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Structure and function of zooplankton-associated bacterial communities in a temperate estuary change more with time than with zooplankton species

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ABSTRACT: Zooplankton support distinct bacterial communities in high concentrations relative to the surrounding water, but little is known about how the compositions and functionalities of these bacterial communities change through time in relation to environmental conditions. We conducted a year-long field study of bacterial communities associated with common zooplankton groups as well as free-living bacterial communities in the York River, a tributary of Chesapeake Bay. Bacterial community genetic fingerprints and their carbon substrate usage were examined by denaturing gradient gel electrophoresis (DGGE) of amplified 16S rDNA and by Biolog EcoPlates, respectively. Zooplankton-associated communities were genetically distinct from free-living bacterial communities but utilized a similar array of carbon substrates. On average, bacteria associated with different zooplankton groups were genetically more similar to each other within each month (65.4% similarity) than to bacterial communities of the same zooplankton group from different months (28 to 30% similarity), which suggests the importance of ambient environmental conditions in shaping resident zooplankton-associated bacterial communities. Monthly changes in carbon substrate utilization were less variable for zooplankton-associated bacteria than for free-living bacteria, suggesting that the zooplankton microhabitat is more stable than the surrounding water and supports specific bacterial groups in the otherwise unfavorable conditions in the water column.

KEY WORDS: Zooplankton · Bacterial communities · Carbon substrates · Biolog EcoPlates · York River

INTRODUCTION

Zooplankton represent dynamic microhabitats for bacteria within aquatic systems, often supporting bacterial concentrations ranging from 10^7 to 10^{11} cells ml^{-1} body volume which match or even exceed those in the surrounding water (Tang et al. 2010). Live zooplankton continually deliver organic matter into their guts through feeding, and produce dissolved organic matter through sloppy feeding and excretions, all of which can supplement the growth of zooplankton-associated bacteria (Carman 1994, Tang et al. 2001, Tang 2005, Møller et al. 2007). Zooplankton-associated bacterial communities may be seeded via the attachment of free-living bacteria or ingestion of free-living and food-associated bacteria (Hansen & Bech 1996), whereby the physical conditions created within the zooplankton microenvironment may select for a specific bacterial community, i.e. a specific subset of the free-living and food-associated bacterial communities (Tang et al. 2010). While similar bacterial groups may be found on zooplankton and in the water column (Møller et al. 2007), the zooplankton-associated bacterial community, as a whole, can be

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quite dissimilar from the free-living one (Grossart et al. 2009). Prior studies have focused primarily on bacteria associated with bulk zooplankton (Heidelberg et al. 2002, Parveen et al. 2011) or a single zooplankton species (Møller et al. 2007, Tang et al. 2009a, Freese & Schink 2011, Homonnay et al. 2012). The few studies that have investigated co-occurring zooplankton found each zooplankton group supporting a different bacterial community (Niswati et al. 2005, Grossart et al. 2009, Brandt et al. 2010). Nevertheless, little is known about how bacterial community compositions (BCCs) of zooplankton co-occurring in the same habitat compare to each other and to free-living bacterial communities, or how their compositions change through time.

A number of studies have investigated temporal variability of free-living or total BCCs and the factors driving these changes. A recent review and meta-analysis showed that freshwater bacterial communities were highly correlated with pH and the ratio of dissolved organic carbon (DOC) to total phosphorus (Newton et al. 2011). Temporal patterns of estuarine and riverine free-living BCC have been related to temperature and chl *a* concentration (Kan et al. 2006) and river discharge (Crump & Hobbie 2005). In their long-term study in lakes, Rösel et al. (2012) found consistent differences among re-occurring patterns of free-living and particle-associated bacteria. In particular, particle-associated bacteria were much more variable over time and their community composition was often directly related to phytoplankton and zooplankton dynamics. Moreover, Fuhrman et al. (2006) concluded that the distribution and abundance of specific microbial groups in a marine system can be predicted from environmental conditions such as temperature, oxygen, salinity, virus abundance and dissolved nitrate. Given that environmental parameters strongly influence the free-living bacterial community structure and that there is a constant exchange between zooplankton-associated and free-living bacteria (Møller et al. 2007, Grossart et al. 2009, 2010), zooplankton-associated bacterial communities may be directly or indirectly shaped by environmental conditions as well and consequently exhibit seasonal changes.

In addition to environmental conditions, each zooplankton group may shape its own BCC due to differences in their lifestyle. For example, copepods and cladocerans collected from the same lake at the same time exhibited very different bacterial communities (Grossart et al. 2009). When the same cladocerans were transplanted into a different lake, they retained >83% of their BCC, indicating a rather stable bacterial assemblage regardless of the environment. In contrast, the copepod-associated bacteria were greatly influenced by the surrounding environment (Grossart et al. 2009). There are likely complex interactions between the environment and zooplankton themselves which may help to select for specific bacterial communities.

Generally, zooplankton-associated bacteria have higher production rates than their free-living counterparts (Carman 1994, Møller et al. 2007), but the underlying mechanisms such as carbon substrate utilization supporting this elevated production are largely unknown. Biolog EcoPlates™ offer an efficient method for assessing the ability of a mixed microbial assemblage to utilize 31 common carbon substrates. EcoPlates have been used to delineate carbon substrates utilized by free-living estuarine bacteria and bacteria associated with organic aggregates which function as microbial hotspots in aquatic systems and support bacteria that are more metabolically active and diverse than their free-living counterparts (Tang et al. 2006, Tang & Grossart 2007, Lyons et al. 2010, Lyons & Dobbs 2012).

The goal of this study was to assess the community compositions and functionalities of bacterial communities associated with multiple zooplankton groups and the free-living bacterial community over time within a temperate estuary. We hypothesized that each zooplankton group would support a genetically and functionally distinct bacterial community. Additionally, we sought to determine whether and which environmental conditions influence zooplankton-associated bacterial community composition and functionality, causing seasonal changes. To address these goals, we conducted a year-long field study in the York River, a tributary of Chesapeake Bay on the east coast of the United States. The genetic and functional components of bacteria associated with the dominant meroplanktonic and holoplanktonic zooplankton groups were assessed each month, compared to the free-living bacteria, and related to environmental conditions.

MATERIALS AND METHODS

Sample collection

Zooplankton were collected on a monthly basis from May 2010 to April 2011 at a fixed station in the York River, Virginia (37° 14′ 50.36″ N, 76° 29′ 58.03″ W), with a 0.5 m mouth diameter, 200 µm mesh net. All samples were collected at high or near high tide dur-

ing daylight hours, and transported immediately back to the laboratory. In the lab, each sample was split into 4 equal fractions. Each fraction was gently concentrated onto a 200 µm mesh sieve and transferred to sterile filtered Instant Ocean® artificial seawater (ASW), where the zooplankton were allowed to clear their guts overnight to eliminate food-associated bacteria. After gut clearance, sub-samples were used to assess (1) zooplankton-associated bacterial genetic fingerprint via denaturing gradient gel electrophoresis (DGGE) of 16S rDNA amplified using eubacterial primers, (2) zooplankton-associated bacterial functionality via carbon substrate usage measured by Biolog EcoPlates, and (3) zooplankton community composition. The fourth subsample was used for additional zooplankton-associated bacterial measurements and will be reported elsewhere.

