Vol. 78: 1–9, 2016
doi: 10.3354/ame01799

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online November 17

Different microbiomes associated with the copepods *Acartia tonsa* and *Temora longicornis* from the same marine environment

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ABSTRACT: Microbiomes of the neritic copepod species *Acartia tonsa* and *Temora longicornis* collected in coastal Danish waters were investigated by use of 16S rRNA gene-amplicon high-throughput sequencing. In contrast to current assumptions and findings, microbiomes of the 2 copepod species from the same environment were significantly different in bacterial species composition, particularly in terms of relative abundance of bacteria associated with each copepod species. The microbiomes of both species, likewise, differed from that of *A. tonsa* reared in laboratory culture, but similarities were also observed between wild and laboratory-reared *A. tonsa*. *Alphaproteobacteria* dominated *A. tonsa* microbiomes, whereas *Gammaproteobacteria* were most abundant in *T. longicornis* microbiomes. The overall diversity of bacteria associated with the 2 copepod species was to some extent in harmony with previous findings, but several of the most abundant operational taxonomic units correspond to bacteria that have not previously been found in association with marine copepods. The results thus add to the knowledge of the diversity of microbiomes of marine zooplankton organisms and document the fact that different planktonic copepod species from the exact same environment may host significantly different microbiomes. Hence, different copepod species are either not colonized by the same bacteria assemblages or support colonization/growth of different bacteria populations, leading to distinct copepod microbiomes.

KEY WORDS: Bacterial composition · MiSq sequencing · Illumina

INTRODUCTION

Planktonic copepods interact with bacteria in marine plankton in various ways. Copepod carcasses are effectively colonized and exploited by bacteria and thereby function as microbial hotspots in the water column (Tang 2005, Tang et al. 2006a,b). Also, live copepods are associated with bacteria, and their microbiomes comprise a wide range of bacterial species (Brandt et al. 2010, Dziallas et al. 2013, Gerdts et al. 2013), of which some are also common components of ambient microbial plankton. An active exchange of bacteria takes place between the copepod body and the surrounding water (Møller et al. 2007, De Corte et al. 2014). Bacteria associated with copepods are to a large extent attached to the exoskeleton (Nagasawa 1989), but some are located in the gut (Tang et al. 2010, 2011, Skovgaard et al. 2015), and the latter may consist of both resident bacteria and transient bacteria that follow the food passing through the gut (Tang et al. 2010).

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Little is known about the function of the copepod microbiome and the possible interactions between copepods and their associated bacteria. Copepods serve as physical substrate for bacteria and may, at the same time, offer organic substances to become exploited by bacteria, thereby providing growth conditions that are superior to the condition experienced by bacteria living free in the surrounding water (Møller et al. 2007). However, whether there are effects on the hosts, the live copepods, is still an issue that needs to be studied. The function of the copepod microbiome is not only of ecological interest, but also has ramifications for human health. Copepods in coastal waters have the capability to function as reservoirs for the human-pathogenic bacteria *Vibrio cholerae* and *Enterococcus faecalis* (Huq et al. 1983, Colwell & Huq 2001, Signoretto et al. 2004). Similarly, copepods are associated with *Vibrio* spp. pathogenic to oysters (Vezzulli et al. 2015), thereby possibly serving as environmental reservoirs for these pathogens.

Copepod microbiomes are very diverse, and numerous studies have aimed to identify these microorganisms by use of culture-dependent techniques (Sochard et al. 1979, Hansen & Bech 1996) as well as through more recently developed sequencing-based methodology (Møller et al. 2007, Dziallas et al. 2013, Gerdts et al. 2013). Only very recently has high-throughput sequencing technology been applied for studying microbiomes of copepods (De Corte et al. 2014, Skovgaard et al. 2015), thereby increasing the resolution of sequence-based studies on copepod microbiomes.

