Copepods promote bacterial community changes in surrounding seawater through farming and nutrient enrichment

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Summary

Bacteria living in the oligotrophic open ocean have various ways to survive under the pressure of nutrient limitation. Copepods, an abundant portion of the mesozooplankton, release nutrients through excretion and sloppy feeding that can support growth of surrounding bacteria. We conducted incubation experiments in the North Atlantic Subtropical Gyre to investigate the response of bacterial communities in the presence of copepods. Bacterial community composition and abundance measurements indicate that copepods have the potential to influence the microbial communities surrounding and associating with them – their 'zoosphere', in two ways. First, copepods may attract and support the growth of copiotrophic bacteria including representatives of Vibrionaceae, Oceanospirillales and Rhodobacteraceae in waters surrounding them. Second, copepods appear to grow specific groups of bacteria in or on the copepod body, particularly Flavobacteriaceae and Pseudoalteromonadaceae, effectively 'farming' them and subsequently releasing them. These distinct mechanisms provide a new view into how copepods may shape microbial communities in the open ocean. Microbial processes in the copepod zoosphere may influence estimates of oceanic bacterial biomass and in part control bacterial community composition and distribution in seawater.

Introduction

Bacteria have a variety of mechanisms to navigate extreme nutrient limitation in the oligotrophic open ocean. One such mechanism is attraction to marine snow particles that release carbon and nutrients into their surroundings (Alldredge and Gotschalk, 1990; Azam, 1998; Dang and Lovell, 2016). Bacteria attached to living marine organisms may use released nutrients in much the same ways as the bacteria in association with marine snow (Carman and Dobbs, 1997, Fouilland & Mostajir 2010). Copepods, a group of abundant marine mesozooplankton, can liberate nutrients into the surrounding seawater in at least three ways: (i) dissolved organic matter (DOM) is released via copepod sloppy feeding (Roy *et al*., 1989; Moller *et al*., 2007), (ii) dissolved and particulate organic carbon (DOC) and urea are released from rapidly sinking faecal pellets (Urban-Rich, 1999; Thor *et al*., 2003), and (iii) ammonium, urea and DOC are directly released with the copepod waste (Saba *et al*., 2011). Copepods may excrete up to 40% of the nitrogen they consume as ammonium, the preferred nitrogen source for microbes, to the surrounding seawater (Saba *et al*., 2011). Copiotrophic bacteria attracted to these nutrients could potentially either attach to the copepods or remain as free-living in the surrounding waters. Overall, copepod-associated bacterial communities contain both stable and temporarily attached groups, one major source of the variability in the microbiome being the copepod diet (Hansen and Bech, 1996; Moisander *et al*., 2015). Yet, the potential for copepods to serve as sites of active *in situ* microbial growth is not well understood. Such host-sustained growth could be induced by at least two distinct mechanisms. First, the nutrients released from the copepod could support free-living bacteria in the surrounding seawater, actively attracting opportunistic groups and promoting their growth. Second, copepods could be 'farming' certain bacteria in their gut or on their exoskeleton by providing beneficial growth conditions for these groups, and then potentially release some of these bacteria to the copepod surroundings. The nutrient-enriched microenvironment in/on and surrounding the copepod should provide distinct, beneficial environments for opportunistic or host-specific bacteria. Separating the *in situ* growth in the
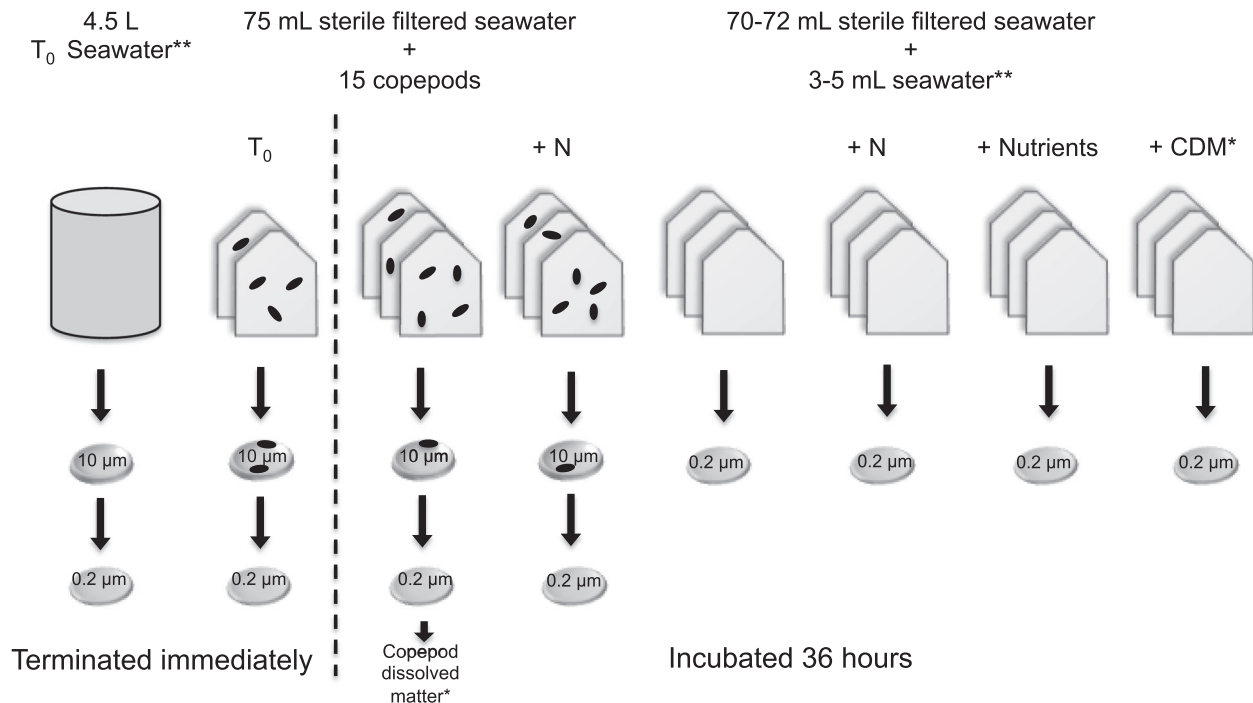
Fig. 1. Experimental design of copepod and seawater incubation experiments. Rinsed copepods, depicted as black ovals, were added to copepod treatment bottles. T0 samples were collected for the seawater inoculum and for copepod treatments. Nitrogen and carbonate addition (+N) were done for a parallel study (see Moisander *et al*., 2018) and included 0.5 μM each of NO_2^-, NO_3^-, NH_4^+ and CO_3^{-2}. Nutrients added included NO_3^- (0.5 μM), NH_4^+(0.5 μM), dextrose (0.5 μM), FeCl (0.5 μM), EDTA (0.5 μM) and PO_4^{-3} (1 μM). After incubation, triplicate samples were filtered directly onto 0.2 μm for seawater, and sequentially onto 10 and 0.2 μm filters for copepod treatments. *In Experiment 1, CDM was collected at the end of the experiment and used as the addition in Experiment 3. **Unfiltered seawater was used as a bacterial inoculum in all seawater treatments.

copepod association from growth under general nutrient amendments should help build mechanistic understanding of the host–symbiont interactions in and on copepods. Many of the dominant bacterial community members reported in copepod association are considered copiotrophs, promoted by organic matter and nutrients, and are commonly reported in marine particles (Dang and Lovell, 2016). Yet, the copepod association could potentially promote related but distinct bacterial groups.

Specifically, the goal of this study was to separately investigate the influence of copepods on the surrounding microbial communities through the two distinct mechanisms described (nutrient enrichment of surrounding free-living communities vs. farming in/on the copepod). We conducted incubation experiments with large calanoid

copepods sampled in the North Atlantic subtropical gyre during late summer. Bacterial communities developing during bottle incubations with and without copepods were studied, and communities on copepods and in the surrounding water in bottle incubations were investigated separately. In addition, we investigated the importance of nutrient enrichment versus farming by combining information from bacterial cell counts with relative abundances from sequence analyses.