The sub-sample for zooplankton community composition was filtered onto a 200 µm nitex mesh dish and frozen at −40°C until analysis. Zooplankton were identified to the lowest practical taxon. Relative percentages of each zooplankton group within the sampled community were calculated for each month.

Water samples were collected at the same time as the zooplankton; water temperature, salinity, chl *a*, ammonium, phosphate, and abundance and community composition of free-living bacteria were determined. Chl *a* was extracted from the filters with 90% acetone and measured fluorometrically. Ammonium concentrations were measured in duplicate on a Shimadzu UV-1601 spectrophotometer following the phenol hypochlorite method (detection limit 0.05 µmol N l^{-1}; Koroleff 1983). Phosphate concentrations were run in duplicate on a Lachat QuikChem 8500 autoanalyzer (detection limit 0.05 µmol l^{-1}; Parsons et al. 1984). Free-living bacterial abundance was counted in triplicate by DAPI direct counts (Porter & Feig 1980). To assess the genetic composition of free-living bacteria, approximately 60 ml of 5 µm prefiltered York River water was filtered on to a 0.2 µm pore size polycarbonate membrane filter and stored at −40°C until analysis.

DNA extraction and DGGE

After gut clearance, zooplankton were gently concentrated onto a sterile 200 µm mesh sieve and rinsed 3 times with sterile filtered ASW to remove any free-living or loosely attached bacteria. Zooplankton were back-rinsed into a sterile Petri dish and narcotized with sodium bicarbonate. Preliminary experiments indicated that narcotization with sodium bicarbonate did not influence the abundance of zooplankton-associated bacteria. Two or 3 replicates of 5 to 10 individuals of the same zooplankton species were transferred to a sterile microcentrifuge tube, preserved in 95% molecular biology grade ethanol and stored at −40°C until analysis. Consistent DGGE banding patterns have been attained previously regardless of the number of individuals used for analysis (Brandt et al. 2010). Zooplankton samples were centrifuged for 2 min at room temperature at 17 000 × *g* to pellet out the zooplankton and any bacteria that may have detached from the zooplankter during the preservation process. Excess ethanol was pipetted off after centrifugation and discarded. Zooplankton-associated and free-living bacterial DNA was extracted using the phenol-chloroform-isoamylalcohol method with smoldered zirconia beads (Zhou et al. 1996). Extracted DNA was checked for quality and quantity on a UV/VIS Spectrophotometer (NanoPhotometer™ Implen). PCR amplified for DGGE using eubacterial primers 341f-gc with a 5′ GC clamp (*5′* CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG 3′) and 907r (*5′* CCG TCA ATT CMT TTG AGT TT 3′) (Muyzer & Ramsing 1995). Each 50 µl PCR reaction contained 5 µl 10× PCR buffer, 2.5 µl 50 mM $MgCl_2$, 5 µl of 2.5 mM dNTP, 10 pmol of each primer, 0.5 µl BSA, 0.5 µl red-*Taq* DNA polymerase (Bioline), 2 to 3 µl of template DNA (approximately 20 ng DNA template) and was brought to volume with PCR water. The PCR cycling program was as follows: initial denaturation for 3 min at 95°C followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 54°C, 2 min extension at 72°C with a final extension at 72°C for 10 min.

DGGE was performed according to Tang et al. (2009b). An average of 540 ng PCR product was loaded into each well of a 7% acrylamide gel with 40 to 70% denaturing gradients (formamide and urea). Gels were run at 100 V for 18 h then stained with 1X SYBR-gold for 30 min, destained with Milli-Q water and imaged on a UV light table. Due to limited space on each DGGE gel, only 1 replicate of each sample was run on the analyzed gels. Preliminary analyses indicated a high degree of similarity among replicate samples (average of 90.5% similarity), with the exception of samples from June.

Carbon substrate utilization

For samples collected from August 2010 to April 2011, Biolog EcoPlates were used to assess each bacterial community's ability to utilize a variety of car-

bon substrates. EcoPlates contain triplicate wells of 31 carbon substrates, and control wells with no substrate addition to account for any carbon substrates added with the inocula. Each well also contains minimal growth media and the redox dye, tetrazolium violet, which changes from colorless to purple in the presence of electron transfer, indicating bacterial usage of the respective substrate (Bochner 1989). Carbon sources can be grouped into the larger biochemical categories of polymers, carbohydrates, carboxylic acids, amino acids, amines and phenolic compounds (see Table 3 below; Choi & Dobbs 1999).

After they cleared their guts, zooplankton were concentrated onto a sterile sieve, rinsed and backrinsed into a sterile Petri dish as described previously but without narcotization. Twenty-five to 35 individuals of each of the most abundant zooplankton groups were picked and transferred to sterile 15 ml centrifuge tubes with 5 ml of sterile filtered, autoclaved York River water. Preliminary experiments indicated the number of individuals used did not impact substrate usage patterns (S. Bickel, unpubl. data). To assess the free-living bacterial community, 5 ml of 5 µm filtered York River water was added to a sterile 15 ml centrifuge tube. Each sample was sonicated for 40 s on ice with an ultrasonic homogenizer at 4 W output power to break apart zooplankton bodies and dislodge any attached bacteria (modified from Tang 2005). Microscopic inspection verified that sonication effectively dislodged bacteria from the zooplankton tissue. Samples were brought to 15 ml final volume with sterile filtered, autoclaved York River water and centrifuged for 10 min at 102 × *g* to precipitate any zooplankton debris. The supernatant was gently pipetted into a sterile loading chamber and 150 µl of supernatant was added to each well of the EcoPlate. Free-living bacterial samples were processed in the same manner as zooplankton samples. Optical density (OD) (λ= 590 nm) of each well was measured immediately with a BioTek EXL800 plate reader and again after a 7-d incubation at 19°C in the dark. Final OD measurements of each well were adjusted by subtracting initial OD for each well and the average absorbance of the control was subtracted from the average absorbance of each substrate. Individual substrate usage was expressed as the average substrate color development (ASCD). The ASCD was calculated by dividing the OD of the substrate by the sum of ODs from all substrates and averaging the triplicate values for each substrate. ASCD was then expressed as a percent. This accounts for differences in inoculum densities, the triplicate measurements of each substrate on the Ecoplate, and any color development in control wells (Montserrat Sala et al. 2006). A substrate was considered used if it contributed to at least 2% of the total absorbance of all substrates (Montserrat Sala et al. 2006, Lyons & Dobbs 2012). The total number of substrates utilized by the microbial community was used as a measure of functional potential of the heterotrophic community (Zak et al. 1994). Each individual zooplankter supported approximately 10^5 bacteria (see 'Results') which led to an initial inoculum density of $l0^5$ cells ml^{-1} for zooplankton-associated bacteria. Likewise, free-living bacteria inoculum densities were 10^5 ml^{-1}. While this is at the very low end of recommended inoculum densities (Konopka et al. 1998), Christian & Lind (2006) demonstrated that inoculum density had no impact on average well color development after 72 h of incubation.

