the Calanoida- and Cyclopoida-associated bacteria, respectively (Fig. 2a). This relatively high abundance of the *Betaproteobacteria* in cyclopoid copepods was mainly caused by 2 samples collected at Stn 4 where *Betaproteobacteria* contributed 53 % to Cyclopoida-associated bacteria (Fig. 2a). Generally, the copepod-associated community was characterized by a relatively high contribution of chloroplasts, probably derived from ingested phytoplankton ( $7 \pm 8$  %; Fig. 2a).

Although the composition of the bacterial community of the ambient water was rather uniform among the different stations, as was the composition of the copepod-associated bacterial community, 2 samples of Cyclopoida-associated bacterial communities were strikingly different from all other samples (Fig. 2a), specifically the bacteria associated with the families Oncaeidae and Lubbockiidae at Stn 4, although members of the Oncaeidae were also collected at other stations. These 2 samples were composed mainly of *Betaproteobacteria* (genus *Burkholderiales*, 51 %) and *Flavobacteria* (genus *Flavobacteriales*, 16 %; Fig. 2a,b).

The bacterioplankton community of the 100 m depth layer mainly consisted of *Alphaproteobacteria* (30 %), *Cyanobacteria* (32 %; mainly composed of *Synechococcales*, 31 %), and *Actinobacteria* (17 %). The bacterial community of the 750 m layer was mainly composed of members of *Deltaproteobacteria* (29 %), *Alphaproteobacteria* (20 %), *Chloroflexi* (10 %), SAR406 (10 %), and *Gammaproteobacteria* (6 %; Fig. 2a, Table 2).

Table 2. Phylogenetic affiliation and relative contribution (%  $\pm$  SD) of the individual bacterial families to the total number of sequences obtained from the communities associated with 2 copepod orders and from samples of ambient water collected from 2 different depth layers