Results

Bacterial abundance

In all of the experiments (Fig. 1 and Table 1), at the end of incubation, bacterial abundance in the incubation water was 1–3 orders of magnitude greater when copepods were

Table 1. Bottle incubation experimental parameters used in this study.

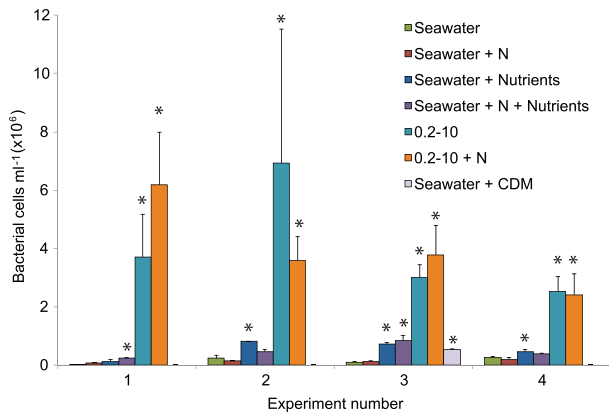| Exp #	Bottle type	Type of copepods	Incubation temperature°C	Length of incubation (h)	SW inoculum depth (m)	Amount of SW inoculum (ml)
1	125-ml polycarbonate	15 Mixed	27–34	36	20	5
2	125-ml polycarbonate	15 Mixed	27–44	36	10	5
3	75-ml glass	15 *Undinula*	12–31	36	80	3
4	125-ml polycarbonate	15 Mixed	12–24	36	40	5

Fig. 2. Bacterial cell abundances in the incubation water at the end of the experiments. Error bars are the standard deviation around the mean between triplicate experimental samples. Asterisks indicate significantly higher abundance over seawater control incubations within each experiment. See Fig. 1 for explanation of treatments. The treatment '0.2-10' indicates the seawater from the bottle containing the copepods. [Color figure can be viewed at wileyonlinelibrary.com]

present than in the samples with seawater incubated without copepods (Fig. 2). In all cases, bacteria were much more abundant in the presence of copepods than in seawater with or without any nutrient additions (analysis of variance [ANOVA], $p < 0.05$).

Overall variation in community composition

Amplicon sequencing of the 16S rRNA gene for Experiment 3 was conducted on samples from the beginning and end of
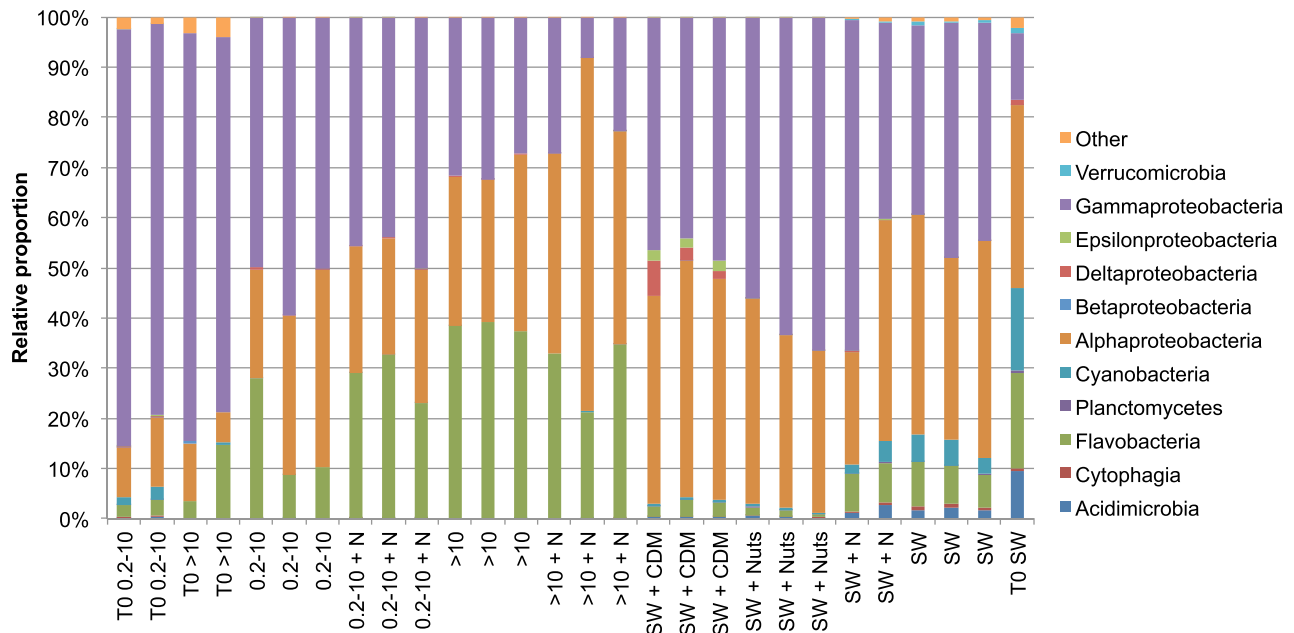
the incubation, to investigate differences in bacterial communities in the presence and absence of copepods. Sequencing resulted in an average of 82,175 reads per sample (S.D. = ± 41,615; Supporting Information Table S1). Alpha diversity was significantly greater in the seawater incubated with no nutrient additions than the copepod incubation treatments, or seawater with nutrients or copepod dissolved matter (CDM) added (ANOVA, $p < 0.01$, Supporting Information Table S1).

The bacterial community was notably consistent between treatment replicates, indicating repeatable sampling and sequencing efforts (Fig. 3). Several treatment-specific trends were observed in the communities. First, the bacterial community in the copepod size fraction (>10 μm) was distinct from the smaller size fraction (0.2–10 μm representing the 'free-living bacteria') from the same incubations (Fig. 4 – filled vs. open orange and blue circles, ANOSIM, $p < 0.01$). The communities were similar in copepod incubations without additional nutrients, and in copepod incubations in the presence of added N. Incubations with whole seawater inoculum without copepods (triangles in Fig. 4) were distinct from communities from both size fractions of copepod incubations (ANOSIM, $p < 0.001$). The 0.2–10- and >10-μm size fraction communities from the copepod incubations clustered more closely together than the communities from the seawater inoculum treatments. Interestingly, seawater inoculum incubations with the addition of artificial nutrients and CDM (pink and purple triangles respectively) were distinct, yet clustered relatively closely together (ANOSIM, $p = 0.10$) and were

Fig. 3. Relative abundance of major bacterial groups in Experiment 3. Each bar represents the bacterial community composition of a sample normalized to 100%. Treatments with copepods are listed as either '>10' for the copepod fraction or '0.2–10' for the 0.2–10-μm size fraction of seawater surrounding the copepods in the incubation bottles. The bars with the same legend are experimental replicates of that treatment. FSW samples with a whole seawater (bacterial) inoculum are labelled with 'SW', while 'T0 SW' represents the original untreated seawater sample used as inoculum. 'N' represents samples with nitrogen and carbonate added, 'Nuts' indicates nutrients added and 'CDM' indicates copepod dissolved matter was added. [Color figure can be viewed at wileyonlinelibrary.com]
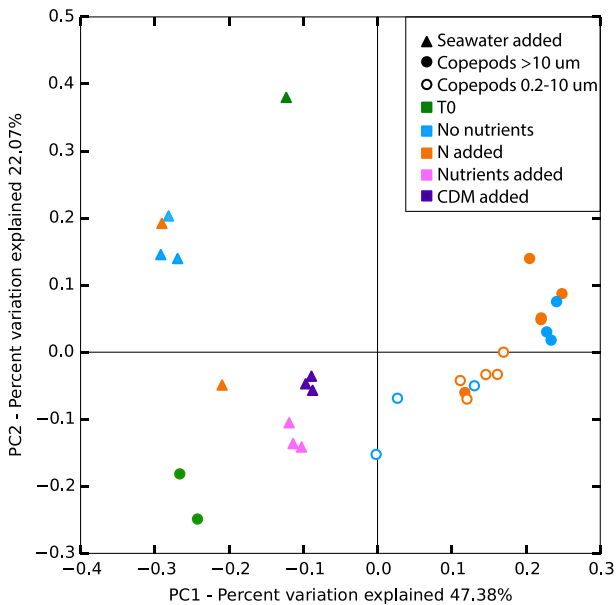
Fig. 4. Principle coordinates analysis of community composition within each sample from Experiment 3 based on 16S rRNA amplicon sequencing. Treatments are shown by colour. The 0.2–10-µm size fraction water from the copepod treatments are denoted with an open circle, and 10-µm size fraction (the copepod fraction) are denoted with a closed circle. Seawater treatments (no copepods in the incubation) are marked with a triangle. Due to the low sequencing depth of the 0.2–10-µm size fraction from the T0 copepods, the samples were removed from this analysis. [Color figure can be viewed at wileyonlinelibrary.com]