Statistical analyses

Cluster analysis of DGGE banding patterns was performed with GELCOMPAR II, v.3.5 (Applied Maths) using the unweighted pair group method with arithmetic averages. Cluster analysis of carbon substrate utilization patterns was performed in PRIMER6 (PRIMER-E), also using the unweighted pair group method with arithmetic mean (UPGMA). Pairwise similarity matrices were calculated for presence/absence of both DGGE banding patterns and carbon substrate utilization patterns using the Dice similarity index (Dice 1945). Multidimensional scaling (MDS) was performed in PRIMER6 using the Dice similarity matrices to determine the genetic and functional similarities of the different bacterial communities based on their distances from each other on a 2-dimensional plot. Significance of the observed similarities and differences was assessed with analysis of similarity (ANOSIM) in PRIMER6. Water quality parameters were analyzed with the multivariate statistical method of canonical correspondence analysis (CCA) to determine which environmental parameters contributed to the presence or absence of specific DGGE bands, or use of particular substrates among zooplankton-associated and free-living bacterial communities. The environmental parameters included temperature, salinity, chl *a*, ammonium, phosphate, free-living bacterial abundance and zooplankton-associated bacterial abundance. All CCA analyses were performed with the vegan package (Oksanen et al. 2012) in R statistical software (version 2.15.2, R Core Team 2012). All explanatory variables were initially included in CCA analyses and then tested for collinearity by examining variance infla-

tion factors (VIF) in R. If VIFs were greater than 10, the variable with the highest VIF was removed from the model until all VIFs were less than 10 (Borcard et al. 2011).

RESULTS

Environmental parameters

Water temperatures were lowest in winter (3.5°C in January), increased through spring and early summer to a peak of 30.5°C in July, and then decreased again in fall (Table 1). Salinity was typically between 20 and 24.5, with the exception of May 2010, when it was only 17.5 (Table 1). Ammonium reached its highest concentration in late summer (6.92 µmol l^{-1} in August). In contrast, phosphate was lowest in late spring and peaked in December (0.56 µmol l^{-1}). Chl *a* concentration was lowest in December, increased during late winter and peaked in early spring. Free-living bacterial abundance followed a trend similar to temperature, with highest abundances in summer, decreasing through fall to lowest values in winter (Table 1).

Table 1. Monthly water quality measurements and free-living bacterial abundances for the York River, Virginia, USA, during the field study. BLD: below level of detection

Date	Water temperature (°C)	Salinity	Ammonium (µmol l^{-1})	Phosphate (µmol l^{-1})	Chl *a* (µg l^{-1})	Free-living bacterial abundance (10^6 cells ml^{-1})
May 2010	22.5	17.5	0.56	BLD	2.69	3.65
Jun 2010	28.0	22.0	3.87	BLD	3.52	3.27
Jul 2010	30.5	22.0	1.11	0.21	2.48	1.42
Aug 2010	28.5	23.5	6.92	0.36	2.71	3.90
Sep 2010	26.0	24.0	3.76	0.22	0.71	3.76
Oct 2010	16.0	23.0	3.08	0.31	0.18	2.31
Nov 2010	13.0	22.0	1.62	0.49	4.15	1.71
Dec 2010	4.0	24.5	1.64	0.56	0.03	1.20
Jan 2011	3.5	24.0	0.39	0.45	0.38	0.94
Feb 2011	9.0	23.0	0.45	0.03	3.69	1.04
Mar 2011	11.0	21.0	1.03	0.08	3.22	1.05
Apr 2011	16.0	20.0	0.54	0.03	6.34	0.91

Zooplankton community composition and associated bacterial abundances

The calanoid copepod *Acartia* sp. was present year-round and comprised 23 to 99% of the zooplankton community (Table 2). The barnacle nauplius *Balanus* sp. was the second most common zooplankter, present in 10 of 12 mo and comprising 5 to 55% of the zooplankton community. Other common zooplankton included polychaete larvae, harpacticoid copepods, the cladoceran *Podon* sp., and the calanoid copepods *Parvocalanus* sp., *Pseudodiaptomus* sp. and *Centropages* sp. (Table 2). The average number of bacteria per individual zooplankter was on the order of 10^5 with peaks in abundances observed in August and December/January. The highest average abundance per individual was 8.25×10^5 on harpacticoid copepods in August and the lowest was 0.15×10^5 on the calanoid copepod *Pseudodiaptomus* sp. in January. The average number of bacteria per *Acartia* ranged from 0.67×10^5 in January to 5.71×10^5 in August. The average number of bacteria per individual *Balanus* ranged from 0.25×10^5 in May to 7.41×10^5 in January.

Table 2. Zooplankton community composition in the York River, Virginia, USA, in monthly samples taken between May 2010 and April 2011

Taxon/group	% total zooplankton											
	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr
Acartia	27.12	87.71	99.08	75.18	50.68	30.34	94.34	91.74	67.47	48.23	23.09	38.26
Balanus	55.02	5.53	0.00	16.31	23.24	8.72	0.00	4.96	24.15	15.71	52.79	53.62
Polychaete larvae	5.50	0.00	0.00	0.00	0.25	1.25	0.21	0.41	1.45	8.65	0.29	0.50
Podon	9.27	0.00	0.00	0.00	5.19	0.09	0.00	0.21	0.16	0.24	0.00	1.19
Harpacticoids	0.58	0.00	0.00	0.71	0.00	7.38	0.00	0.41	0.64	0.12	0.00	0.40
Parvocalanus	0.48	0.00	0.23	1.42	12.61	13.79	0.00	0.41	1.45	5.36	3.82	0.50
Pseudodiaptomus	0.39	0.00	0.69	2.84	4.45	35.77	4.19	1.65	0.48	1.83	7.79	4.46
Centropages	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.38	19.00	8.68	0.50
Other	1.64	6.76	0.00	3.55	3.58	2.67	1.26	0.21	0.81	0.85	3.53	0.59

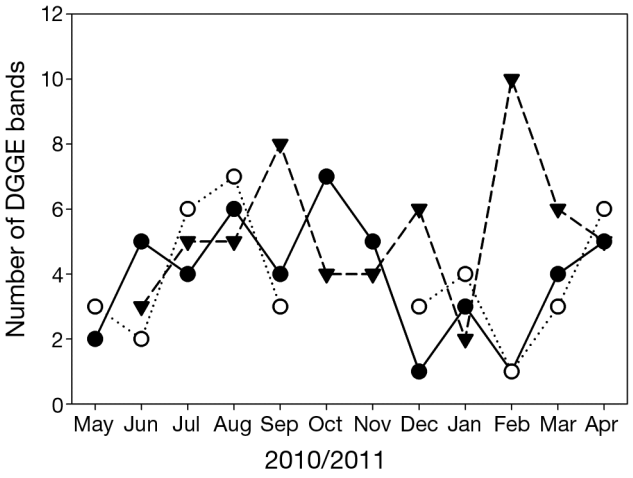


Fig. 1. Total number of denaturing gradient gel electrophoresis (DGGE) bands present in monthly samples of zooplankton-associated and free-living bacterial communities in the York River, Virginia, USA: (●) *Acartia*; (○) *Balanus*; (▼) free-living bacteria