		Calanoida				Cyclopoida				Surface				Deep			
		100 m	750 m	100 m	750 m	100 m	750 m	100 m	750 m	100 m	750 m	100 m	750 m	100 m	750 m	100 m	750 m
<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	0.6 $\pm$ 0.4	0.2 $\pm$ 0.1	1.0 $\pm$ 0.8	0.7 $\pm$ 0.5	0.2 $\pm$ 0.2	2.2 $\pm$ 3.9	0.2 $\pm$ 0.1	1.5 $\pm$ 0.9	0.2 $\pm$ 0.3	0.2 $\pm$ 0.1	0.2 $\pm$ 0.3	0.2 $\pm$ 0.1	0.2 $\pm$ 0.3	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
	<i>Oceanospirillales</i>	0.6 $\pm$ 0.4	0.2 $\pm$ 0.1	1.0 $\pm$ 0.8	0.7 $\pm$ 0.5	0.2 $\pm$ 0.2	2.2 $\pm$ 3.9	0.2 $\pm$ 0.1	1.5 $\pm$ 0.9	0.2 $\pm$ 0.3	0.2 $\pm$ 0.1	0.2 $\pm$ 0.3	0.2 $\pm$ 0.1	0.2 $\pm$ 0.3	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
	<i>Chromatiales</i>	0.6 $\pm$ 0.4	0.2 $\pm$ 0.1	1.0 $\pm$ 0.8	0.7 $\pm$ 0.5	0.2 $\pm$ 0.2	2.2 $\pm$ 3.9	0.2 $\pm$ 0.1	1.5 $\pm$ 0.9	0.2 $\pm$ 0.3	0.2 $\pm$ 0.1	0.2 $\pm$ 0.3	0.2 $\pm$ 0.1	0.2 $\pm$ 0.3	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
<i>Cyanobacteria</i>	<i>Synechococcales</i>	16.1 $\pm$ 15.3	0.3 $\pm$ 0.2	4.4 $\pm$ 4.9	0.2 $\pm$ 0.2	0.3 $\pm$ 0.1	31.4 $\pm$ 11.3	0.1 $\pm$ 0.2	0.1 $\pm$ 0.2	0.3 $\pm$ 0.1	31.4 $\pm$ 11.3	0.1 $\pm$ 0.2	0.1 $\pm$ 0.2	0.3 $\pm$ 0.1	31.4 $\pm$ 11.3	0.1 $\pm$ 0.2	0.1 $\pm$ 0.2
	Chloroplasts	16.1 $\pm$ 15.3	0.3 $\pm$ 0.2	4.4 $\pm$ 4.9	0.2 $\pm$ 0.2	0.3 $\pm$ 0.1	31.4 $\pm$ 11.3	0.1 $\pm$ 0.2	0.1 $\pm$ 0.2	0.3 $\pm$ 0.1	31.4 $\pm$ 11.3	0.1 $\pm$ 0.2	0.1 $\pm$ 0.2	0.3 $\pm$ 0.1	31.4 $\pm$ 11.3	0.1 $\pm$ 0.2	0.1 $\pm$ 0.2
<i>Firmicutes</i>	<i>Clostridia</i>	8.1 $\pm$ 1.8	8.5 $\pm$ 2.1	6.7 $\pm$ 3.9	8.6 $\pm$ 5.2	14.9 $\pm$ 9.4	3.5 $\pm$ 4.1	8.1 $\pm$ 1.8	8.5 $\pm$ 2.1	6.7 $\pm$ 3.9	8.6 $\pm$ 5.2	14.9 $\pm$ 9.4	3.5 $\pm$ 4.1	8.1 $\pm$ 1.8	8.5 $\pm$ 2.1	6.7 $\pm$ 3.9	8.6 $\pm$ 5.2
	<i>Lactobacillales</i>	8.1 $\pm$ 1.8	8.5 $\pm$ 2.1	6.7 $\pm$ 3.9	8.6 $\pm$ 5.2	14.9 $\pm$ 9.4	3.5 $\pm$ 4.1	8.1 $\pm$ 1.8	8.5 $\pm$ 2.1	6.7 $\pm$ 3.9	8.6 $\pm$ 5.2	14.9 $\pm$ 9.4	3.5 $\pm$ 4.1	8.1 $\pm$ 1.8	8.5 $\pm$ 2.1	6.7 $\pm$ 3.9	8.6 $\pm$ 5.2
	<i>Bacilli</i>	8.1 $\pm$ 1.8	8.5 $\pm$ 2.1	6.7 $\pm$ 3.9	8.6 $\pm$ 5.2	14.9 $\pm$ 9.4	3.5 $\pm$ 4.1	8.1 $\pm$ 1.8	8.5 $\pm$ 2.1	6.7 $\pm$ 3.9	8.6 $\pm$ 5.2	14.9 $\pm$ 9.4	3.5 $\pm$ 4.1	8.1 $\pm$ 1.8	8.5 $\pm$ 2.1	6.7 $\pm$ 3.9	8.6 $\pm$ 5.2
<i>Actinobacteria</i>	<i>Koll 13</i>	21.7 $\pm$ 1.8	0.3 $\pm$ 0.4	19.1 $\pm$ 11.4	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	15.0 $\pm$ 6.9	21.7 $\pm$ 1.8	0.3 $\pm$ 0.4	19.1 $\pm$ 11.4	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	15.0 $\pm$ 6.9	21.7 $\pm$ 1.8	0.3 $\pm$ 0.4	19.1 $\pm$ 11.4	0.1 $\pm$ 0.1
	<i>Acidimicrobiales</i>	21.7 $\pm$ 1.8	0.3 $\pm$ 0.4	19.1 $\pm$ 11.4	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	15.0 $\pm$ 6.9	21.7 $\pm$ 1.8	0.3 $\pm$ 0.4	19.1 $\pm$ 11.4	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	15.0 $\pm$ 6.9	21.7 $\pm$ 1.8	0.3 $\pm$ 0.4	19.1 $\pm$ 11.4	0.1 $\pm$ 0.1
	<i>Actinomycetes</i>	21.7 $\pm$ 1.8	0.3 $\pm$ 0.4	19.1 $\pm$ 11.4	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	15.0 $\pm$ 6.9	21.7 $\pm$ 1.8	0.3 $\pm$ 0.4	19.1 $\pm$ 11.4	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	15.0 $\pm$ 6.9	21.7 $\pm$ 1.8	0.3 $\pm$ 0.4	19.1 $\pm$ 11.4	0.1 $\pm$ 0.1
<i>Alphaproteobacteria</i>	<i>Rickettsiales</i>	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6	3.4 $\pm$ 2.6	0.7 $\pm$ 0.5	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6	3.4 $\pm$ 2.6	0.7 $\pm$ 0.5	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6
	<i>Caulobacteriales</i>	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6	3.4 $\pm$ 2.6	0.7 $\pm$ 0.5	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6	3.4 $\pm$ 2.6	0.7 $\pm$ 0.5	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6
	<i>Rhodobacteriales</i>	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6	3.4 $\pm$ 2.6	0.7 $\pm$ 0.5	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6	3.4 $\pm$ 2.6	0.7 $\pm$ 0.5	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6
	<i>Rhizobiales</i>	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6	3.4 $\pm$ 2.6	0.7 $\pm$ 0.5	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6	3.4 $\pm$ 2.6	0.7 $\pm$ 0.5	5.3 $\pm$ 2.7	11.4 $\pm$ 12.1	1.5 $\pm$ 0.1	3.6 $\pm$ 2.6