more similar to the communities associated with water surrounding the copepods than to the seawater controls incubated without nutrients or CDM (Figs. 4 and 5).

Taxonomic variability

On average, Gamma- and Alphaproteobacteria were the groups with the highest proportion in all of the treatments, averaging 47% ($\pm$13.6%) and 32% ($\pm$10.7%) of the total community respectively (Fig. 3). Overall, the average relative abundance of Flavobacteria made it the third most dominant group, but there was a large difference between copepod and seawater treatments. The Flavobacteria proportion averaged 28% ($\pm$ 9.2%) of the total community in copepod treatments and 4.6% ($\pm$ 3.1%) in seawater treatments. At times, Flavobacteria formed a greater part of the community than Alpha- or Gammaproteobacteria. There was a significantly greater proportion of Flavobacteria (predominantly family Flavobacteriaceae) in copepod treatments than seawater inoculum treatments (Kruskal–Wallis [KW] $p < 0.001$; Figs. 3 and 5 and Supporting Information Fig. S1). Additionally, the Flavobacteria abundances in seawater treatments with either artificial nutrient or CDM amendments were equally low, having abundances within the same order of magnitude as the seawater without nutrients (Supporting Information Fig. S1). The majority of Flavobacteria sequences from copepod treatments (~90%)

could be grouped into five operational taxonomic units (OTUs) (Fig. 6). Although the relative proportion of these five OTUs was slightly different among samples, all were present in all copepod treatment samples, including both 0.2–10- and >10-µm size fractions, at the end of the incubation.

Cyanobacteria and Acidimicrobiia were present in the T0 samples and the incubated seawater controls (filtered seawater [FSW] + SW inoculum) but were greatly reduced or disappeared in nutrient and copepod treatments during the incubation. Delta- and Epsilonproteobacteria were present in the CDM treatment at significantly higher relative abundances than in any other treatment (KW $p < 0.01$; see Fig. 3).

Within Gammaproteobacteria, there was a clear shift in proportions of Pseudoalteromonadaceae and Alteromonadaceae in the incubation water, when comparing treatments with and without copepods (Fig. 7). The abundances of these key bacterial groups were also calculated using the flow cytometry data and relative proportions in the amplicon sequencing data in a direct one-to-one ratio, without factoring in possible differences in 16S rRNA copy number per cell. These count-based data show that bacteria from Alteromonadaceae were present relatively consistently in all incubations, although appeared to respond less in the presence of copepods than in the presence of artificial nutrients or CDM (Fig. 7 and Supporting Information Fig. S2). Calculated abundances of Alteromonadaceae in the incubation water were actually higher in the absence of copepods when artificial nutrients or CDM were added to seawater than in the presence of copepods (KW, $p = 0.001$). In contrast, members of Pseudoalteromonadaceae thrived in the presence of copepods but formed a smaller proportion of the community in seawater inoculum treatments, even when nutrients or CDM were added (Figs. 5–7). Five major OTUs were identified in both size fractions of the copepod incubations that clustered closely with *P. spongiae*, *P. rubra* and *P. tunicata* (Fig. 6). These OTUs were also present at high proportions in the T0 copepods.

The abundances of Vibrionaceae and Oceanospirillaceae increased with the addition of either nutrients, presence of copepods or addition of CDM (Fig. 7). Within Oceanospirillaceae, *Marinomonas* and *Oleibacter* were present in all incubations. When nutrients or CDM were added, the dominant Oceanospirillaceae was *Neptunibacter* (Fig. 8). The seawater treatments with nutrient or CDM additions maintained a high level of similarity to each other, with some important differences. Two Vibrionales OTUs with nucleotide identities closely related to *Vibrio tubiashii* and *Vibrio brasiliensis* were present in both treatments, while rare in other treatments (Fig. 6). A key difference between the SW + nutrients and SW + CDM treatments was the presence of Epsilon- and Deltaproteobacteria in the SW + CDM treatment, absent in the SW + nutrients treatment (Fig. 3). While these groups were detected in copepod treatments
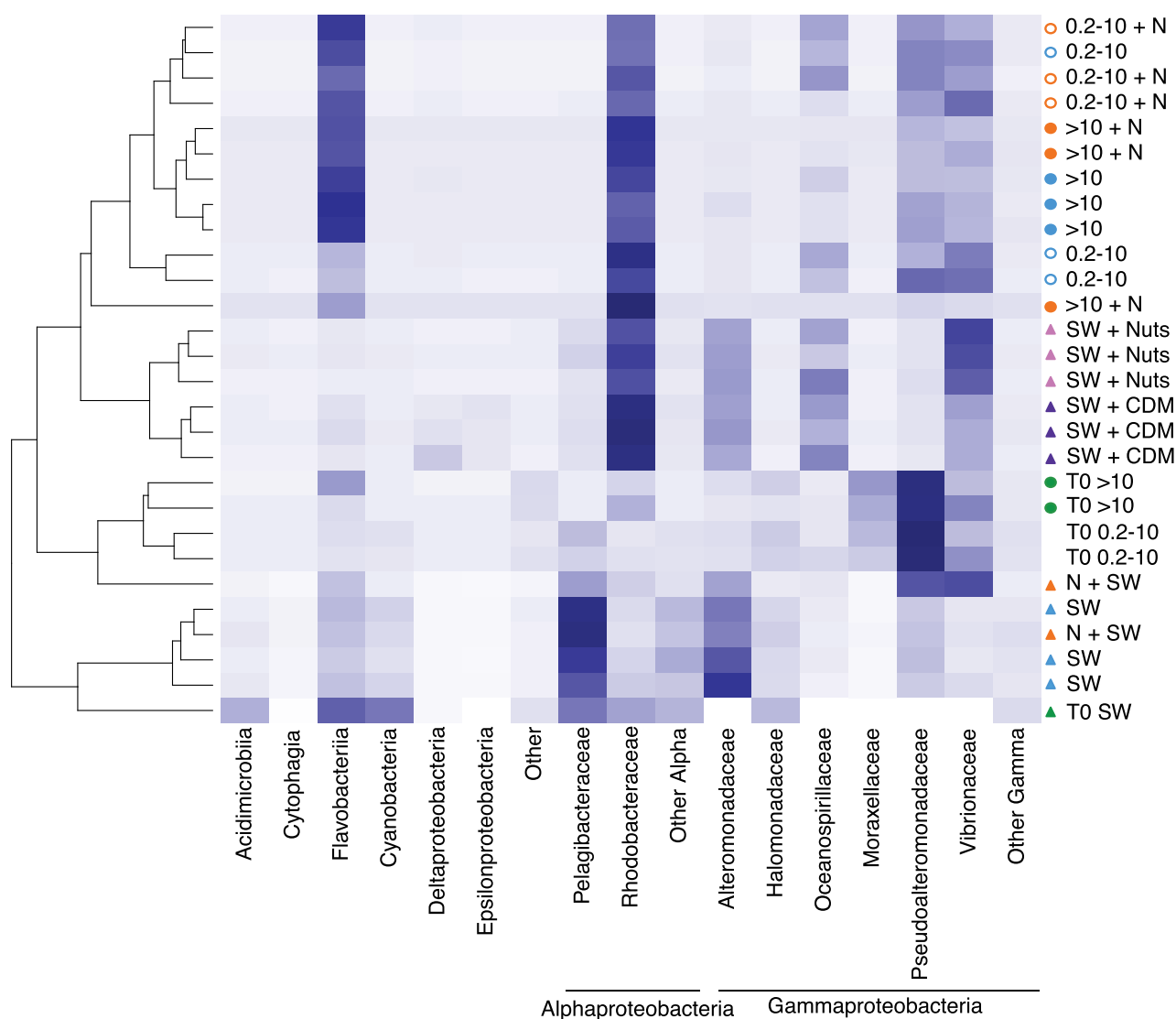
Fig. 5. Relative proportions of the entire community for the key abundant groups present in Experiment 3. Alpha- and Gammaproteobacteria were the dominant groups present throughout the experiment. Some of the most dominant Gammaproteobacterial families are shown. Samples are arranged on the Y-axis according to the dendrogram created using a Bray–Curtis dissimilarity matrix. Sample labels and symbols are the same as in Fig. 3. [Color figure can be viewed at wileyonlinelibrary.com]