Bacterial community composition

There was a large month to month variation in the number of DGGE bands in each of the zooplankton-associated bacterial communities. The *Acartia*-associated bacterial community ranged from only 1 band in December and February to 7 bands in October. Similarly, *Balanus*-associated bacteria ranged from 1 band in February to 7 bands in August. Free-living bacteria ranged from 2 bands in January to 10 bands in February (Fig. 1). The patterns of DGGE band abundance were similar for *Acartia* and *Balanus*, with the highest number of DGGE bands observed in the late summer and fall and the lowest numbers observed in winter, with the exception of June *Balanus* (Fig. 1). During some months, different zooplankton groups supported a similar number of DGGE bands (e.g. mysid, crab zoea and *Acartia* each supported 4 DGGE bands in July; data not shown), while the number of bands per zooplankton group were drastically different in other months (e.g. *Pseudodiaptomus* contained 4 DGGE bands and *Acartia* supported 7 bands in October; data not shown). On an annual average, the free-living bacterial community contained more DGGE bands, with 5.27 bands per month, while *Acartia*- and *Balanus*-associated bacterial communities had 3.91 and 3.80 bands, respectively.

Although the zooplankton-associated and free-living bacterial communities contained similar numbers of DGGE bands, the composition of the bacterial community (determined by the position of the DGGE bands within the gel) was notably different. Cluster analysis (Fig. 2A) and MDS (Fig. 2B) indicated that free-living bacterial communities were dissimilar (<10% similar) from all zooplankton-associated bacterial communities. ANOSIM confirmed the dissimilarity between zooplankton-associated bacteria and free-living bacteria ($R = 0.372$, $p < 0.001$). Within the free-living bacteria there were 2 main groups: a winter/spring group (December through April) and a summer/fall group (May through November). Within each month, similarities among bacterial communities associated with different zooplankton groups ranged from 22% in June to 100% in February (Fig. 2A, average 65.4%), and

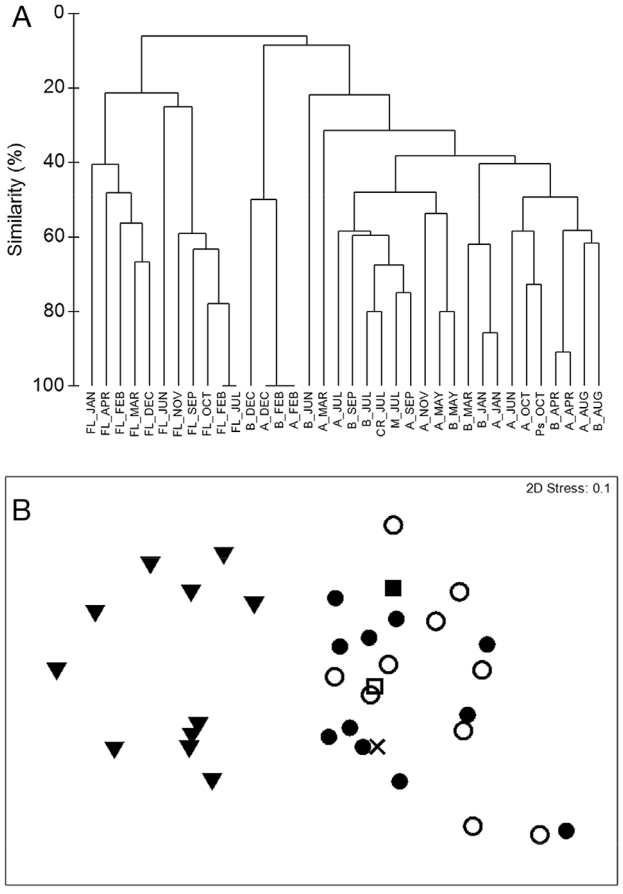


Fig. 2. (A) UPGMA dendrogram of similarities among DGGE banding patterns for monthly samples of zooplankton-associated and free-living bacterial communities collected from the York River, Virginia, USA, from May 2010 to April 2011. Dendrogram abbreviations show taxon/group and month of sampling. CR = crab zoea; all other abbreviations are the same as those used in Table 3. (B) MDS plot for DGGE banding patterns of zooplankton-associated and free-living bacteria. ●: *Acartia* (n = 12); ○: *Balanus* (n = 10); ▼: free-living bacteria (n = 11); □: crab zoea (n = 1); ■: *Pseudodiaptomus* (n = 1); ×: mysid (n = 1)

the differences in bacterial communities of different zooplankton were not significant (ANOSIM, global R = −0.116, p = 0.939). Although there were no distinct seasonal groupings among the zooplankton-associated bacteria, there were significant difference in zooplankton-associated bacterial communities among months (ANOSIM, global R = 0.835, p = 0.001).

Bacterial substrate usage

Substrate usage was highest in October for *Acartia*-associated, *Balanus*-associated and polychaete larvae-associated bacteria (28, 29 and 27 substrates, respectively; Fig. 3) and in November for free-living bacteria (20 substrates; Fig. 3). Substrate usage was lowest in August for both *Acartia*-associated (14) and *Balanus*-associated bacteria (13); polychaete larvae-associated bacteria used 15 substrates in September. Free-living bacteria used 17 substrates in March and April (Fig. 3). Within each month, zooplankton-associated bacteria used a similar number of substrates as free-living bacteria (Fig. 3). On an annual average, *Acartia*-associated bacteria used 18.4 substrates, *Balanus*-associated bacteria 17.1 substrates, polychaete larvae-associated bacteria 18.6 substrates, and free-living bacteria 18.0 substrates. Free-living bacteria and *Acartia*-associated bacteria regularly utilized substrates from all of the tested biochemical categories. *Balanus* and polychaete-associated bacteria also regularly utilized all biochemical categories except phenolic compounds (Table 3). The most commonly used substrates among all samples were the carboxylic acid pyruvic acid methyl ester, the polymer Tween 40 and the carbohydrate N-acetyl-D-glucosamine (Table 3). There was some overlap of carbon substrate utilization patterns between free-living bacteria and zooplankton-associated bacteria (Fig. 4B). Substrate utilization patterns of free-living bacteria from February and November were 72 to 74% similar to substrate usage of all zooplankton collected in October (Fig. 4A). Substrate usages by free-living bacteria from January, March and April were approximately 59% similar to those of *Acartia*-associated bacteria in April and November (Fig. 4A). Within a particular month, the average similarity of substrate usage between free-living bacteria and any zooplankton-associated bacteria was 57.7%. ANOSIM indicated no significant difference in substrate usage profiles between free-living and zooplankton-associated bacteria (global R = 0.055, p = 0.242). Within each month, substrate usage profiles for bacteria associated with different zooplankton groups were not significantly different (global R = −0.077, p = 0.804), with 35.3 to 94.0% similarity (average 69.4%; Fig. 4). However, substrate usage by zooplankton-associated bacterial communities was significantly different among months (global R = 0.301, p = 0.002). When both DGGE and substrate usage patterns were considered, both *Acartia*- and *Balanus*-associated bacteria had fewer DGGE bands but used a similar number of substrates as free-living bacteria.