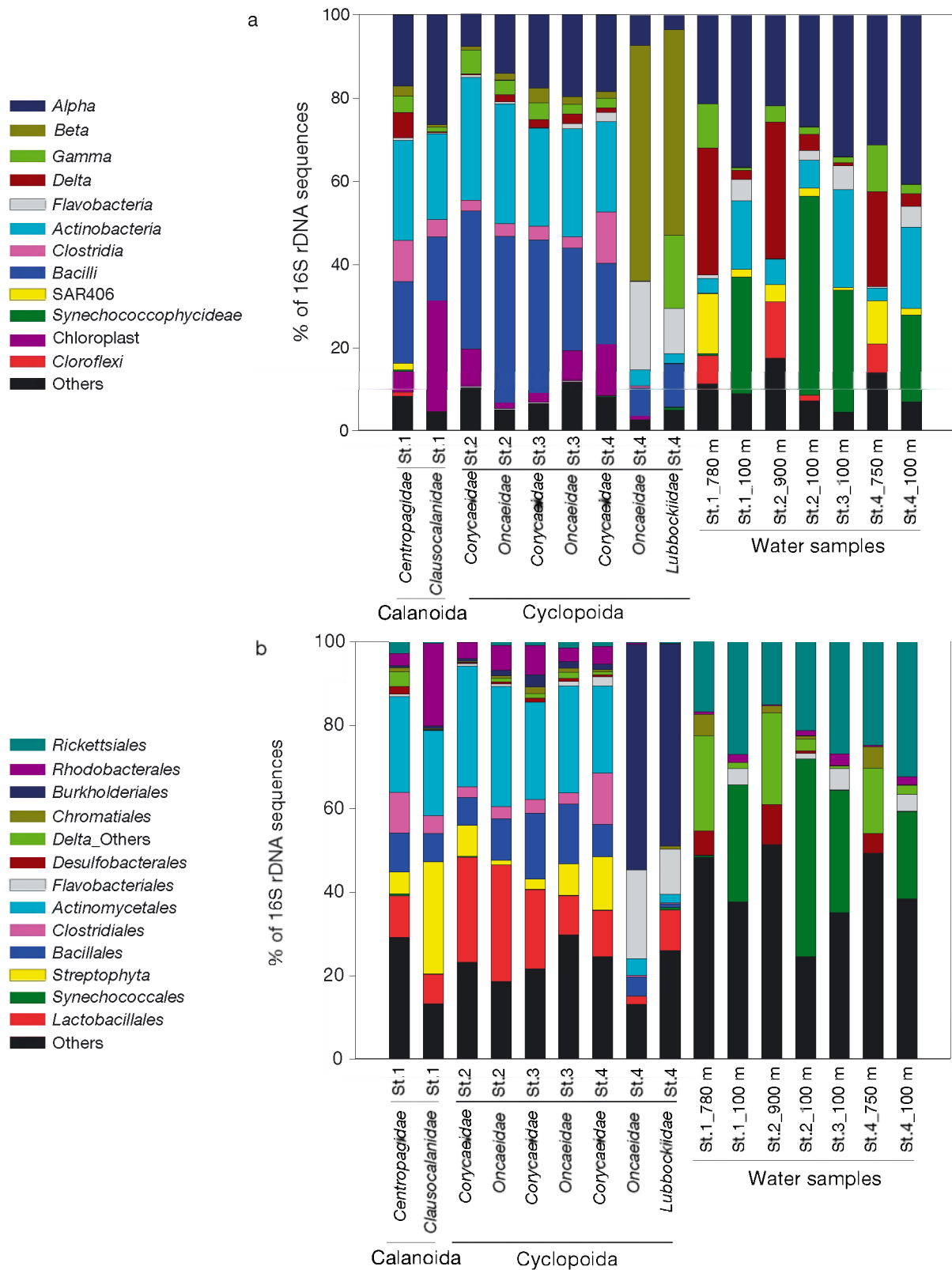


Fig. 2. Relative contribution of the more abundant phylogenetic (a) classes and (b) orders to the total number of 16S rDNA sequences obtained from copepod-associated and ambient-water bacterial communities