The copepod microbiome has typically been considered a more or less homogeneous compartment of bacteria in the marine plankton (Heidelberg et al. 2002a), and studies have failed to confirm differences in the composition of microbiomes of different copepod species (Dziallas et al. 2013, Gerdts et al. 2013, Bickel et al. 2014). Previously, observed differences in the composition of copepod microbiomes have been attributed to external parameters, such as season and habitat rather than to copepod species (Gerdts et al. 2013, Bickel et al. 2014), i.e. no host-specificity among copepod-associated bacteria has been demonstrated. As copepod microbiomes have been increasingly explored, a larger diversity of bacteria has been discovered and evidence has started to build up that the composition of the copepod microbiome is highly dependent on the environment in which the copepods live. The response of the composition of the copepod microbiome changes on a time scale of months (Bickel et al. 2014), but also on time scales down to a few days (Skovgaard et al. 2015).

The existence of host specificity among bacteria in copepod microbiomes has been explored through molecular biology-based techniques such as PCR-DGGE (Brandt et al. 2010, Dziallas et al. 2013, Gerdts et al. 2013, Bickel et al. 2014) and high-throughput sequencing (De Corte et al. 2014, Skovgaard et al. 2015), but so far, no studies have achieved quantitative proof of dissimilarities between microbiomes of different copepod species living in the same environment. However, indications have occurred recently suggesting the existence of host specificity among bacteria associated with marine copepods. In a PCR-DGGE study of copepod microbiomes from Greenlandic waters, an apparent difference in the microbiomes of 2 Arctic copepods, *Calanus finmarchicus* and *Metridia longa*, was noted (Dziallas et al. 2013). Unfortunately, the results could not be documented quantitatively because the bacterial sequences were disguised by abundant phytoplankton chloroplast sequences. More recently, differences were found between the microbiomes of dominant copepod families from the Atlantic Ocean (De Corte et al. 2014). The latter study was, however, based on bulk samples, thus hindering microbiomes of specific copepod species from being distinguished. Consequently, it remains unresolved whether host specificity exists in the microbiomes of different copepod species from the same environment.

The present study was dedicated specifically to investigating whether 2 copepod species, *Acartia tonsa* and *Temora longicornis*, from the same habitat, host different microbiomes, and whether a single copepod species (*A. tonsa*) hosts different microbiomes in the wild as compared to a copepod culture kept under laboratory conditions for decades.

MATERIALS AND METHODS

Sampling

Zooplankton was collected on November 26, 2013, in the estuarine strait of Øresund (The Sound), separating the northeastern part of Zealand, Denmark, from southwestern Sweden. Sampling was conducted from RV 'Ophelia' (Marine Biological Laboratory, Helsingør) using a plankton net with a mesh size of 250 µm. Samples were taken just below the thermocline at a depth of approximately 15 m where the water had a salinity of 30 ppt and a temperature of 7.7°C.

Copepods were transferred to 1–2 l of filtered seawater in a thermo box containing seawater from the sampling station and then brought directly to the lab-

oratory. Within 1–3 h, adult and late copepodite-stage copepods were isolated under a dissection microscope. Separate samples were made for the 2 numerically dominating copepod species, *Acartia tonsa* and *Temora longicornis*. Copepods were isolated and each animal was washed individually 3 times in freshly 0.2 µm filtered seawater. Five replicates of 25 specimens of each species were transferred to sterile 1.5 ml Eppendorf tubes. The water was then removed from the tubes with a drawn-out Pasteur pipette, carefully avoiding sucking up any copepods, and the samples were placed immediately at –20°C until DNA extraction. Laboratory-reared *A. tonsa* was sampled in a similar manner in 4 replicates. These laboratory-reared copepods originated from the permanent copepod culture at the National Institute of Aquatic Resources, Technical University of Denmark, in which copepods have been cultivated for decades. The copepods were cultivated in water with a salinity of 30 ppt and were fed the cryptophyte *Rhodomonas salina*. Copepods were sampled from the culture by retaining them on a net with a mesh size of 200 µm and were then transferred to 1 l of 0.2 µm filtered tank water and sampled as described above, except they were rinsed in 0.2 µm filtered tank water. No attempt was made to remove possible internal bacteria. Copepods were sampled for DNA extraction within 2–3 h after collection.