and the seawater inoculum, they were present in much lower proportions (<1% total community compared to 5.7% of the CDM treatment). The dominant Deltaproteobacteria was from the family Bacteriovoraceae (order Bdello-vibrionales), and the primary Epsilonproteobacteria was genus *Arcobacter* (order Campylobacterales).

Relative abundances of community members in the sequence data show similar trends between the 0.2–10- and >10-µm (copepod) size fractions for copepod incubations, with an overall slightly diminished relative abundance of Gammaproteobacteria in the copepod fraction (Supporting Information Fig. S3, 34%–22% respectively). This reduction in the Gammaproteobacterial proportion in the copepod size fraction is primarily due to a lower proportion of *Marinomonas* (Fig. 8) and an overall increase in the

proportion of Flavobacteria in the copepod fraction compared with the 0.2–10-µm fraction surrounding the copepods.

A higher proportion of Alphaproteobacteria was present at the end of the incubation than at the beginning for both copepod- and seawater-treated samples (Fig. 3). The dominant Alphaproteobacteria in all copepod treatments was Rhodobacteraceae, while the seawater treatments contained both Rhodobacteraceae and Pelagibacteraceae. The Pelagibacteraceae proportion diminished during incubation in seawater treatments with nutrients or CDM (Fig. 5).

Discussion

The results from this and other recent studies from copepod–bacteria interactions are starting to shape a
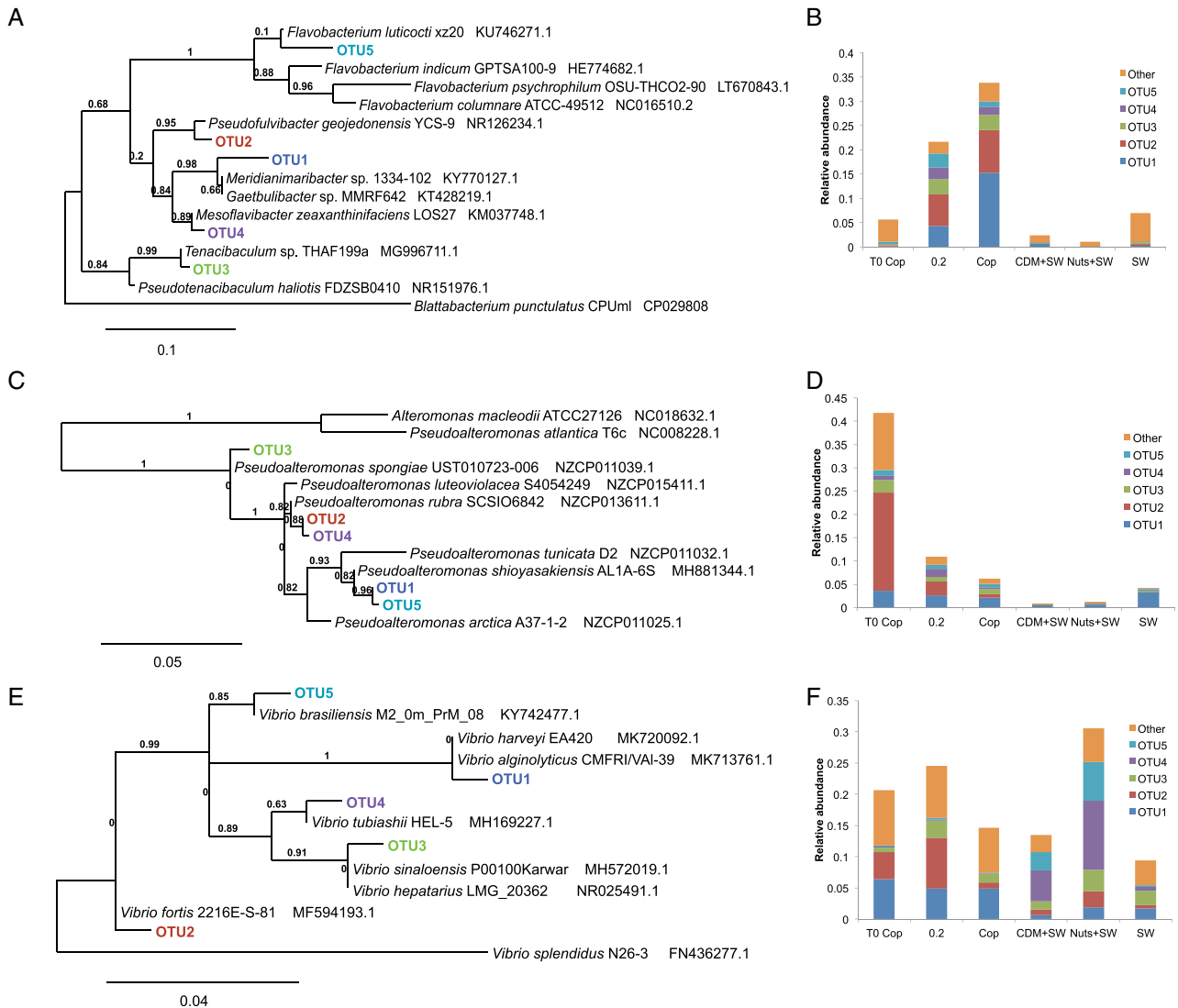
Fig. 6. Maximum-likelihood trees containing representative sequences of abundant OTUs and their average relative abundance in each treatment. These OTUs were present in all copepod experiments at the end of the incubation. 'Other' OTUs are all of the combined OTUs within each group that are not represented on the tree. Sequences from this study are in bold, and reference 16S rRNA sequences are from NCBI. Sequences were aligned with MUSCLE and the tree constructed using the maximum likelihood method PhyML.
A. The five most abundant OTUs within Flavobacteriaceae.
B. The relative contribution of each Flavobacteriaceae OTU to the overall bacterial community in each treatment.
C. The five most abundant OTUs within Pseudoalteromonadaceae, all of which are members of the genus *Pseudoalteromonas*.
D. Relative proportion of each *Pseudoalteromonas* spp. OTU to the total community.
E. The five most abundant OTUs identified as *Vibrio*.
F. The relative contribution of each *Vibrio* spp. OTU to the overall bacterial community.
Relative abundances are averaged within treatments. Due to high similarities, in (B), (D) and (F), treatments with N addition are combined with the corresponding treatment with no nutrient addition. [Color figure can be viewed at wileyonlinelibrary.com]

picture of marine mesozooplankton that are engaged in dynamic interactions with the bacterial communities surrounding them. We propose the bacterial community surrounding copepods be termed the copepod 'zoosphere', following terminology for other systems (Fig. 9). The microhabitats provided by these living, small crustaceans appear to have exciting parallels with other microhabitats in the marine and terrestrial environments. The phytoplankton phycosphere hosts intriguing phytoplankton–bacteria

interactions (Bell and Mitchell, 1972; Amin *et al.*, 2012; Segev *et al.*, 2016; Seymour *et al.*, 2017), and in terrestrial environments, extensive research on plant rhizospheres has shown a range of dynamic and mutualistic plant–microbe associations occurring in these systems (Gottel *et al.*, 2011; Bulgarelli *et al.*, 2013; Farrar *et al.*, 2014).