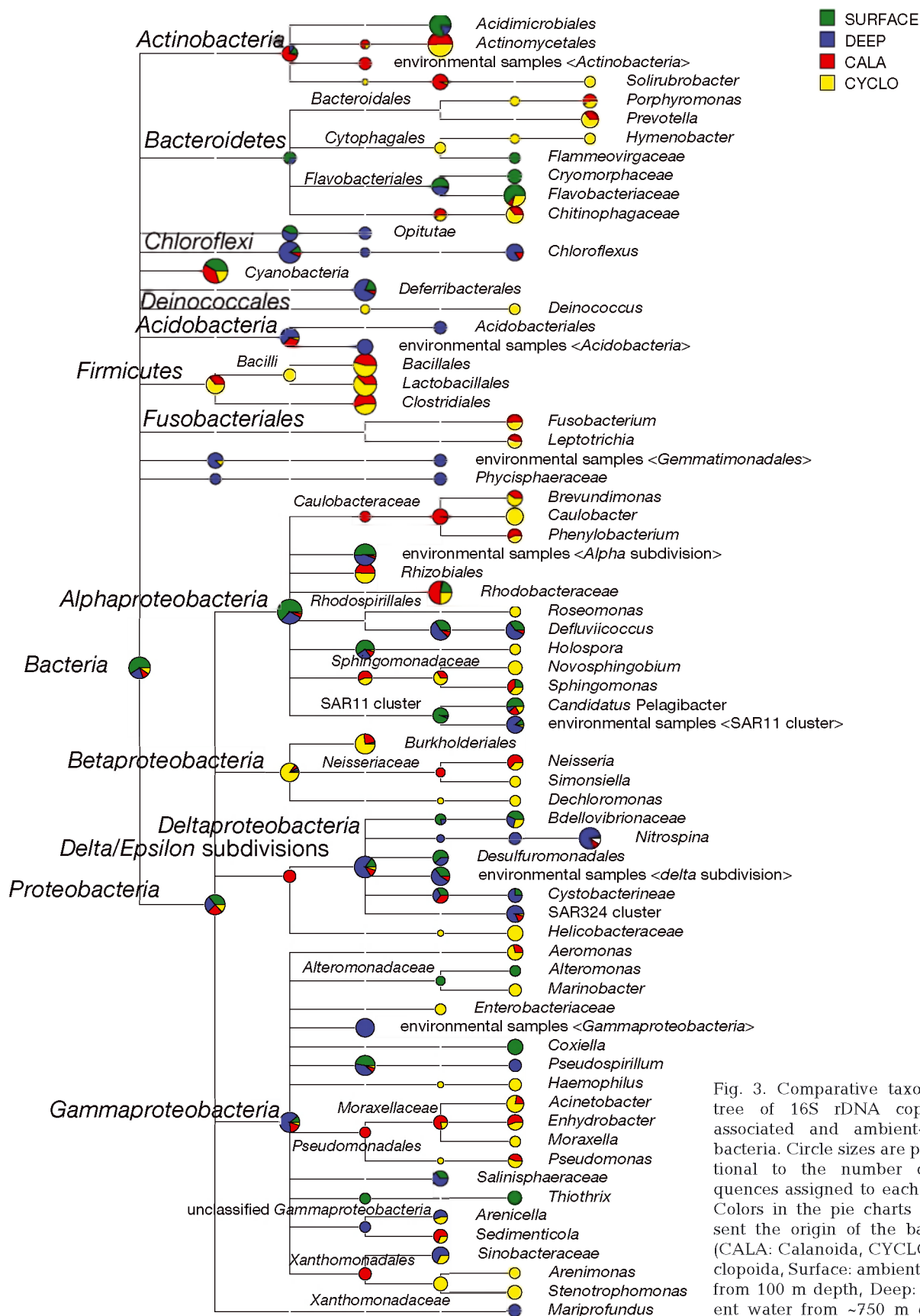


Fig. 3. Comparative taxonomic tree of 16S rDNA copepod-associated and ambient-water bacteria. Circle sizes are proportional to the number of sequences assigned to each node. Colors in the pie charts represent the origin of the bacteria (CALA: Calanoida, CYCLO: Cyclopoida, Surface: ambient water from 100 m depth, Deep: ambient water from ~750 m depth)