16S rRNA gene amplicon Illumina sequencing and pre-processing

Prior to DNA extraction, samples were homogenized by use of a battery-driven Kontes™ Pellet Pestle™ (VWR — Bie & Berntsen) using sterile disposable pestles. DNA was then extracted using the QIAamp® DNA Mini Kit (QIAGEN) following the manufacturer's instructions. The 16S rRNA gene region V3–V4 was amplified using primers designed with adapters for the Nextera Index Kit® (Illumina), which primarily target bacterial communities. The primer sequences (NXt_V3-V4_F 5′-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** CCT AYG GGR BGC ASC AG-3′ and NXt_V3-V4_R 5′-**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GG ACT ACN NGG GTA TCT AAT-3′; adapters in **bold**), amplification settings (1st PCR round), indexing (2nd PCR round), libraries' purification and sequencing (MiSeq platform) were performed as outlined by Pyndt Jørgensen et al. (2014). Pair-ended reads (and quality scores) were trimmed and merged with the CLC Genomic Workbench (CLC bio, version 7.0.4). Subsequently, UPARSE software (Edgar 2013) was used for de-replicating, operational taxonomic units (OTUs) clustering (97% sequence similarity) and filtering chimeric sequences. Taxonomic classification of reads was carried through the Greengenes database, version 12.10 (McDonald et al. 2012), and further diversity and statistical analyses were performed using the open software Quantitative Insights Into Microbial Ecology (QIIME, version 1.7.0) (Caporaso et al. 2010).

Statistics

For all analyses, the datasets were subsampled to 90% of the reads contained within the most indigent sample (53 000 reads, -e value for alpha and beta diversity; -d value for multiple rarefactions). Alpha diversity estimates (expressed as observed species, 97% similarity) were determined with the alpha rarefaction workflow, computed with 10 subsampled OTU tables and compared by a non-parametric *t*-test (Monte Carlo, 999 permutations). Beta diversity was determined with the jackknife beta diversity workflow. Briefly, 10 OTU tables and a phylogenetic tree (UniFrac method) were used to generate 10 rarefied distance matrices from which principal coordinates for each sample were determined, plotted and visualized as principal coordinate analysis. The differences between categories or classes within the distance matrices were evaluated with ANOSIM. Changes in relative distribution of taxonomic groups were determined through 1-way ANOVA.

RESULTS

Sequences obtained by high-throughput sequencing

Amplification and sequencing of the 16S rRNA gene (V3–V4 region) from 14 samples yielded 2 187 748 raw reads that had a mean sequence length of 382 base pairs (bp). The number of high-quality reads retrieved after de-replicating, sorting and filtering of chimeric sequences accounted for 1 880 180, with an average of 134 299 sequences per sample (minimum: 58 801, maximum: 215 148). The raw dataset is available through the European Nucleotide Archive (ENA) under accession number PRJEB-14826; sample information can be found in Table S1 in the Supplement at www.int-res.com/articles/suppl/a078p001_supp.pdf.

Comparison of microbiomes across copepod species and environment

The numbers of observed OTUs calculated in microbiomes associated with the 2 wild copepod species *Temora longicornis* (mean ± SD, 385 ± 26) and *Acartia tonsa* (320 ± 37) were not statistically different (*t*-test, p = 0.07), but were significantly higher than the estimated number of species in the microbiome of *A. tonsa* grown under laboratory conditions (145 ± 20, *t*-test, p < 0.05). Beta diversity analysis based on weighted UniFrac-distance matrices showed clear separation (ANOSIM, p < 0.05) of bacterial communities derived from the 3 groups of copepods (Fig. 1). The dissimilarity between the microbiomes of laboratory-reared *A. tonsa* and wild copepods was greater (ANOSIM R-value > 0.98) than the dissimilarity between the microbiomes of *T. longicornis* and *A. tonsa* from coastal water (ANOSIM R-value = 0.81).