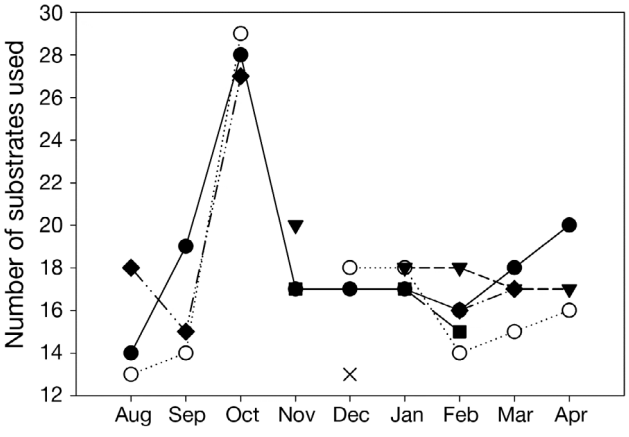
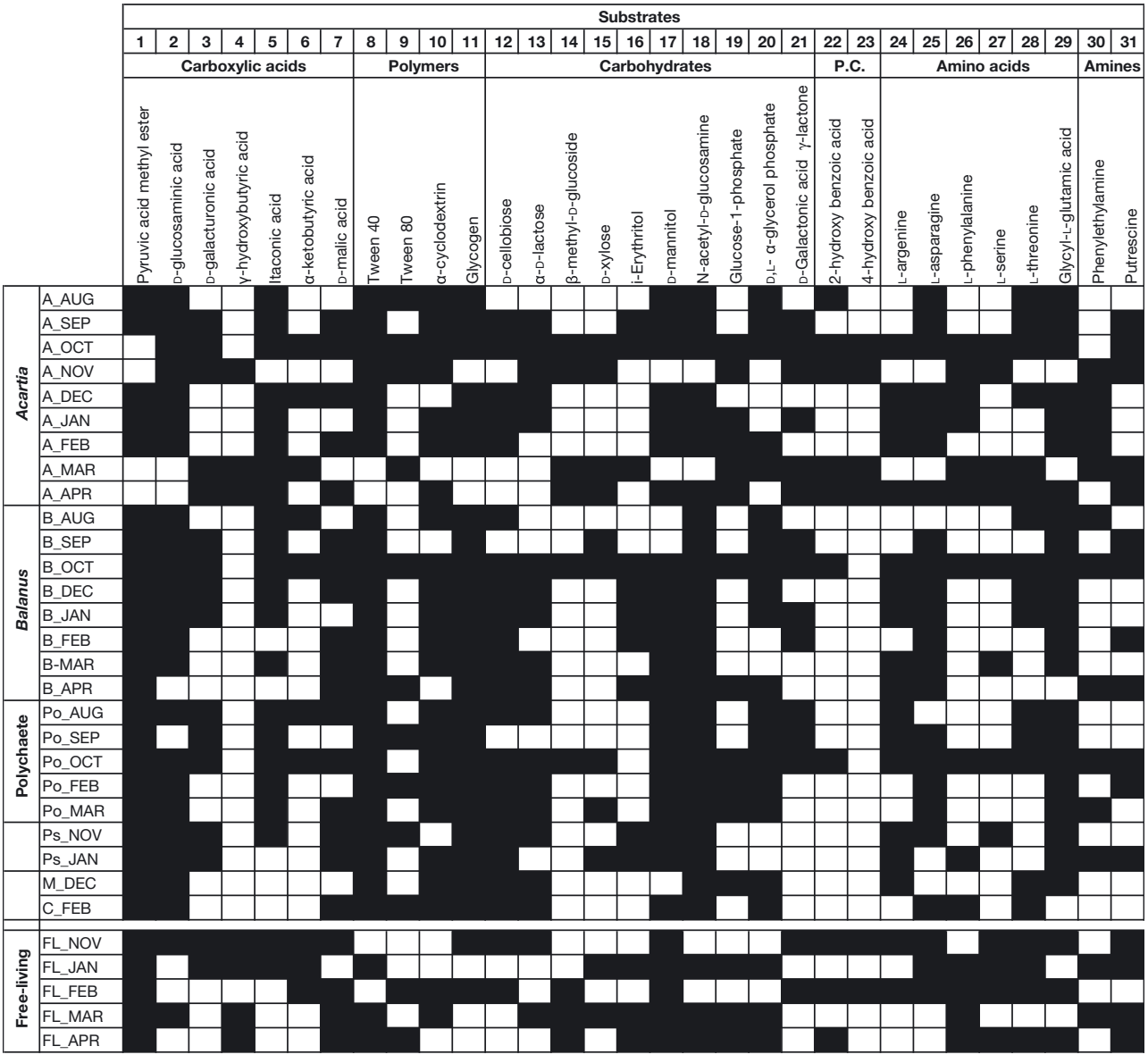


Fig. 3. Total number of carbon substrates utilized by zooplankton-associated and free-living bacteria communities collected between August 2010 and April 2011. ●: *Acartia*; ○: *Balanus*; ▼: free-living bacteria; ■: calanoid copepods; ◆: polychaete larvae; ×: mysid

Relationship with environmental factors

Measured environmental parameters accounted for 37.6% of genetic variability in all zooplankton-associated bacteria (Fig. 5A). The first 2 canonical axes accounted for 10.8 and 9.7% of the variability, respectively. Three DGGE bands (18, 21 and 26) were related to high salinity, high phosphate and high zooplankton-associated bacterial abundance. Many of the bands were not related to measured environmental parameters. A higher proportion of the variation (65.8%) among *Acartia*-associated bacteria (Fig. 5B) was explained by environmental conditions. Again, Bands 21 and 26 were linked to high salinity and high phosphate, and a number of bands were not explained by measured environmental variables. Free-living bacterial abundance was removed from CCA analyses of DGGE banding for free-living bacteria due to collinearity. Among the free-living

Table 3. Monthly carbon substrate utilization by zooplankton-associated and free-living bacteria of the York River, Virginia, USA, sampled between August 2010 and April 2011. Black squares indicate the substrate was used. Carbon substrates are grouped according to their biochemical category ('P.C.' indicates phenolic compounds). Substrate numbers correspond to numbers used in canonical correspondence analysis (CCA) of the EcoPlate data (see Fig. 6). Samples are coded by taxon/group and month of sampling; not all zooplankton groups were present in all months. A: *Acartia*; B: *Balanus*; Po: polychaete larvae; Ps: *Pseudodiaptomus*; C: *Centropages*; M: mysid; FL: free-living



bacteria (Fig. 5C), environmental parameters accounted for 52.2% of the variability in the bacterial community composition, with the first and second axes contributing 24.7% and 11.6%, respectively. Four DGGE bands (4, 5, 11 and 20) were linked to high chl *a* concentrations. Another group of bacteria (Bands 2, 17 and 35) was linked to high temperatures.