To investigate an alternative to the QIIME classifier and to directly visualize the distribution of individual bacterial taxa among the 2 copepod orders and the 2 depth layers, the samples were blasted against the SILVA and Greengenes database (data not shown). These results were visualized by MEGAN (Huson et al. 2007) using the BLAST hit-score to assign the taxonomy. As indicated in Fig. 3, some bacterial taxa associated with the 2 copepod orders were not present in the water column, including members of *Firmicutes*, *Fusobacteriales*, and most of the *Betaproteobacteria*. At the genus level, bacteria associated with copepods but absent in the ambient water belonged to the *Actinomycetales*, *Bifidobacteriales*, *Bacteroidales*, *Deinococcus*, *Bacillales*, *Lactobacillales*, *Clostridiales*, *Fusobacterium*, *Leptotrichia*, *Caulobacteraceae*, *Neisseriaceae*, and *Pseudomonadales* (Fig. 3).

Conversely, a few taxa of the bacteria were specific to the ambient water. Ambient water-specific bacteria at the genus level belonged to the *Acidimicrobiales*, *Flammeovirgaceae*, *Cryomorphaceae*, *Cystobacterineae*, *Alteromonas*, *Coxiella*, *Pseudospirillum*, *Thiothrix*, and *Mariprofundus* (Fig. 3).

Most of the bacterial taxa were present in both the ambient water and associated with copepods; however, their contribution to the respective bacterial community differed among the 2 contrasting environments (Fig. 3). Although our data were analyzed with 2 different databases (SILVA and Greengenes), the phylogenetic affiliation of the bacterial 16S rRNA gene obtained with both databases was comparable. The results from the BLAST hit-score using the NCBI taxonomy of the 2 different databases (see Fig. S3 in the Supplement) were significantly correlated ( $p < 0.01$ ,  $r^2 = 0.94$ ) with a slope close to unity. The only remarkable discrepancies between the 2 databases were detected for *Actinomycetales* and *Rickettsiales*, probably due to the lower number of sequences from these groups available in the SILVA database as compared to the Greengenes database (Fig. S3). The bacterial community composition of the ambient water and that associated with copepods were significantly different (Unifrac significance test,  $p < 0.001$ , Bonferroni corrected). The PCoA clearly separated ambient-water and copepod-associated bacterial communities (Fig. 4), with the first coordinate accounting for 42.6% and the second for 25.5% of the samples' variance. Furthermore, the bacterial communities of the ambient water clustered according to depth, and the copepod-associated bacteria according to the copepod order, but with a higher variability than the ambient-water bacterial communities (Fig. 4). In particular, the bacterial communities of 2 Cyclopoida samples were well

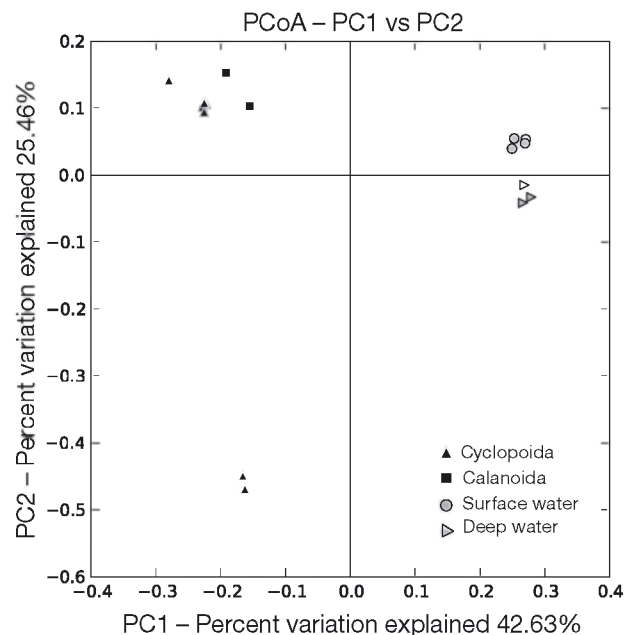
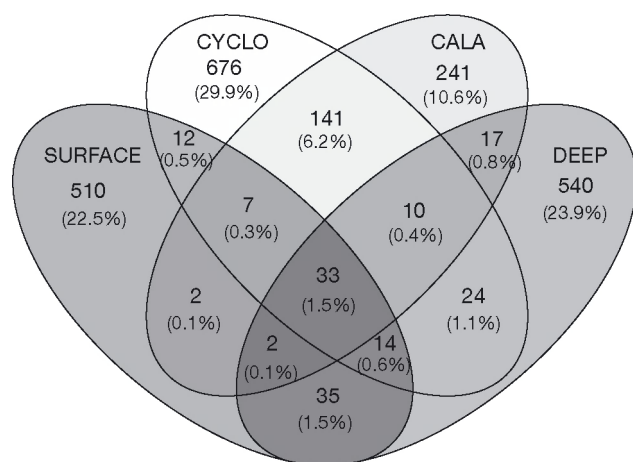