Dominating OTUs of copepod microbiomes

The microbiomes of copepods exhibited profound differences in taxonomic distribution as a function of their species and living environment (Table 1, Fig. 2). In the laboratory-reared *A. tonsa*, microbiomes were dominated by 1 OTU belonging to the family *Rhodobacteraceae* (up to 89% relative abundance; see phylogenetic distances in Fig. S1 in the Supplement), with another unclassified OTU of *Rhodobacteraceae* (a member related to *Citreicella* sp.) being second-most abundant (up to 44% relative abundance). These 2 OTUs together comprised 83–92% of all reads in the samples from laboratory-reared *A. tonsa*. In the wild *A. tonsa* microbiomes, *Rhodobacter* sp. (*Alphaproteobacteria*, up to 66% relative abundance) and a non-classified OTU of *Sinobacteraceae* (*Gammaproteobacteria*, up to 37% relative abundance; see phylogenetic distances in Fig. S2 in the Supplement) were the most common bacteria, together constituting 69–89% of the reads. In contrast to both laboratory-reared and wild *A. tonsa*, bacteria belonging to *Rhodobacteraceae/Rhodobacter* sp. did not represent more than 2% of total reads in the *T. longicornis* microbiomes (Table 1, Fig. 2). In this copepod species, the dominating OTUs were instead *Flavobacterium* (phylum *Bacteroidetes*, up to 19% relative abundance) and the unclassified genus from *Sinobacteraceae* (up to 76% relative abundance) that was also present in the wild *A. tonsa*. These 2 OTUs together constituted 9–77% of the reads. In one *T. longicornis* sample, an OTU belonging to the genus *Comamonas* (*Betaproteobacteria*) accounted for 53% of the microbiome. This OTU did not contribute to more than 0.02% of the microbiome in any other sample.

A striking characteristic of the *T. longicornis* microbiome was that it contained several numerically dominating bacterial sequences that were absent or rare in all *A. tonsa* samples, i.e. both wild and laboratory-reared *A. tonsa* (Fig. 2). This was particularly the case for *Flavobacterium* sp. and unclassified genera of *Acidimicrobiales* (*Actinobacteria*), and *Pelagibacteraceae* and *Rickettsiales* (both *Alphaproteobacteria*). Furthermore, in comparison to *A. tonsa*, the *T. longicornis* microbiome tended to comprise a relatively larger number of OTUs (up to 15% of total sequence reads) that occurred in low abundance, i.e. constituting less than 1% of the total microbiome (Fig. 2). In general, all microbiomes were characterized by a very low number of chloroplast-related sequences, indicating that practically all observed OTUs originated from bacteria and not from ingested phytoplankton cells.

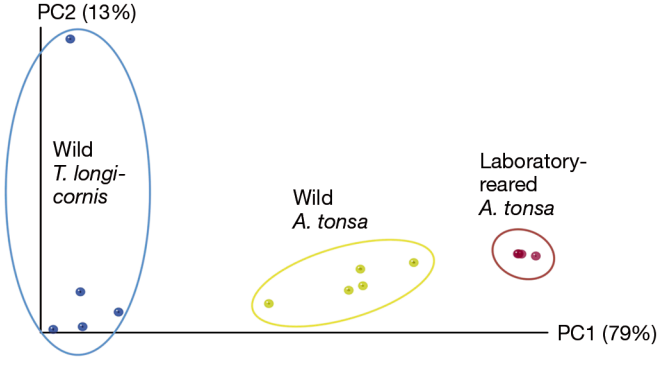

Fig. 1. Principal coordinate (PC) analysis plot of the microbiomes of laboratory-reared *Acartia tonsa*, wild *A. tonsa*, and wild *Temora longicornis*, based on weighted UniFrac distance matrices. Every sample of the dataset was subsampled using 53 000 random sequences

DISCUSSION

This study has revealed a hitherto unrecognized diversity of bacterial taxa associated with marine planktonic copepods; the environmental samples collected in the Øresund, with 25 specimens each of *Acartia tonsa* and *Temora longicornis*, disclosed on average 320 and 385 different bacterial OTUs, respectively. This sequence diversity is markedly higher in comparison to previous reports of copepod microbiomes, regardless of whether these were based on culture-dependent techniques (Sochard et al. 1979,

Table 1. Significant variations in relative distribution of bacterial genera in the microbiomes of 2 copepod species from 2 different environments: laboratory-reared *Acartia tonsa* (Lab *A. tonsa*), and *A. tonsa* and *Temora longicornis* isolated from environmental samples from Øresund, Denmark. The p- and q-values (false discovery rate correction method) were determined with 1-way ANOVA (using 53 000 random sequences per sample)