This study appears to be the first to pair high-throughput amplicon sequencing with an attempt to understand the influence of copepods on the microbial communities
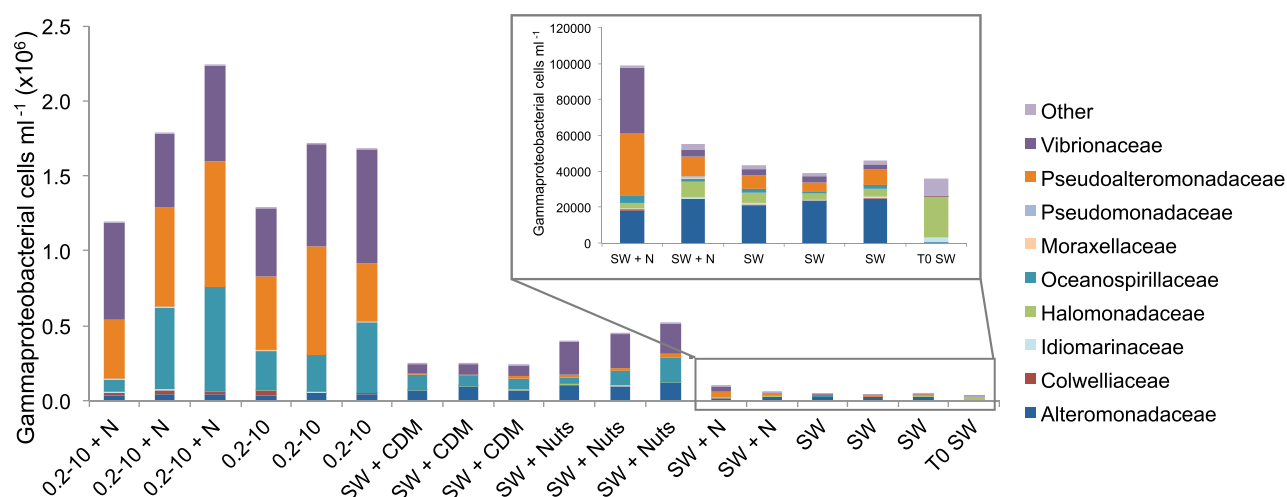
Fig. 7. Abundance (cells ml^{-1} × 10^6) of Gammaproteobacterial groups at the end of the Experiment 3 incubation. The numbers were determined based on proportion of the community in sequencing and the total bacterial cell numbers from flow cytometry. X-axis labels are the same as in Fig. 3. T0 represents the bacterial numbers in the seawater inoculum before adding to the SW incubations. Samples with low Gammaproteobacteria abundance are shown in the insert. [Color figure can be viewed at wileyonlinelibrary.com]

surrounding them, in addition to the bacteria physically attached to them (latter generally referred to as their microbiome). A physical association, even if loose and temporary, may be an important mechanism for maintaining bacterial access to nutrients from copepods. Moving copepods are likely to replace the surrounding fluid rapidly (Kiørboe and Visser, 1999; Jiang, 2002), but bacteria may be able to associate with the viscous boundary layer surrounding even swimming copepods (Catton *et al*., 2007) to maintain exposure to nutrients and other copepod-sourced signals. During periods of reduced copepod movement, nutrient plumes surrounding copepods could potentially persist longer. However, detailed assessment via modelling the small-scale fluid-dynamics coupled to nutrient fluxes, bacterial growth rates and swimming speeds are needed to understand the importance of the copepod zoosphere *in situ*. Nevertheless, the bottle incubations conducted here provide evidence that copepods have the

potential to selectively promote bacterial growth in their surroundings *in situ*. Our data show evidence for two distinct mechanisms by which copepods influence the bacterial communities surrounding them. Our results suggest that copepods (i) recruit and induce the growth of a pool of bacteria from the surrounding seawater through chemical and physical stimuli and (ii) incubate/farm a different pool of bacteria in or on the copepod body; some of these bacteria are then released to the surrounding seawater (Fig. 9).

In this study, the bacterial community on the copepods before the bottle incubation was very similar to the community we previously reported on the same copepod taxa (*Undinula* sp.) at the same study site (Shoemaker and Moisander, 2015), with the dominant classes being Gammaproteobacteria, followed by Alphaproteobacteria and Flavobacteria. One of the major trends observed here was that during incubation, the relative proportions of taxonomic groups in the bacterial community within the copepod size fraction shifted to a higher proportion of Flavobacteria and Alphaproteobacteria, with a reduction of Gammaproteobacterial groups. Although the relative proportion of Gammaproteobacteria, specifically Pseudoalteromonadaceae, was reduced on the copepods from T0 to the end of the incubation, there was substantial net growth of Pseudoalteromonadaceae during incubation.

The results suggest that some groups detected within the copepod zoosphere are specifically benefitting from the presence of copepod host, beyond general nutrient or other chemical responses. Based on calculated abundances from cell counts surrounding copepods and relative species composition in both size fractions in copepod incubations, Pseudoalteromonadaceae and Flavobacteriaceae, and to a lesser extent Vibrionaceae and Oceanospirillaceae, thrive in the physical presence of copepods, and likely grow on the
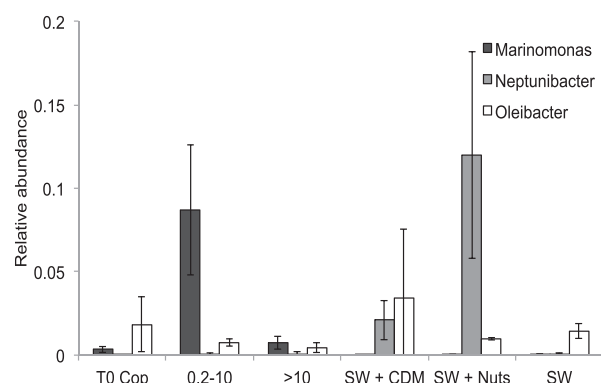

