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PRIMARY RESEARCH PAPER

Zooplankton-associated and free-living bacteria in the York River, Chesapeake Bay: comparison of seasonal variations and controlling factors

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Abstract Zooplankton provide microhabitats for bacteria, but factors which influence zooplankton-associated bacterial abundance are not well known. Through a year-long field study, we measured the concentration of free-living bacteria and bacteria associated with the dominant mesozooplankters *Acartia tonsa* and *Balanus* sp. Free-living bacteria peaked in the summer, while zooplankton-associated bacteria peaked in summer and winter. No relationships were found between bacterial abundance per individual and zooplankter width, length, surface area or body volume. Multiple regression analyses indicated that free-living and *Acartia*-associated bacterial concentrations were explained by temperature, salinity, ammonium, chl *a*, and all term interactions. *Balanus*-associated bacterial concentration was explained by ammonium and phosphate. Ammonium significantly influenced all sampled bacterial communities. In laboratory experiments, copepods raised under high ammonium concentration had higher bacterial concentrations than those raised under low ammonium condition. Transplant experiments showed that high ammonium favored loosely attached bacteria, whereas low ammonium selected for firmly attached bacteria, suggesting greater exchange between free-living and zooplankton-associated bacterial communities in nutrient-rich systems. Additional sampling of other zooplankton taxa all showed high bacterial concentrations, supporting the notion that zooplankton function as microbial hotspots and may play an important, yet overlooked, role in marine biogeochemical cycles.

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Introduction

Bacteria play an important role in organic matter decomposition and regulating biogeochemical cycles within aquatic systems. They exist either as free-living cells or can be associated with particles and other organisms (Simon et al., 2002). Copepods and other crustacean zooplankton are highly abundant in the ocean, and some bacteria directly attach to a zooplankter's chitinous exoskeleton and gut (reviewed in Tang et al., 2010), highlighting the importance of zooplankton as microhabitats for bacteria. Zooplankton-associated bacteria occur in very high concentrations on a cells-per-unit-biovolume basis (Tang et al., 2010), and they can account for up to 40% of the total bacteria in aquatic systems (Heidelberg et al., 2002). Consequently, studies which examine only free-living bacteria (FLB) may grossly underestimate bacterial abundance, production,

and relevant microbial processes. Investigation into possible relationships between zooplankton-associated bacterial abundance and environmental or zooplankton-specific parameters will shed light into which factors regulate this bacterial community.

Positive correlations between potential habitat size and organism abundance are common (e.g., Gaston & Lawton, 1990) even on a microscopic scale: Larger marine aggregates provided a larger SA for bacterial colonization, and as a result supported more bacteria (Alldredge & Gotschalk, 1990). Therefore, we hypothesized that larger zooplankton, both within and across species, would support higher bacterial abundances.

Ambient environmental conditions may also play a role in regulating zooplankton-associated bacterial abundances. A multitude of studies have shown that free-living bacterial abundance and activity are strongly influenced by temperature (Hoch & Kirchman, 1993; Felip et al., 1996; Peierls & Paerl, 2010) and nutrients (Kirchman, 1994; Felip et al., 1996). Other important environmental factors include salinity (Amon & Benner, 1998; Revilla et al., 2000) and primary production (Hoch & Kirchman, 1993; Amon & Benner, 1998), which is the primary source of labile dissolved organic carbon for FLB (Kirchman, 1994; Peierls & Paerl, 2010). Because these environmental factors do not act in isolation, it is important to consider the interactions of multiple environmental factors (Pomeroy & Wiebe, 2001; Peierls & Paerl, 2010). For example, Pomeroy & Wiebe (2001) highlighted the fact that excess nutrients may override temperature limitations on bacterial growth. To our knowledge, the impact of environmental factors on zooplankton-associated bacterial abundances has not been investigated. Zooplankton can produce large amounts of dissolved organic matter (DOM) via sloppy feeding and excretions (Møller, 2005; Møller et al., 2007), allowing attached bacteria to exploit the nutrient-rich environment at the zooplankton surface. Association with zooplankton may give attached bacteria access to resources not available to FLB, thereby moderating their responses to environmental conditions. We hypothesized that zooplankton-associated bacteria exploit zooplankton-derived nutrients, and therefore would be less sensitive to ambient nutrient concentrations.