Fig. 4. Principal coordinates analysis (PCoA) of copepod-associated and ambient-water bacteria from individual samples. Bacterial communities from the same group of samples are represented by the same color

separated from the rest of the communities (Fig. 4) and had a lower bacterial diversity as compared to the other Cyclopoida samples (Table 1). These 2 Cyclopoida-associated bacterial communities were significantly different from the other Cyclopoida- and Calanoida-associated communities ( $p < 0.001$  Bonferroni corrected), and correspond to the communities associated with Oncaeidae and Lubbockiidae collected at Stn 4 as shown in Fig. 2.

The number of OTUs shared between the bacterial communities of the 2 depth layers and the copepod orders is indicated in Fig. 5. The 2 different copepod orders shared 191 (8.3%) OTUs while the 2 depth layers (100 m and 750 m) shared only 84 (3.7%) OTUs. Therefore, the number of shared OTUs was higher within the copepod-associated than within ambient-water bacterial communities. Only 33 (1.5%) OTUs were ubiquitously present, i.e. in all 4 sample categories. These ubiquitously present OTUs consisted of the most abundant ambient-water OTUs such as SAR11, SAR324, *Chloroflexi*, *Desulfobacteriales*, *Rhodobacteraceae*, and *Synechococcophycideae*. Furthermore, the bacterial communities from the ambient water and Cyclopoida-associated samples harbored a larger number of unique OTUs (510 [22.5%] OTUs at 100 m depth, 540 [23.9%] OTUs at 750 m depth, 676 [29.9%] OTUs in Cyclopoida) than the community associated with Calanoida (241 OTUs, 10.6%).





## DISCUSSION

In this study, we used a 454 high-throughput sequencing approach to characterize the bacterial community associated with 2 orders of copepods and to compare them to the bacterioplankton community of the ambient water collected at the same location. Generally, the bacterial community composition obtained in this study with 454 pyrosequencing differed from those reported in other marine and freshwater zooplankton studies using different techniques such as cloning and sequencing, agar plating, and CARD-FISH (Sochard et al. 1979, Hansen & Bech 1996, Peter & Sommaruga 2008, Grossart et al. 2009, Tang et al. 2010, Freese & Schink 2011). These differences are likely attributable to the different approaches used to investigate the bacterial community associated with the zooplankton. Early studies on zooplankton-associated bacteria used agar-plating approaches and consequently underestimated bacterial diversity (Sochard et al. 1979, Hansen & Bech 1996). However, the bacterial community associated with copepods obtained with the pyrosequencing technique was comparable to a study conducted on freshwater zooplankton-associated bacterial communities using denaturing gradient gel electrophoresis (DGGE) combined with sequencing (Grossart et al. 2009, Tang et al. 2010). In the latter study, the bacterial community associated with *Thermocyclops oithonoides* (a marine and brackish cyclopoid copepod) was dominated by *Betaproteobacteria*, *Bacteroidetes*, and *Actinobacteria*, followed by *Alpha*-, and

The limited amount of data available and the different methods used to determine the zooplankton-associated bacterial community composition preclude a thorough assessment of compositional differences in bacterial communities between zooplankton species or different oceanic provinces. However, the available data indicate considerable interspecific variability in the composition of the zooplankton-associated bacterial community (Tang et al. 2010). This microbial community is mainly associated with the exoskeleton and gut, which provide a favorable environment for bacterial attachment and growth (Nagasawa & Nemoto 1988, Pruzzo et al. 1996, Carman & Dobbs 1997).