Fig. 8. Relative abundance of members within the Gammaproteobacterial group Oceanospirillaceae (treatment average +/− s.d.).
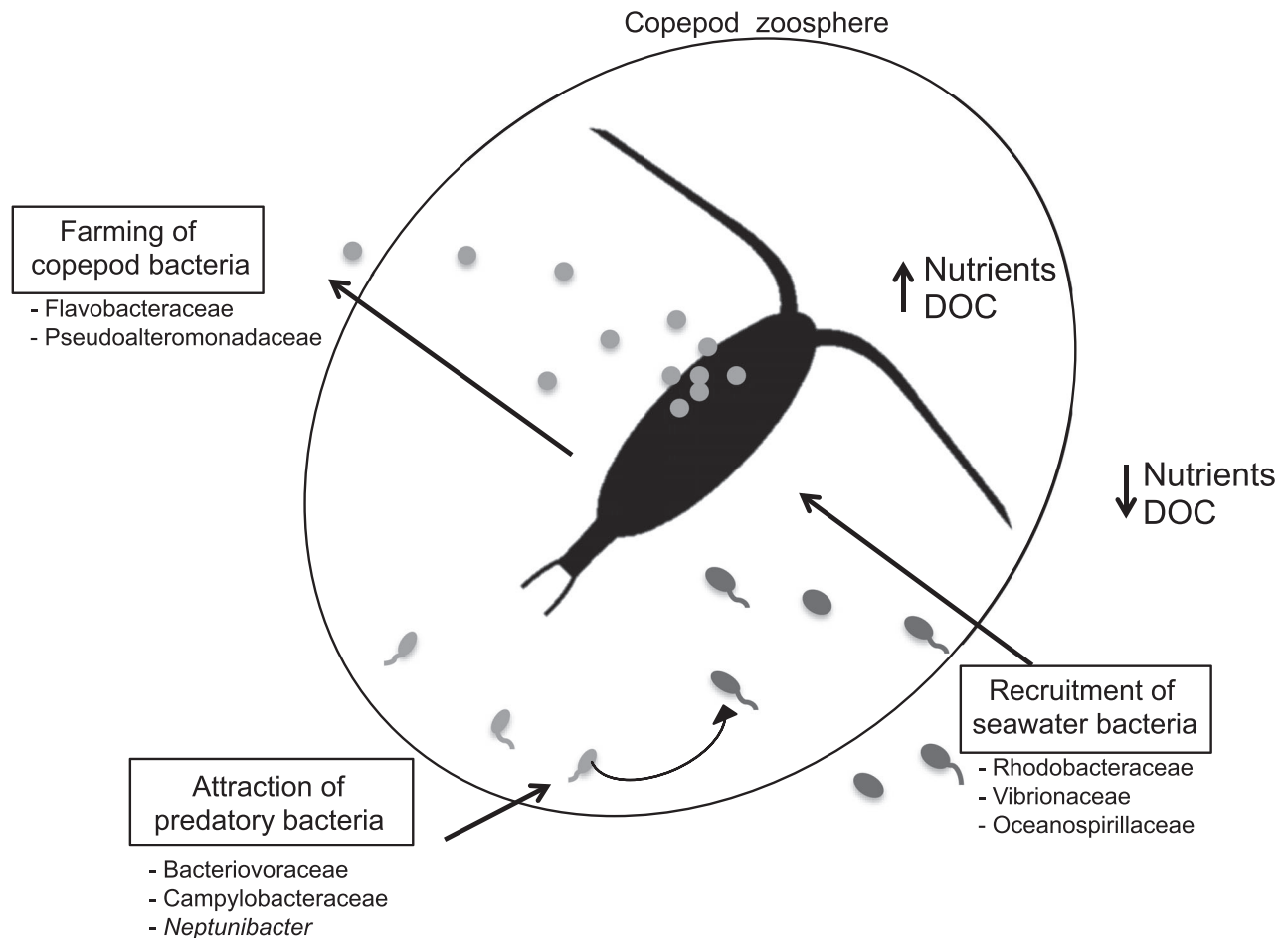
Fig. 9. Copepod zoosphere: a schematic proposed model of the influence of copepods on surrounding bacterial communities. Nutrients released from copepods recruit and support growth of bacteria from the surrounding seawater. Secondary colonizers that include predatory bacteria utilize the CDM but are poor competitors on the copepod. Some bacterial groups grow in or on the copepod in loose associations, and may detach readily (copepod farming) to either colonize other living or dead particles or remain in the free-living state for a period of time.

copepod body. Pseudoalteromonadaceae and Flavobacteriaceae were poor competitors in the absence of copepods, even if nutrients or CDM were provided, which indicates that the mere presence of copepod-derived nutrients was not sufficient to promote growth of these groups. Both groups have previously been reported on copepods from subtropical, temperate and polar waters – at times as major contributors to the community (Hansen and Bech, 1996; Gerdts *et al*., 2013; Moisander *et al*., 2015; Shoemaker and Moisander, 2015). Flavobacteriaceae is a large and diverse family, although traits including gliding motility and the ability to degrade high–molecular-weight compounds, including chitin, a structural component in the copepod exoskeleton, are conserved throughout the order (Woese *et al*., 1990; Cottrell and Kirchman, 2000). Flavobacteriaceae are also abundant members of marine snow-associated microbial communities (Delong *et al*., 1993; Rath *et al*., 1998; Crump *et al*., 1999), suspended detrital particle communities (Duret *et al*., 2019) and also associate with phytoplankton (Grossart *et al*., 2005; Sapp *et al*., 2007).

Flavobacteriaceae had a higher abundance in the T0 seawater than at the end of the bottle incubations of seawater, which could be due to a requirement for substrate attachment or to being in close proximity to a nutrient source such as zooplankton. In parallel, members of Pseudoalteromonadaceae may exhibit rapid chemotaxis towards DOM released from marine snow (Stocker *et al*., 2008). It appears that both Pseudoalteromonadaceae and Flavobacteriaceae are released from the copepods at high abundances and could contribute to the overall microbial composition of the seawater, similarly to the cells released from marine snow (discussed in Kirchman, 2002). The data here suggest that these bacterial 'farming' associations on copepods are relatively loose and that these bacteria subsequently proliferate in the copepod surroundings, if given the opportunity.

Groups including Rhodobacteraceae, Oceanospirillaceae and Vibrionaceae grew well both on and in the vicinity of the copepod host, and were also stimulated by the addition of copepod-derived nutrients and artificial nutrients in the

absence of the host. These groups appear to show a more general nutrient enrichment response, although some OTU-level specificity among treatments was observed as well. Steinberg *et al*. (2002) showed that the dissolved N in the dissolved material derived from *Pleuromamma* copepods was composed of a 3:1 ratio of ammonium:DON, and rapidly recycled in the euphotic zone. Our previous, parallel study reported that all treatments containing copepods in these experiments resulted in high ammonium concentrations (31.4 $\pm$ 13.1 μM), while all seawater treatments, regardless of N addition, contained an average of 1.8 $\pm$ 0.99 μM NH_4^+ by the end of the incubation (Moisander *et al*., 2018). In this study, bacterial abundances were 5–8 times higher in seawater amended with nutrients or CDM than seawater controls. This rapid proliferation of bacteria appears to be dependent on the added nutrients. Part of the observed community response may be comparable under the presence of copepods and any other nutrient source, including detrital matter. When considering the data on SW + CDM enrichment and copepod farming on surrounding bacteria, it should be noted that PON excretion is reduced in non-feeding copepods, so the N present in the copepod treatments was likely less than would normally be released by a copepod in nature (Saba *et al*., 2011). Bacterial abundance of farmed groups and those induced by the exposure to nutrients/CDM may thus be underestimates of the total numbers of bacterial cells that could be induced by these nutrient sources (in addition, see discussion below about the trophic cascade).

In the same copepod incubations discussed here, dissimilatory nitrate reduction to nitrite was detected in the presence of oxygen in the incubations (Moisander *et al*., 2018). Sequencing and expression data of genes involved in dissimilatory nitrate reduction suggested that *Vibrio* spp., *Alteromonas* spp. and *Pseudoalteromonas* spp. were respiring nitrate in the copepod incubations, and this appears to be one of their fitness strategies in copepod associations (Moisander *et al*., 2018). The sustained nutrient concentrations in the copepod treatments were likely higher than in the seawater samples amended at the beginning of the experiment, which could partially explain the lower competitive fitness of Alteromonadaceae in copepod association than seawater treatments amended with nutrients or CDM (Supporting Information Fig. S2). Members of Alteromonadaceae and Pseudoalteromonadaceae often occupy a similar niche, and the proliferation of Pseudoalteromonadaceae in the presence of higher nutrient conditions could ultimately be linked to their relative efficiencies in nutrient acquisition. McCarren *et al*. (2010) found that *Alteromonas* spp. rapidly responded to a DOM spike in a seawater microcosm, but competing bacteria, including other Gammaproteobacteria, ultimately outcompeted *Alteromonas.* The steady release of nutrients from the copepod may give an advantage to competitors of

Alteromonas that remain within the zoosphere for sustained nutrient acquisition.