To address our hypotheses, we used the zooplankton-associated and FLB of the York River, Chesapeake Bay as a test case. Chesapeake Bay is the largest estuary in the United States and has been experiencing eutrophication due to human activities in the surrounding watershed (Kemp et al., 2005). Free-living bacterial growth and abundance in Chesapeake Bay has been linked to temperature and substrate supply (Shiah & Ducklow, 1994) and anywhere between 0.01 and 40% of the total bacterial abundance can be associated with bulk zooplankton (Heidelberg et al., 2002). Through a year-long, monthly field sampling, we assessed how zooplankton-associated bacterial abundance was related to zooplankton body length, width, SA, and volume. In addition, we compared temporal changes among zooplankton-associated and free-living bacterial concentrations and assessed how the respective concentrations were related to environmental conditions. Complementary laboratory experiments were conducted to further explore the effects of inorganic nitrogen availability on zooplankton-associated bacterial abundances.

Materials and methods

Field sampling

Environmental conditions and FLB

Monthly samples were collected from May 2010 to April 2011 at a fixed station located in the York River estuary near Gloucester Point, VA (37°14′50.36″N, 76°29′58.03W). All samples were collected at or near high tide during daylight hours. Surface water was collected to measure ambient water environmental parameters including temperature, salinity, chlorophyll *a* (chl *a*) concentration, ammonium, phosphate, and free-living bacterial concentration. For chl *a* concentrations, approximately 100 ml of water was filtered through a GF/F filter. Chl *a* was extracted from the filters with 90% acetone and measured fluorometrically***. Fifty ml of water was filtered through 0.2-μm filters for ammonium and phosphate analyses. Ammonium concentrations were measured in duplicate on a Shimadzu UV-1601 spectrophotometer following the phenol hypochlorite method (detection limit 0.05 μmol N/L; Koroleff, 1983). Phosphate concentrations were run in duplicate on a Lachat QuikChem 8500 autoanalyzer (detection limit 0.05 μmol/l; Parsons et al., 1984). Triplicate 1 ml aliquots of whole water were filtered onto 0.2-μm pore size filters and stained with DAPI nucleic acid stain to enumerate FLB (Porter & Feig, 1980). Ten fields of view were counted within each replicate under 1,000× total magnification.

Zooplankton-associated bacteria

Zooplankton were collected via multiple tows with a plankton net (200 µm mesh, ½ m mouth diameter) with non-filtering cod end. Tow samples were combined in a 5-gallon container with ambient water and immediately taken back to the laboratory. In the lab, the zooplankton sample was gently concentrated down to approximately 1 l, and split into four equal fractions with a plankton splitter. Each fraction was transferred to a sterilized glass jar and brought to a final volume of 1 l with 0.2-µm filtered artificial seawater (ASW). Zooplankton were allowed to clear their guts overnight to eliminate any food-associated bacteria. After gut clearance, one fraction was used to determine zooplankton community composition and another was used to assess zooplankton-associated bacterial abundance. The remaining two fractions were used to assess the genetic and functional diversities of zooplankton-associated bacteria which will be reported elsewhere.

All members of the zooplankton community were counted and identified. Calanoid and cyclopoid copepods, and barnacle nauplii were identified to genus level, while other zooplankton were placed in larger zooplankton groups. The relative abundance of each zooplankton group was determined. The calanoid copepod *Acartia tonsa* Dana and the naupliar form of the barnacle *Balanus* sp. are commonly found in the York River estuary (Steinberg & Condon, 2009) and were the dominant zooplankters found in our samples. They were therefore chosen as the representative organisms for this study. Other zooplankton groups were present intermittently throughout the year and were sampled when available; these included polychaete larvae, harpacticoid copepods, crab zoea, mysid shrimp, fish eggs, the cladoceran *Podon* sp., the cyclopoid copepod *Oithona* sp., and the calanoid copepods *Pseudodiaptomus* sp., *Centropages* sp., *Eurytemora affinis*, *Parvocalanus* sp., and *Temora* sp.