Previous studies indicated that the bacterial community associated with the gut of crustacean zooplankton consists of 2 different bacterial communities: the resident bacteria persistently living in the gut and hence representing the stable component of the gut community, and the transient bacteria representing the variable gut community just passing through the digestive system of the host (Harris 1993, Tang et al. 2010). In our study, some bacterial phylogenotypes were consistently and abundantly found associated with the copepods and absent or only present in low abundances in the surrounding water (Fig. 3). However, some taxa of the copepod-associated bacteria varied considerably in abundance between the individual copepod samples, particularly in 2 out of the 9 copepod samples (Fig. 2, Table 2). These 2 Cyclopoida-associated bacterial communities deviated substantially from the community composition of the other 7 samples of copepod-associated bacteria and were characterized by a very low abundance of chloroplasts (0.5% of total sequences; Fig. 2a). This may suggest that the gut was empty at the moment of the extraction of the sample. These pronounced differences between the rather similar bacterial community composition of the 2 Calanoida and 5 Cyclopoida samples, on the one hand, and the 2 *Betaproteobacteria*-dominated communities in the other 2 Cyclopoida samples, on the other hand, might reflect differences between the bacterial community composition with a filled gut (resident plus transient bacterial community) and after gut evacuation (resi-

dent bacteria; Grossart et al. 2009). After gut evacuation, only the resident bacteria adapted to live and persist in the short gut of the copepods are detectable (Grossart et al. 2009, Tang et al. 2010). An alternative explanation for these pronounced differences in the bacterial community composition between the 2 Cyclopoida samples and the other zooplankton samples might be due to differences in the food sources, physiological state, or environmental conditions to which the zooplankton were exposed prior to sampling.

Intriguingly, we did not obtain *Vibrio* spp. sequences in our copepod samples, in contrast to other studies that analyzed copepod-associated pathogens in costal and estuarine environments (Huq et al. 1983, Heidelberg et al. 2002, Vezzulli et al. 2010). Although *Vibrio* spp. play an important role in the mineralization of chitin (Huq et al. 1983, Bassler et al. 1991, Heidelberg et al. 2002) and account for a significant proportion of the zooplankton-associated microbial community (Huq et al. 1983, Heidelberg et al. 2002), their role in the open ocean zooplankton remains unknown.

#### Comparison between the copepod-associated and the ambient-water bacterial communities

Grossart et al. (2009) suggested that the diversity of bacteria associated with zooplankton is mainly dependent on host–symbiont interactions, food, and the ambient bacterial community to which the host is exposed. We compared the copepod-associated bacteria to the bacterioplankton to determine the linkage between the 2 bacterial communities. Despite the significant differences between the copepod-associated and the ambient-water bacterial communities (Figs. 2 & 3), the presence of shared OTUs (Fig. 5) between all the samples (1.5% of the total OTUs) suggests a limited exchange of bacteria between the ambient water and the copepods.

The long tail of rare OTUs obtained for the ambient water in the rank-frequency distribution (Fig. S2) might provide a seed-bank of OTUs adapted to environmental conditions different from those prevailing in the ambient water (Pedrós-Alió 2006). However, we were not able to detect OTUs of the resident copepod-associated bacterial community in the ambient water, most likely because of the insufficient coverage of the ambient-water community (Fig. 1).

Taken together, our findings point toward a dynamic relationship between bacteria, zooplankton, and the environment where the dispersal of the copepod-associated transient bacterial community is mainly

related to the ingestion and egestion of the food. The development of the resident copepod-associated bacterial community is likely governed by the specific microenvironmental conditions in copepods. The extent to which the transient, and particularly the resident, copepod-associated bacterial communities vary in their composition due to the quality of food sources and periodicity in feeding activity remains to be shown.

#### CONCLUSION

We found significant differences between bacterial communities associated with copepods and those of the ambient water. However, our data suggest a dynamic linkage between these 2 communities. This interaction most likely affects the copepod-associated bacterial activity and diversity. Moreover, the bacterial diversity associated with zooplankton greatly diverged in 2 out of 9 samples, with specific phylogenetic groups dominating in these 2 samples, suggesting that the food sources and feeding status of the zooplankton might influence the bacterial community composition associated with the guts of copepods.

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