Phylum	Class	Order	Family	Genus	Significance		Relative abundance (%)	
					p-value	q-value	Lab *A. tonsa*	Wild *T. longicornis*
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	C111	Unclassified	0.033	0.104	0.02	3.69
Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	0.033	0.106	<0.01	10.91
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	Unclassified #2	0.001	0.038	64.74	1.12
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	Unclassified #3[a]	0.039	0.110	24.51	0.11
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	Unclassified	Unclassified	0.001	0.048	4.51	0.02
Proteobacteria	*Deltaproteobacteria*	*Bdellovibrionales*	*Bdellovibrionaceae*	*Bdellovibrio*	0.001	0.032	1.25	<0.01
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Sinobacteraceae*	Unclassified	0.005	0.062	2.56	46.90
Unassigned					0.018	0.100	0.02	2.28

Phylum	Class	Order	Family	Genus	p-value	q-value	Lab *A. tonsa*	Wild *A. tonsa*
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	Unclassified #2	0.001	0.011	64.74	2.78
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Rhodobacter*	0.000	0.004	0.07	57.33
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	Unclassified	Unclassified	0.001	0.019	4.51	0.03
Proteobacteria	*Deltaproteobacteria*	*Bdellovibrionales*	*Bdellovibrionaceae*	*Bdellovibrio*	0.001	0.010	1.25	<0.01
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Sinobacteraceae*	Unclassified	0.002	0.020	2.56	24.28

Phylum	Class	Order	Family	Genus	p-value	q-value	Wild *A. tonsa*	Wild *T. longicornis*
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	C111	Unclassified	0.047	0.342	0.71	3.69
Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	0.020	0.350	0.37	10.91
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	Unclassified #2	0.039	0.372	2.78	1.12
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Rhodobacter*	0.000	0.001	57.33	0.50
Unassigned					0.015	0.312	0.27	2.28

[a]Initially assigned to *Anaerospora* by the Greengenes database (McDonald et al. 2012), but classified as *Citreicella* by the EzTaxon database (www.ezbiocloud.net)