Due to high degrees of collinearity with measured environmental parameters, free-living bacterial abundance and zooplankton-associated bacterial abundance were removed from CCA analyses of carbon substrate usage. Measured environmental parameters explained only 25.2% of variability in substrate usage among all zooplankton-associated bacteria (Fig. 6A). The first 2 canonical axes accounted for 14.7% and 4.4%, respectively. The use of 8 substrates (2 carbohydrates, 2 phenolic compounds, 2 amino acids, 1 carboxylic acid and 1 amine) was

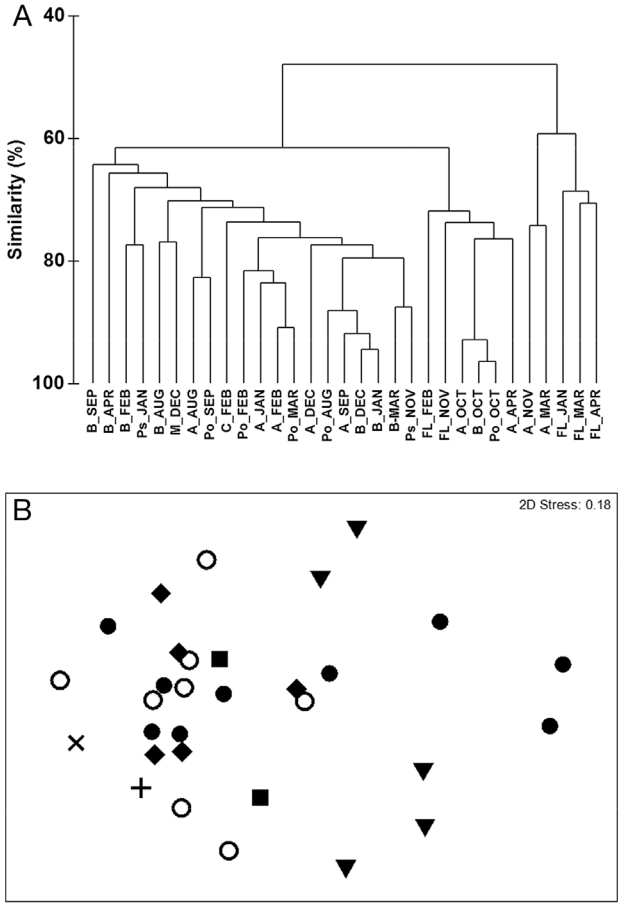


Fig. 4. (A) UPGMA dendrogram of carbon substrate usage profiles for zooplankton-associated and free-living bacterial communities collected from the York River, Virginia, USA, between August 2010 and April 2011. Dendrogram abbreviations show taxon/group and month and are the same as those used in Table 3. (B) MDS plot of carbon substrate usage profiles for zooplankton-associated and free-living bacteria: ●: *Acartia* (n = 9); ○: *Balanus* (n = 8); ▼: free-living bacteria (n = 5); +: *Centropages* (n = 1); ■: *Pseudodiaptomus* (n = 2); ×: mysid (n = 1); ◆: polychaete larvae (n = 5)

linked to high chl *a* concentrations, while the use of 4 substrates (2 carboxylic acids, 1 amino acid and 1 carbohydrate) was linked to high phosphate levels. When only *Acartia*-associated bacteria were examined (Fig. 6B), 78.5% of variation in substrate usage was explained by measured environmental conditions, with the first 2 axes accounting for 44.0% and

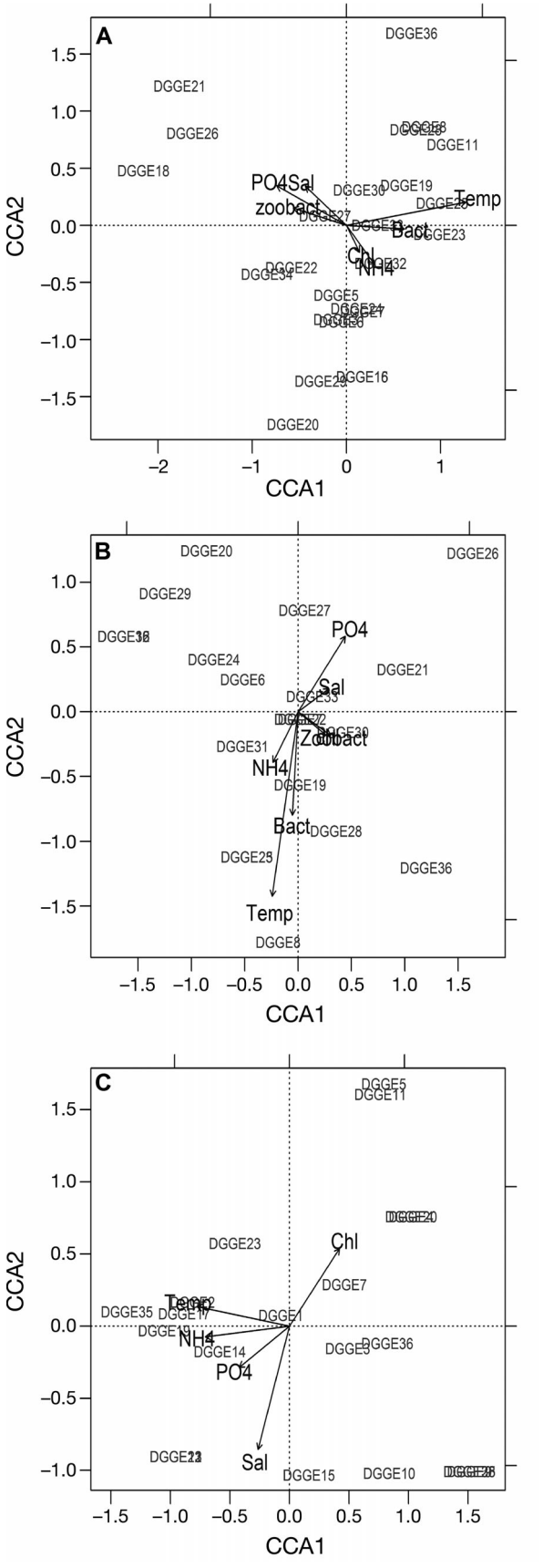


Fig. 5. CCA biplots illustrating the relationship between environmental variables and presence of DGGE bands from (A) all zooplankton-associated bacteria, (B) *Acartia*-associated bacteria and (C) free-living bacteria. DGGE bands present in the gel were arbitrarily numbered 1 through 36. Bact: free-living bacterial abundance; Zoobact: zooplankton-associated bacterial abundance; Sal: salinity; Chl: chlorophyll *a*, PO4: phosphate; NH4: ammonium; Temp: temperature