To further study the effect of the physical presence of copepods versus nutrients alone on the surrounding microbial communities, separate treatments of seawater + nutrients, and seawater + CDM were included, but were incubated with the physical absence of copepods. We hypothesized that the SW + CDM treatment would closely resemble the communities arising in the incubation water with nutrients (SW + nutrients) or copepods present. To some degree, this was true; communities resembling each other developed, yet showed some distinct patterns. Nutrient and CDM amendment to seawater appeared to encourage the growth of some *Vibrio* spp. (OTU4 and OTU5) that were not seen in high proportions in either the copepod treatments or the seawater treatments without nutrients. These *Vibrio* spp. thus seem to have a better fitness in the absence of copepods, while other *Vibrio* spp. appeared to thrive in their presence and were diminished in the SW + nutrients and SW + CDM incubations (Fig. 6). Thus, among *Vibrio* spp., there was a selective response to copepod physical presence. The SW + nutrients and SW + CDM treatments had a high similarity to each other and were consistent throughout replicates, but a few groups arose in the CDM treatment that were not stimulated by the nutrients alone. These groups included one OTU each of *Bacteriovorax* (order Bdellovibrionales) and *Arcobacter* (order Campylobacteriales), and two groups that are rarely observed in surface seawater communities. *Bacteriovorax* and some additional groups within the Bdellovibrionales are periplasmic parasites of gram-negative bacteria (Sockett, 2009; Pasternak *et al*., 2014). *Bacteriovorax* sp. may be responding to specific signals in the CDM and/or be drawn to the bacteria released from copepods. One potential reason these parasitic bacteria were not observed in high numbers associated with the copepods could be the presence of antibacterial defence mechanisms in the zoosphere bacteria. In the terrestrial world, the well-studied plant rhizosphere is known to harbour bacterial community members that offer various modes of protection to their host plant from pathogens, including production of antimicrobial compounds (Qin *et al*., 2011; Farrar *et al*., 2014). Various species of *Pseudoalteromonas* have anti-bacterial and bacteriolytic properties when attached to a eukaryotic host (Holmström and Kjelleberg, 1999). Bacteria colonizing or surrounding copepods in their zoosphere could be actively using such mechanisms as well.

The zoosphere-associated bacteria are among the most commonly reported bacteria in copepods (Shoemaker and Moisander, 2015; Datta *et al*., 2018). These groups are also regularly reported from oceanic particle-associating bacterial communities (López-Pérez *et al*., 2016; Pelve *et al*., 2017). Yet, it is challenging to make quantitative

estimates about the importance of substrate-associated bacteria given the transient nature of the associations. Among the zoosphere groups that were the most abundant in seawater were Rhodobacteriaceae (11% of the seawater community in this study) and Halomonadaceae (8.5% of the seawater community). Flavobacteriaceae, a dominant zoosphere group, did not grow well in the seawater incubations, but is routinely found in the surface layers at Bermuda Atlantic Time Series Station (BATS; 5%–17% of the seawater community seasonally; Shoemaker and Moisander, 2017). This group is often associated with marine snow and phytoplankton (Kirchman, 2002; Buchan *et al.*, 2014). Flavobacteriaceae found in seawater (~17% of the unincubated T0 seawater community in this study) could potentially be supported by a range of different particle associations *in situ*, including those with copepods.

The abundance of bacteria in the copepod incubation water at the end of the incubation was 4.5×10^6 cells ml^{-1}. While we could not enumerate bacteria via flow cytometry on the copepods, a previous study reported copepod-attached bacterial cell abundances to be around 4×10^5 cells copepod^{-1} (Almada and Tarrant, 2016). Since the copepods were placed in 0.2-μm FSW at the start of the experiment, the bacteria present in the surrounding water at the end of the experiment presumably originated from an inoculum added with copepods. These superficially attached bacteria could then have subsisted on a combination of the nutrients released by the copepods and the low levels of nutrients present in the seawater. Based on the bacterial numbers at the end of incubations and the number of copepods per bottle and assuming the T0 time point had no bacteria in the surrounding seawater, it can be estimated that each copepod contributed a net growth of approximately 2.7×10^5 bacteria per ml of surrounding water over the 36-h incubation period. Presumably the bacterial load on the copepods was similar at the start and end of the incubation. In seawater controls with no added nutrients, there was an average of 1.0×10^4 cells ml^{-1} at the beginning and 1.6×10^5 cells ml^{-1} at the end of the 36-h incubation in the experimental bottles, corresponding to a net growth rate of $0.065 \ h^{-1}$. In seawater incubations with nutrients added, there was an average of 5.3×10^5 cells ml^{-1} at the end of the incubation corresponding to an average net growth rate of $0.10 \ h^{-1}$. A small number of protists, nanoflagellates and microzooplankton may have been added to the bottles with the rinsed copepods and the seawater inoculum. Therefore, some of the bacterial communities reported here may have also arisen from associations with them rather than with the copepods. These results represent bacterial abundances arising from the presence of copepods, with potential loss due to nanoflagellate grazing not considered. Although not measured, potential differences of the influence of nanoplankton

among treatments could have contributed to some of the observed differences (Zollner *et al.*, 2009; Sandaa *et al.*, 2017). Zoosphere bacteria supported by copepods in nature likely recruit nanoflagellates, which in turn recruit microzooplankton that the copepods may feed on. Trophic interactions from viruses should also be considered in this cascade. Since viral particles were not excluded from the incubation water, viral lysis could also have contributed to cell losses (Sandaa *et al.*, 2009).

Copepods are effectively shaping the surrounding bacterial community through both the release of nutrients and a release of bacterial cells. The bacterial communities on living copepods are distinct and numerous. Our findings suggest that copepods likely influence bacterial communities surrounding them via different mechanisms, and these communities form a copepod zoosphere that in turn may be seeding the surrounding seawater beyond. The activities influencing biogeochemical cycles in these communities are likely different from activities in the surrounding free-living organisms (De Corte *et al.*, 2018; Moisander *et al.*, 2018) and merit further study. Given the high abundances and ubiquitous distribution of copepods, the microbial processes occurring within the copepod zoosphere are likely to be globally important.

Experimental procedures

Copepod collection and incubation

Onboard incubation experiments were conducted in the North Atlantic Subtropical Gyre to investigate the influence of copepods on the bacterial abundances and community composition in the surrounding seawater (Fig. 1). To separately investigate the copepod-attached and surrounding communities, the communities were analysed by 16S rRNA amplicon sequencing from both the copepod size fraction and the surrounding water (incubation water) from the copepod treatments. In the same experiments, separate treatments investigated responses in seawater bacterial communities without the presence of copepods (Fig. 1). These seawater samples were incubated either without any additions (seawater controls), or were subjected to nutrient additions, or addition of a dissolved organic and inorganic nutrient mixture originating from copepods (CDM). The goal was to separately investigate the influence of: (i) copepods stimulating surrounding bacteria through excreted nutrients and (ii) copepods farming bacteria.

Zooplankton net tows and bottle incubations were conducted onboard the *R/V Atlantic Explorer* in August 2014. All zooplankton tows and CTD casts were done at or near the BATS in the North Atlantic Ocean (31° 40' N 64° 10' W). A 200-μm mesh zooplankton net was towed between 16:30 and 5:20 local time (further details in Moisander

et al., 2018). The codend was sealed with tape to minimize damage to the zooplankton; the net was lowered to approximately 50–90 m, and then ascended at the slowest possible wire speed (7–10 m min^{-1}). Tow contents were immediately diluted with surface seawater and held in gentle aeration until sorted. Living copepods were sorted with transfer pipettes based on visual identification and rinsed individually at least three times in 0.2-μm FSW. Copepods were kept in FSW in multi-well plates, and chilled with ice packs for 1–4 h until a sufficient number of copepods had been picked. This holding time was expected to be long enough to mostly empty copepod guts, although some faecal pellets may have been produced during incubation. Copepods included species of *Pleuromamma*, *Undinula*, a Poecilostomad *Sapphirina* and other unidentified calanoids, and no distinction was made based on sex or life stage. A total of 15 living copepods were dispersed to each copepod incubation bottle with an even mix of copepod types (Table 1).