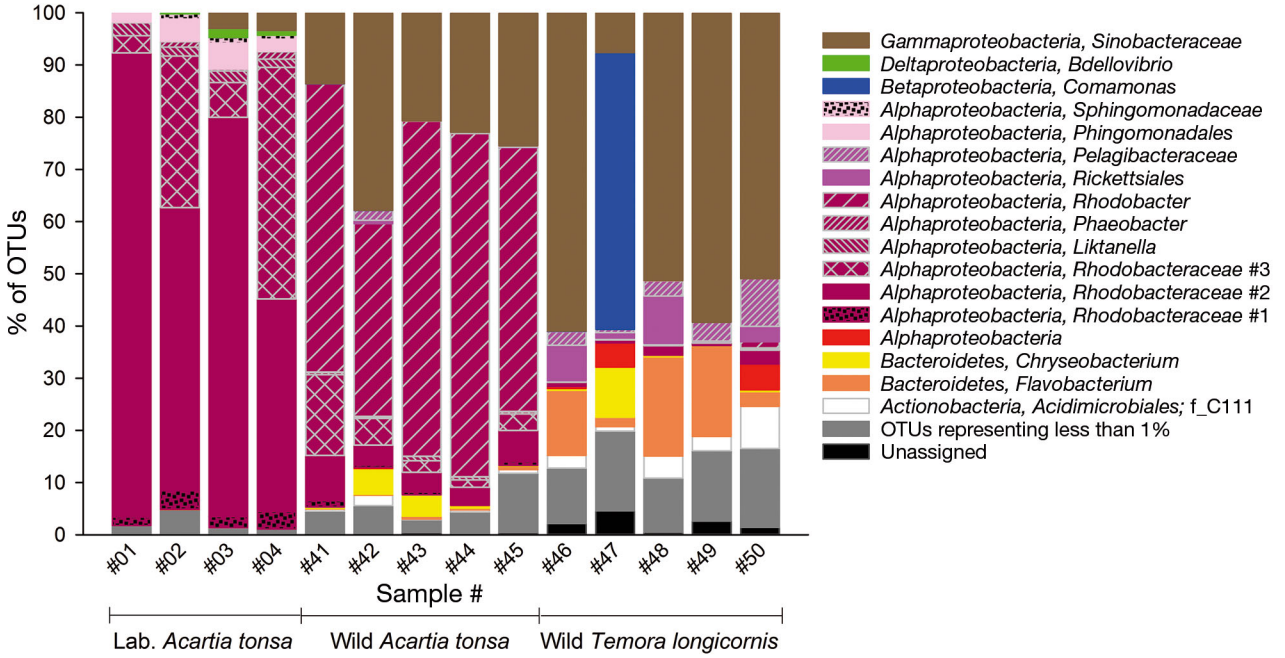

Fig. 2. Relative distribution of bacterial 16S rRNA gene sequence reads in 14 Illumina MiSeq-based 16S rRNA gene amplicon sequencing samples, from laboratory-reared *Acartia tonsa*, wild *A. tonsa*, and wild *Temora longicornis*. Operational taxonomic units (OTUs) belonging to the most abundant class, the *Alphaproteobacteria*, are labeled with various shades of pink/red (with and without patterns). Taxonomic levels below class are indicated in the color key only if such identification was possible. Samples of wild copepods originate from a single net tow in Øresund, Denmark

Hansen & Bech 1996), PCR-DGGE (Møller et al. 2007, Gerdts et al. 2013), or the 454 pyrosequencing platform (De Corte et al. 2014, Skovgaard et al. 2015). Such high diversity may not be surprising, since the Illumina technique is known to yield a higher number of sequences and thereby a higher richness of bacteria in samples.

The qualitative results of the present study are, to some extent, in line with those of De Corte et al. (2014) and Skovgaard et al. (2015), in the sense that all 3 datasets show relatively large numbers of *Alphaproteobacteria* OTUs. Hence, the results presented here demonstrate that *Alphaproteobacteria* are common in the microbiomes of marine planktonic copepods, as has also been shown in several previous studies based on other techniques (Møller et al. 2007, Brandt et al. 2010, Dziallas et al. 2013, Gerdts et al. 2013). However, these bacteria are far from always being the dominating group. Indeed, the microbiome of wild *T. longicornis* from Danish coastal waters contained comparatively high numbers of *Beta-* and *Deltaproteobacteria* as well as *Bacteroidetes*, with a strikingly low contribution of *Alphaproteobacteria* (Fig. 2). Overall, the most abundant bacterial taxa in microbiomes of wild copepod were of the family *Sinobacteraceae* (*Gammaproteobacteria*), *Rhodobacter* sp. (*Alphaproteobacteria*), order *Rickettsiales* (*Alphaproteobacteria*), family *Pelagibacteraceae* (*Alphaproteobacteria*), OTU #2 of family *Rhodobacteraceae* (*Alphaproteobacteria*), *Flavobacterium* sp. and *Chryseobacterium* sp. (*Bacteroidetes*), and an OTU of the order *Acidimicrobiales* (*Actinobacteria*). Among these, the OTUs of *Pelagibacteraceae* stand out, since these bacteria are usually free-living in the water column (e.g. Ortmann & Santos 2016). It is possible that a member of *Pelagibacteraceae* has adapted to a different life strategy than its closest relatives, but one can only speculate on the reason for this apparent discrepancy. The microbiome of laboratory-reared *A. tonsa* was dominated by a member of *Sphingomonadales* (*Alphaproteobacteria*), OTU #1, #2, and #3 of *Rhodobacteraceae*, and *Loktanella* sp. (*Alphaproteobacteria*). Most of these groups and genera are already known to be associated with marine planktonic copepods (Gerdts et al. 2013, De Corte et al. 2014, Skovgaard et al. 2015). However, among the most abundant OTUs, those corresponding to *Comamonas* sp., *Loktanella* sp., and *Chryseobacterium* sp. have not previously been found associated with marine copepods. The OTU corresponding with *Comamonas* sp. was abundant in one single sample only (wild *T. longicornis*), in which it was the dominating

OTU, accounting for approximately half of all OTUs (Fig. 2). It cannot be ruled out, however, that accidental sampling of a moribund copepod could have biased this sample. Even though a certain degree of variation between the copepods' microbiomes was observed (Fig. 2), the overall structure of the microbiomes was highly uniform within samples of 'species' and 'environment', respectively, as shown in the principal coordinate analysis (Fig. 1).