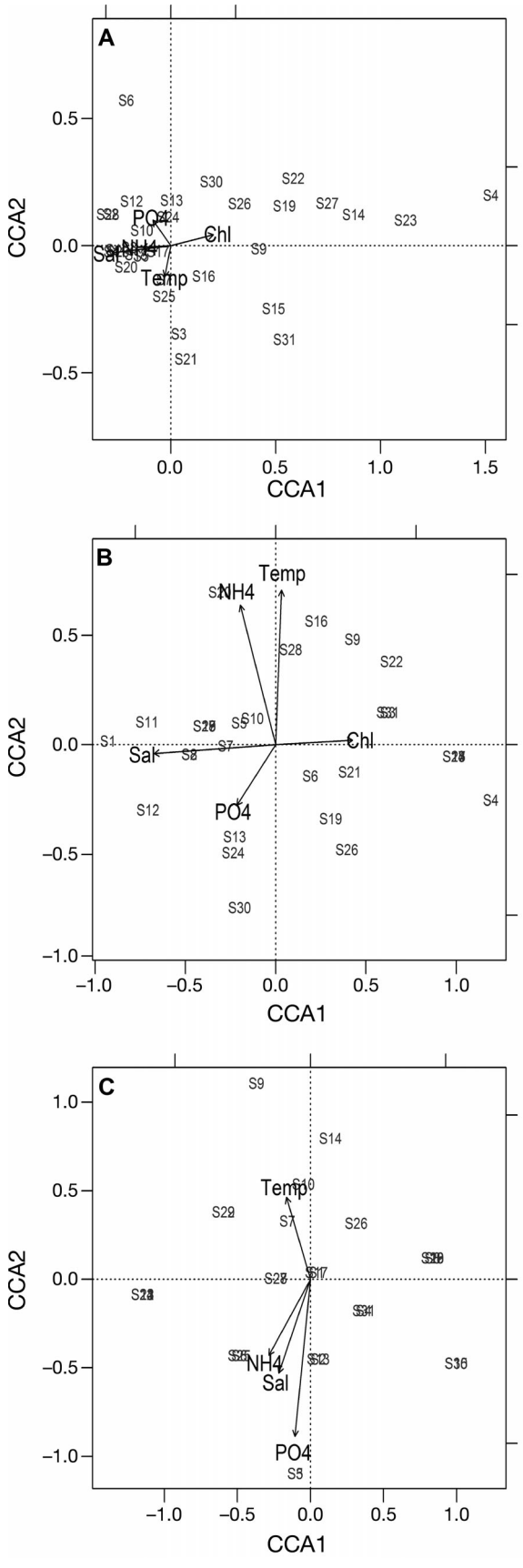


Fig. 6. CCA biplots illustrating relationship between environmental conditions and the usage of specific carbon substrates by (A) all zooplankton-associated bacteria, (B) *Acartia*-associated bacteria and (C) free-living bacteria. The substrates were denoted as S1 through S31 as defined in Table 3. See Fig. 5 legend for definitions of other abbreviations

13.2%, respectively. Again, 8 substrates were linked to chl *a* concentration (2 carbohydrates, 2 phenolic compounds, 2 carboxylic acids 1 amino acid and 1 amine). Usage of L-argenine, α-D- lactose and phenylethylamine was tied to high phosphate concentrations Use of D-L-α glycerol phosphate was linked to ammonium concentrations and higher temperatures. Measured environmental conditions explained all variations (100%) among substrate usage by free-living bacteria (Fig. 6C). Usage of Tween 80 was linked to higher salinity, while the usage of D-glucosaminic acid and Itaconic acid was linked to phosphate. The most commonly used substrates among all sample types (pyruvic acid methyl ester, Tween 40 and N-acetyl D-glucosamine) did not exhibit a strong association with any measured environmental conditions.

DISCUSSION

Comparison of zooplankton-associated bacterial communities

Previous DGGE analyses of zooplankton-associated bacteria showed distinctly different banding patterns among different zooplankton groups (Niswati et al. 2005, Grossart et al. 2009, Brandt et al. 2010); however, none of these studies quantified the level of similarity among the bacterial communities. The results of the current study are similar to those of Gerdts et al. (2013) who found no significant differences in the bacterial communities associated with 4 different calanoid copepods in the North Sea. The small differences in BCC of co-occurring zooplankton suggest that zooplankton-specific characteristics such as food preference or excretion rates may still influence the associated bacterial community composition. During the exchange between free-living, food-associated, and zooplankton-associated communities (Harris 1993, Grossart et al. 2010, Grossart & Tang 2010), the microenvironment created by each zooplankter may act as a selective filter, concentrating a specific, distinct bacterial community, which ultimately depends on the initial bacterial community to which the zooplankter was exposed.

Temporal changes in zooplankton-associated bacteria

With one exception (Gerdts et al. 2013), previous studies provided a limited snapshot of zooplankton-associated bacterial communities. Consequently, temporal changes and impacts of changing environmental conditions have not been fully explored. Bacterial communities associated with calanoid copepods in the North Sea showed no change across seasons (Gerdts et al. 2013); however, environmental conditions within the North Sea region (e.g. temperature and salinity; Reiss & Kröncke 2005) may not fluctuate as widely as in the York River. The large monthly changes we observed in composition (Fig. 2) and functionality (Fig. 4) of bacteria associated with each zooplankton group suggest that the physical, chemical and biological conditions of the ambient environment are largely responsible for shaping the bacterial communities, which are then further refined by the zooplankton microenvironment.

The most commonly used substrates were responsible for the similarities in substrate usage through time and suggest that zooplankton-associated bacteria are able to utilize both ambient and zooplankton-derived substrates. Both pyruvic acid methyl ester and Tween 40 are used by bacteria isolated from coral (Ritchie & Smith 1995) and marine aggregates (Lyons et al. 2010, Lyons & Dobbs 2012), and by free-living bacteria in marine and estuarine systems (Montserrat Sala et al. 2005, 2006, Lyons & Dobbs 2012). N-acetyl-D-glucosamine is the structural monomer of chitin and is used in the formation of peptidoglycan in bacterial cell walls. Autoradiography indicated that N-acetyl-glucosamine is used by bacteria in all freshwater systems studied (Nedoma et al. 1994), and chitinase gene diversity was correlated with crustacean zooplankton biomass in a mesotrophic lake (Beier et al. 2012). Many marine bacteria also have the ability to utilize N-acetyl-D-glucosamine as a potential carbon and nitrogen source (Riemann & Azam 2002).

During October, November, March and April, less commonly utilized substrates such as phenolic compounds 2-hydroxybenzoic acid and 4-hydroxybenzoic acid were responsible for the temporal differences in substrate usage by zooplankton-associated bacteria (Fig. 4, Table 3). Both phenolic compounds are components of lignin contained in the seagrass *Zostera marina* (Klap et al. 2000), which grows in dense beds in the York River near the sampling site (Moore 2009). Seagrass beds in this area typically die back during late September (Moore et al. 2000), but degradation of lignin is slow: only 10% of lignin was mineralized after a 23-d incubation (Benner et al. 1984). A second pulse of lignin may be added to the system via allochthonous material with the spring freshet, which typically occurs in the York River around March and April (Marshall & Alden 1990, Kniskern & Kuehl 2003).