Experiments were done in tandem with measurements for rates of respiratory nitrogen (N) cycle processes, reported elsewhere (Moisander *et al*., 2018). Due to parallel sampling to study these N transformations, the experiments described here were performed with two slightly different procedures (Table 1). Experiment 3 (used for community analysis described below) was carried out in gas-tight 75-ml glass serum vials filled with FSW, while Experiments 1, 2 and 4 were done in 125-ml polycarbonate bottles with 75-ml FSW. Incubation bottles were chosen based on the needs for the parallel study, and to ensure adequate incubation space for the multiple treatments. Seawater treatments (Fig. 1) consisted of either nutrient addition, composed of a range of nutrients including NO_3^- (0.5 μM), NH_4^+ (0.5 μM), dextrose (0.5 μM), FeCl (0.5 μM) chelated with EDTA (0.5 μM) and PO_4^{-3} (1 μM), or a treatment with only N and carbonate addition (NO_2^-, NO_3^-, NH_4^+ and CO_3^{-2}, each at 0.5 μM; referred to as +N). A seawater control with no nutrients was also included, as well as a treatment consisting of both nutrient and + N addition, which was only used for cell counts. All seawater bottles with no copepods received an addition of 3–5 ml of unfiltered seawater, considered the bacterial inoculum (taken with Niskin bottles on the CTD-Rosette, Table 1). Copepod treatments received copepods added into FSW in the vials but no unfiltered seawater inoculum. One treatment in Experiment 3 consisted of the dissolved fraction (<0.2 μm) taken from a copepod-only incubation that ended the previous day (CDM). For this treatment, 36 ml of the CDM was added to 36 ml of FSW (1:1 mixture), and 3 ml of unfiltered seawater was added as a bacterial inoculum. All experimental incubations were done in triplicate.

Several T0 samples were collected at the start of each experiment. For T0 copepod samples, 15 copepods were added to duplicate vials with FSW and were filtered immediately onto a 10 μm polycarbonate filter (General Electric Healthcare Life Sciences, Pittsburgh, PA), rinsed once with FSW and flow through was collected on a 0.2-μm Supor membrane filter (Pall Gelman, Port Washington, NY). The 10-μm filter collected the copepods and any other particles larger than 10 μm. The 0.2-μm filter collected the 0.2–10-μm-sized particles, including bacteria, that were superficially attached to the copepod or quickly detached when the copepods were introduced to the bottle of FSW. Filters were placed in sterile 2-ml bead beater tubes containing a mixture of 0.1 and 0.5 mm glass beads (BioSpec, Bartlesville, OK) and were then frozen at −80°C. For the whole seawater samples at T0, 4.5 l of the seawater inoculum was filtered through a 10 μm filter and onto a 0.2-μm membrane filter and the filter frozen at −80°C. A T0 sample of the seawater inoculum going into the experimental bottles was also collected for bacterial cell enumeration by preserving 2 ml of unfiltered seawater in a 2-ml cryovial, fixed to a final concentration of 0.2% ultrapure formaldehyde (Electron Microscopy Sciences, Hatfield, PA) and frozen at −80°C.

Experimental bottles were incubated according to Table 1 for 36 h. At the end, 2 ml of water was first preserved as above for cell enumeration. The experiment was terminated by filtration, and the samples were stored the same way as the T0 samples. Briefly, for treatments including copepods, both the copepod size fraction (>10 μm) and the 0.2–10-μm size fraction (incubation water representing 'free-living bacteria' and any <10 μm living or dead particles present at the end of the incubation) were separately included. The treatments without copepods were filtered directly on 0.2-μm filters. The water passing through the 0.2-μm filter was collected in acid-washed polyethylene bottles and frozen at −20°C for nutrient analyses (Moisander *et al*., 2018). Samples for flow cytometry and DNA analysis were returned to the University of Massachusetts Dartmouth in a liquid N dry shipper and stored at −80°C.

16S rRNA gene amplicon library preparation and sequence analysis

Bacterial community analysis shifts in Experiment 3 were investigated by sequence analysis. DNA was extracted from the 0.2- and 10-μm filters using reagents from the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA) with a modified protocol (Supplementary Methods). The V3–V4 region of the 16S rRNA gene was amplified by bacteria-specific primers, Bact-0341F/Bact-0785R (Klindworth *et al*., 2013). Amplicon libraries were created following a previously published protocol (Shoemaker and Moisander, 2017). Paired-end sequencing was performed at the Tufts University

Core Facility for Genomics (Boston, MA) using the Illumina MiSeq v300 method.

MacQIIME v1.8.0 (Caporaso *et al.*, 2010) was used to join the paired-end reads and to call OTUs at the 97% similarity level. Taxonomies were assigned using the GreenGenes database (DeSantis *et al.*, 2006). OTUs with more than one sequence present in the negative control were removed from all samples. Of the negative sequences, 98% matched an unclassified genus of Bradyrhizobiaceae. This OTU did not represent more than 1% of the community in any sample except the copepod T0 samples, which had a low sequence coverage. Shannon diversity indices and principle components were calculated in MacQIIME, and the non-parametric KW tests were done with the R stats package in R Studio (v. 3.1.1). Sequences have been deposited into the Sequence Read Archive (SRA) under the accession number SRP089732.

Cell enumeration

Total cell counts from experimental water at the end of incubations and the seawater inoculum were obtained with a BD Influx flow cytometer (San Jose, CA). Samples were thawed at room temperature, stained with SYBR Green I to a final concentration of 0.01% and incubated for 10 min in the dark at room temperature. Particles were excited at 488 nm and forward (<15°) scatter (FSC), side (90°) scatter (SSC) and green fluorescence (530/40 nm) were recorded. Calibration and alignment were done using 1-μm yellow-green microspheres (Polysciences). Bacterial cells were gated and enumerated using the FCS Express 6 software (De Novo Software, Los Angeles, CA) based on green fluorescence and forward scatter, avoiding dimly green fluorescent particles that were likely dead or broken cells (debris) at the time of sample fixation. Normality was assessed with quantile–quantile plots, and ANOVA tests were done in R Studio (v. 3.1.1) to compare bacterial counts.

Bacterial cell numbers at the end of the experiments in the incubation water were counted flow cytometrically. Taxon-specific cell abundances were calculated based on relative proportions in sequence data, assuming the same relative proportion of total bacterial cell numbers. Bacteria remaining on copepods at the end of the incubation could not be enumerated with this method.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Sequence read yield and Shannon Diversity Index for individual samples from Experiment 3. Treatment labels are the same as in Fig. 3, with copepod treatments shown for the 0.2–10 μm size fraction ('0.2–10') and for the >10 μm size fraction containing the whole copepods ('>10'). Raw read number is the number of unprocessed reads from the MiSeq amplicon sequencing. Trimmed reads is the final number of reads remaining after quality control.

Figure S1. Calculated bacterial abundance of the three major bacterial groups present at the end of the experiment in the incubation water. Error bars represent standard deviation around the mean (n = 3). The label '0.2–10' indicates the 0.2–10 μm size fraction from the copepod incubations.

Figure S2. Calculated abundance of Alteromonadaceae in the incubation water based on proportion of the total community in sequencing and the total bacterial cell abundances from flow cytometry (average +/− s.d.).

Figure S3. Relative proportions of Gammaproteobacteria groups in copepods and in their incubation water in Experiment 3. Six replicate samples for each size fraction (treatments with no nutrients added and treatments with N added) were pooled to show the average relative abundance of major Gammaproteobacteria families. The label '0.2–10' is the size fraction from the incubation water surrounding the copepods, and '>10' is the size fraction containing the copepods at the end of the incubation.