Interestingly, *Vibrio* spp. and *Enterococcus* OTUs were practically absent in the copepod microbiomes in the present study. *Vibrio* spp. and *E. faecalis* are known to be commonly associated with marine plankton in coastal waters (Huq et al. 1983, Signoretto et al. 2005), and copepods are considered as reservoir hosts for both *V. cholera* and *E. faecalis* (Colwell & Huq 2001, Signoretto et al. 2004). Likewise, the fish-pathogenic bacterium *V. anguillarum* has been found to be associated with marine particles (Larsen 1990), suggesting a potential association with copepods. When identifying copepod-associated bacteria by use of more traditional techniques, such as culturing-dependent identification or fluorescent *in situ* hybridization, *Vibrio* spp. and '*Vibrio*-like bacteria' often appear to be among the most common bacteria identified (reviewed by Tang et al. 2010). Yet, even though *Vibrio*-related OTUs did appear in the current sequence dataset, the relative abundance of *Vibrio* OTUs never exceeded 0.5% in any sample (data not shown). Preliminary work showed that the combination of primers and methodology used here was adequate for detecting and quantifying *V. anguillarum* from a laboratory culture (data not shown). The present study is not the first observation of limited association between marine copepods and *Vibrio* spp. Similar observations were made by De Corte et al. (2014) and Skovgaard et al. (2015). In fact, their investigations did not detect any *Vibrio*-related sequences in association with copepods. One must stress, however, that the presence of *Vibrio* spp. in microbiomes of marine planktonic copepods has been confirmed repeatedly in various regions of the world through different techniques (Huq et al. 1983, Heidelberg et al. 2002b, Gugliandolo et al. 2008). The cause of the apparent discrepancy between different investigations is presently unknown, but it appears that *Vibrio* spp. are particularly common in warm coastal waters, in which these bacteria have most frequently been identified in association with zooplankton.

The microbiomes of *A. tonsa* and *T. longicornis* isolated from the exact same sample collected in the Øresund had clear differences (Figs. 1 & 2). This is, to the best of our knowledge, the first time data show with such clarity and with statistical significance that 2 copepod species living in the same marine environment, and originating from the exact same sample, are associated with distinct bacterial communities. While host specificity of bacteria in copepod microbiomes has been studied before, previous investigations have encountered methodological hurdles, hindering definite conclusions. The high efficiency and sensitivity of high-throughput sequencing technologies enhances possibilities to detect and quantitatively analyze differences in copepod microbiomes, which more traditional techniques often reveal less satisfactorily (Dziallas et al. 2013, Gerdts et al. 2013). A couple of recent investigations showed indications of host specificity in copepod microbiomes based on high-throughput sequencing methodology (De Corte et al. 2014, Skovgaard et al. 2015). One of these studies was based on frozen bulk zooplankton samples from different locations in the north-central Atlantic Ocean (De Corte et al. 2014) and demonstrated significant differences between bacterial communities associated with the dominant copepod families. However, because copepods were identified to family level only and furthermore were collected at different locations, the difference in copepod microbiomes might have been a combination effect of host taxon and sampling location rather than actual host specificity. In addition, when based on frozen samples, the possibility exists that some animals may have been dead at the time of sampling and a few deceased copepods would then induce biased results, because copepod carcasses are extensively exploited by opportunistic bacteria (Tang et al. 2006a) that may differ from the bacteria of the live copepods' microbiomes.

The dissimilarities between the microbiomes of laboratory-reared *A. tonsa* vs. wild copepods (ANOSIM R-value = 0.98) and laboratory-reared *A. tonsa* vs. *T. longicornis* (ANOSIM R-value = 1.0) were greater than the dissimilarity between the microbiomes of *T. longicornis* and *A. tonsa* from coastal water (ANOSIM R-value = 0.81) in weighted UniFrac (presented graphically in Fig. 1). This may not seem surprising, considering that there is a dynamic exchange of bacteria between the water and the copepods (Møller et al. 2007, Grossart et al. 2010, De Corte et al. 2014), and the composition of bacterial communities and the growth conditions for bacteria undoubtedly differ between the water of laboratory cultures and natural coastal waters.