While these carbon substrates may potentially be broken down by the zooplankter's digestive enzymes (Mayzaud 1986), cleavage by digestive enzymes alone would not produce a color change of the redox dye. The colorless tetrazolium violet acts as an alternative electron acceptor in the electron transport chain and is reduced to purple formazan (Seidler 1991), which is then quantified spectrophotometrically. Therefore, unless the substrate is broken down to fuel cellular respiration, a color change will not occur.

Environmental influence on bacterial communities

Temporal changes in zooplankton-associated BCC (Fig. 2) suggested that ambient environmental conditions were a stronger selective force on zooplankton-associated bacteria than zooplankton-specific selective forces. Likewise, Kan et al. (2007) noted a similar bacterial composition throughout Chesapeake Bay at any given point in time. However, large seasonal changes in the bacterial communities indicated that environmental conditions with strong seasonality, such as temperature, played a larger role in shaping the microbial community than any region-specific characteristics. The community composition of aquatic free-living and particle-associated bacteria can be shaped by biological, chemical and physical parameters such as temperature (Muylaert et al. 2002, Fuhrman et al. 2006, Kan et al. 2006, Rösel et al. 2012), chlorophyll concentration (Muylaert et al. 2002, Kan et al. 2006), nitrogen and phosphorus concentrations (Muylaert et al. 2002, Fuhrman et al. 2006, Longmuir et al. 2007, Leflaive et al. 2008, Rösel et al. 2012), and grazing pressure (Muylaert et al. 2002). Our results indicate that environmental conditions also act directly on the zooplankton-associated communities, shaping them as they do free-living bacterial communities. Alternatively, the influence may be indirect, with environmental conditions shaping the free-living and particle-associated bacterial communities, which ultimately serve as sources for zooplankton-associated bacteria.

The variation in BCC explained by measured environmental conditions (37% for all zooplankton-associated bacteria, 65.8% for *Acartia*-associated)

was within the range of previous studies of free-living bacteria in lakes (Lindström 2000, Longmuir et al. 2007, Muylaert et al. 2002). Other unmeasured parameters such as DOC may also impact BCC. However, a large portion of the riverine DOC is likely refractory (Raymond & Bauer 2001), and by comparison DOC produced by zooplankton excretions tends to be of high quality (Møller 2005, Møller et al. 2007). The relative importance of ambient and zooplankton-produced DOC for zooplankton-associated bacteria remains to be investigated.

Physical properties of the study system likely also contributed to changes in zooplankton-associated BCC. Water residence time in the York River near our sampling site is approximately 11 d (Shen & Haas 2004). The rapid movement of water can continually move free-living bacteria into and out of the system, preventing the establishment of a stable estuarine bacterial community (Crump et al. 1999). Since the external surfaces of zooplankton must be recolonized by a subset of the free-living bacteria after each molting event, the turnover of zooplankton-associated bacterial communities in a dynamic estuary may be much faster than in more stable freshwater or marine systems.

Zooplankton-associated versus free-living bacteria

The number of DGGE bands detected in this study within the free-living bacterial community is low compared to literature values for Chesapeake Bay (Kan et al. 2006, 2007). The low DGGE band numbers could be due to the dominance of a few phylotypes within the system, as DGGE only detects phylotypes that contribute >1% of the total DNA (Muyzer et al. 1993). Additionally, DGGE primers may have created a bias against some bacterial groups such as *Gammaproteobacteria* (Alonso-Sáez et al. 2007, Gerdts et al. 2013), which comprised a number of the DGGE bands detected by Kan et al. (2006, 2007) with a different set of primers. Future studies should utilize a different set of primers or use multiple methods to investigate BCC (Alonso-Sáez et al. 2007, Gerdts et al. 2013). Despite the potential bias, the numbers of DGGE bands recovered from zooplankton were similar to those observed in previous studies (Møller et al. 2007, Tang et al. 2009a, Brandt et al. 2010), and comparisons can still be made among the different samples.

The significant difference between zooplankton-associated and free-living bacterial communities (Fig. 2) underpins the idea that zooplankton create microhabitats supporting a bacterial community different from that in the surrounding water (Grossart & Tang 2010). Of the 36 DGGE bands detected, 13 were unique to zooplankton-associated communities, 11 were found only in free-living bacterial communities, and 12 were shared by both communities. These results support the notion of an active exchange between free-living and zooplankton-associated bacterial communities (Møller et al. 2007, Grossart et al. 2010).

The average month-to-month similarity in substrate usage among zooplankton-associated bacteria (63.9%, Fig. 4) was slightly higher than that among free-living bacteria (55.1%). Free-living bacteria in the York River previously showed distinct changes in the carbon substrate usage patterns between winter/spring and summer/fall bacterial communities (Schultz & Ducklow 2000). In the Mediterranean Sea, bacteria within a eutrophic harbor showed stable, consistent substrate usage through time (Montserrat Sala et al. 2006). The authors hypothesized that a stable DOC supply allowed a stable bacterial community to establish and use a limited number of substrates. While the present study indicates that zooplankton-associated bacteria can be influenced by ambient environmental conditions, zooplankton themselves constantly produce large amounts of DOC and nutrients (e.g. Gaudy et al. 2000, Møller 2005), creating a stable baseline microenvironment. Thus, the impacts of ambient environmental fluctuations on bacterial communities may be buffered in the zooplankton microenvironment. This unique zooplankton microenvironment could allow certain bacteria to persist in a system even when ambient water conditions are not conducive for their growth (Tang et al. 2011).

The lower number of DGGE bands but similar number of substrates utilized by zooplankton-associated bacteria suggests that zooplankton-associated bacteria may exhibit a larger degree of functional plasticity, while free-living bacteria are more functionally redundant. Bacterial colonization of the macroalga *Ulva australis* has been described by the competitive lottery model (Burke et al. 2011), where a number of bacterial species with the same functional capacity are present within a source community, and specific niches in the *Ulva* ecosystem are randomly filled by whichever species from the source community arrived first (Burke et al. 2011). Our findings support the suggestion of Gerdts et al. (2013) that zooplankton may be colonized in the same manner, with functional niches filled by a subset of a more genetically diverse free-living bacterial com-

munity. The functionality of free-living bacteria is limited by the availability of substrates in the water column. In contrast, bacteria associated with zooplankton would have access to substrates in the water column as well as substrates generated by the zooplankter via ingestion, excretion and sloppy feeding. Thus, the zooplankton-associated bacterial community would have the opportunity to exploit a wider array of substrates.

CONCLUSIONS

This study demonstrates that seasonal changes in ambient environmental conditions impact the community composition and functionality of zooplankton-associated bacteria. Zooplankton create microenvironments within the water column that allow certain bacterial groups to flourish, increasing their overall presence and importance within an aquatic system. Taking into account zooplankton-associated bacteria will not only lead to better estimations of total bacterial abundance within a system, but also of the system's overall bacterial diversity and functionality.

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