Each zooplankton fraction for bacterial abundance determination was gently concentrated onto a sterile, 200-µm mesh sieve and rinsed four times with 0.2-µm sterile-filtered ASW (20 psu) to remove loosely attached bacteria. The mixed zooplankton assemblage was then back-rinsed into a sterile petri dish with 0.2-µm sterile-filtered ASW and narcotized with a small amount of sodium bicarbonate. Preliminary experiments confirmed that the use of sodium bicarbonate did not significantly affect the counts of zooplankton-associated bacteria. After narcotization, ten individuals each of *A. tonsa*, *Balanus* sp. *nauplii*, and other abundant groups were haphazardly picked from the mixed assemblage and transferred to a new, sterile petri dish with approximately 10 µl of surrounding water. Each individual zooplankter was photographed with a Canon Rebel T1i EOS500D camera attached to a Nikon SMZ1000 dissecting microscope. Length (l) and width (w) of each zooplankter were measured from the digital photographs with ImagePro imaging software. Total body volume (BV) and SA of each zooplankter was approximated from length and width measurements with the respective equations for a cylinder with closed ends. SA of *Acartia* was refined further using a nested cylinder model to account for the tubular gut surface. The equation for *Acartia* surface was derived from the ratio of external + gut surface area: external surface area measured from 44 *Acartia* copepodites and adults with full guts. Gut sizes were not measured on individuals processed for bacterial abundance as zooplankton were allowed to clear their guts prior to measurement, making the guts very difficult to see. The following equations were used for SA and BV calculations:

$$\mathrm{SA} = 2\pi * \left(\frac{w}{2}\right)^2 + 2\pi\left(\frac{w}{2}\right)l$$

$$\textit{Acartia}\ \mathrm{SA} = 1.216 * \left(2\pi * \left(\frac{w}{2}\right)^2 + 2\pi\left(\frac{w}{2}\right)l\right)$$

$$\mathrm{BV} = \pi\left(\frac{w}{2}\right)^2 l$$

After being photographed, each individual was transferred to a microcentrifuge tube containing 600 µl of sterile sea water. To account for any FLB transferred with the zooplankter in the surrounding water, 10 µl of water from the petri dish into which the zooplankton had been rinsed was transferred to a separate microcentrifuge tube for use as a control. Three control replicates were prepared every month and processed in the same manner as the zooplankton samples. All samples were homogenized on ice with a microprobe sonicator (4 W output power, six rounds of 5 s on, 5 s off) to break apart the copepod and release the externally attached and gut bacteria (Tang, 2005). After sonication, the probe was rinsed with 600 µl of sterile seawater into the same

microcentrifuge tube with the sample. A separate, preliminary experiment indicated that the sonication method produced no significant change in the bacterial concentrations of FLB. Each zooplankton homogenate was filtered onto a 0.2-μm black polycarbonate filter, stained with SYBR-gold (Chen et al., 2001) and counted on an epifluorescence microscope with blue light excitation. Twenty fields of view were counted under 600× total magnification. SYBR-gold stain displayed greater contrast than DAPI between bacterial cells and zooplankton detritus. Preliminary experiments indicated the counts with the two staining methods were comparable. Cell counts were normalized to unit BV (μm^3) to account for variations in zooplankter sizes throughout the year; BV was converted from μm^3 to ml to compare zooplankton-associated bacterial concentrations with free-living bacterial concentrations.

Laboratory experiment

Copepod cultures under specific ammonium concentrations

Based on results from the field study, ammonium was the only environmental factor incorporated into the best-fitting multiple linear regression models for all three bacterial communities (Table 1). Therefore, we conducted complementary laboratory experiments to examine the potential impact of ammonium concentration on the abundance and detachment of bacteria associated with *A. tonsa*. Adult *A. tonsa* from a laboratory culture were divided into two experimental groups in 0.2 μm filtered ASW: (1) high ammonium (H; ca. 10 μM) and (2) low ammonium (L; ca. 2 μM). 10 μM represents the high end of ammonium concentrations observed in the York River (Condon et al., 2010). Water was renewed daily with the appropriate nutrient concentration. Copepods were fed a saturating concentration (33,000 cells ml^{-1}; Kiørboe et al., 1985) of a 1:1:1 cell mixture of *Rhodomonas salina*, *Isochrysis galbana*, and *Thalassiosira weissflogii*. To minimize the nutrients added with the phytoplankton, the appropriate volume of each phytoplankton culture was centrifuged for 15 min at 200 RCF. The supernatant was gently pipetted off, and cells were resuspended in a minimal amount of media. The three phytoplankton species were combined and added to the copepods in typically less than 1 ml of growth media. Microscopic inspection verified that centrifugation did not compromise the integrity of the cells. Water samples were taken in duplicate at the beginning and end of each day for the first 7 days to monitor ambient ammonium concentrations. Eggs laid by the adult copepods were collected, hatched, and grown in the same ammonium conditions at 19°C for 2 weeks.