Microbiomes of laboratory-reared *A. tonsa* and wild *A. tonsa* from coastal water samples were clearly different (Fig. 1), but pronounced similarities were

also obvious. *Alphaproteobacteria* of the family *Rhodobacteraceae* were by far the dominating group of bacteria in all *A. tonsa* samples irrespective of origin (i.e. laboratory or coastal samples). In both cases, 2 *Rhodobacteraceae* taxa were represented in relatively high abundances; *Rhodobacteraceae* has previously been found to dominate microbiomes of both laboratory-reared and wild *A. tonsa* (Skovgaard et al. 2015). Hence, copepods appear to constitute a suitable growth substrate for these bacteria regardless of whether the copepods are living free in the sea or kept in culture for many generations. On the other hand, OTUs related to *Rhodobacteraceae* contributed a maximum of 2.5% to the microbiome of *T. longicornis* (Fig. 2), indicating that the high abundance of *Rhodobacteraceae* in copepod microbiomes was not merely a result of these bacteria being dominating in the surrounding environment. The nature of interactions between copepods and bacteria is not limited to which bacteria are present in the surrounding water. The copepod's diet may also play a role, since different food items will be associated with and potentially enhance growth of different bacterial communities. This will in turn influence which bacteria the copepods are exposed to. These mechanisms may be part of the explanation of the difference between the microbiomes of *T. longicornis* and *A. tonsa* from the same environment.

Little is known about the possible function of bacteria associated with copepods, and future research should aim to resolve potential beneficial as well as harmful elements of copepod microbiomes. One may speculate that bacteria attached to a live copepod have competitive advantages upon the death of the copepod as compared to bacteria colonizing recently dead copepods, because the bacteria that were attached while the copepod was alive may be able to respond faster to the sudden presence of suitable growth substrate. Such a strategy remains unstudied, and thereby speculative, for copepod-associated bacteria, but it is a known strategy for certain ciliated protozoa (Grimes & Bradbury 1992), and it is plausible that bacteria may benefit in a similar manner by being passively attached to copepods and then feed on the hosts upon their death. In addition, a wide range of commensal and parasitic eukaryotes are associated with copepods (Skovgaard & Saiz 2006), and it is natural to assume that copepods may also host bacteria that are infectious to the copepods. The existence and possible ecological effect of such pathogenic bacteria have yet to be elucidated in planktonic copepods; so far, a single observation has suggested that bacteria may, in some cases, invade and proliferate in the tissue of live copepods (Ianora et al. 1990). The present study was based on samples in which 25 copepods were pooled. However, judging from the amount of DNA extracted from each sample, it should be possible to also compare the microbiomes of individual copepods, which would allow the inclusion of intraspecific variation in future microbiome studies. Likewise, such future studies may reveal hitherto hidden relationships, such as patterns in the microbiome of a single copepod species from different geographical locations and environments as well as spatial and seasonal variations in these microbiomes. Finally, possible functions of bacteria associated with copepods might be explored by investigating relationships among microbiomes and the physiological state of copepod populations, including parameters such as non-predatory mortality, population growth, and longevity.

Acknowledgements. We are grateful to the crew of RV 'Ophelia' (University of Copenhagen) for assistance in field sampling and to Jack Melbye, DTU-Aqua, for providing the laboratory copepod culture. J.L.C.-M. was supported by a University of Copenhagen Excellence grant for interdisciplinary research (CALM). A.S. was supported through the project IMPAQ — IMProvement of AQuaculture high quality fish fry production, funded by the Danish Council for Strategic Research (grant no. 10-093522), and AMPHICOP, Acartia tonsa Molecular PHysiology — Implementation of novel and fast tools to assess COPepod physiological states, funded by the Villum Foundation (project no. 8960).

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Editorial responsibility: Klaus Jürgens, Rostock, Germany

Submitted: February 8, 2016; Accepted: September 7, 2016
Proofs received from author(s): October 21, 2016