Table 1 The best-fitting model produced for each bacterial community as assessed by AIC

	Free-living bacteria ($\log_{10}$ cells ml^{-1})	*Acartia*-associated bacteria ($\log_{10}$ cells ml^{-1} BV)	*Balanus*-associated bacteria ($\log_{10}$ cells ml^{-1} BV)
Intercept	−6.704*	20.757***	9.045***
Temp	0.548***	−0.381	–
Sal	0.520***	−0.521*	–
NH_4	1.216**	−5.018***	0.173***
PO_4	–	–	2.544***
Chl *a*	1.771***	−2.565**	–
FLB	NA	–	–
temp × Sal	−0.021***	0.016*	–
temp × NH_4	−0.0003	−0.034***	–
temp × PO_4	–	–	–
temp × chl a	−0.0322***	−0.043***	–
temp × FLB	NA	–	–
Sal × NH_4	−0.052**	0.255***	–
Sal × PO_4	–	–	–
Sal × Chl *a*	−0.064***	0.099**	–
Sal × FLB	NA	–	–
NH_4 × PO_4	–	–	−0.245
NH_4 × Chl *a*	0.07**	−0.041	–
NH_4 × FLB	NA	–	–
PO_4 × Chl *a*	–	–	–
PO_4 × FLB	–	–	–
Chl *a* × FLB	–	–	–
R^2	0.913	0.597	0.707
P value	<0.001	<0.001	<0.001
AIC_c	−50.983	123.147	73.454
Weighted probability	0.475	0.359	0.248

Values are the coefficients for each of the predictor variables in the model

Temp temperature, *Sal* salinity, NH_4 ammonium, PO_4 phosphate, *FLB* free-living bacteria, *NA* not applicable. Free-living $N = 120$, *Acartia*-associated $N = 120$, *Balanus*-associated $N = 100$

Asterisks denote significant values: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Transplant experiment

Copepods from each respective experimental group were gently collected onto a sterile 200-μm mesh sieve and back-rinsed into a sterile petri dish. Four replicates, with three copepods in each replicate, were used to assess copepod-associated bacterial abundance before gut clearance. All remaining copepods were transferred to 250 ml of 0.2-μm filtered ASW of the appropriate ammonium concentration and allowed to clear their guts for 3.5 h to eliminate food-associated bacteria. After gut clearance, each experimental group was again concentrated onto a sterilized 200-μm mesh sieve and back-rinsed into a sterile petri dish. Four replicates with three copepods in each were used to assess copepod-associated bacterial abundance after gut clearance. All copepods in the samples were photographed, and processed for copepod-associated bacteria in the same manner as the field samples with the exception that samples were preserved after sonication with formaldehyde (~4% final concentration) to extend their storage time.

Four separate transplant treatments were established using the copepods with clear guts: (1) copepods raised in low ammonium kept in low ammonium (L–L treatment); (2) copepods raised in low ammonium transferred to high ammonium (L–H treatment); (3) copepods raised in high ammonium transferred to low ammonium (H–L treatment); and (4) copepods raised in high ammonium kept in high ammonium (H–H treatment). For each replicate, three copepods with cleared guts were placed in 5 ml of the respective water in a well of a sterile 12-well tissue culture plate. Additional copepod-free controls were established for both high and low ammonium waters. Five ml water samples were taken at the start of the experiment for each ammonium concentration to determine initial free-living bacterial abundance. Four replicates of each treatment and control were performed. All treatments were incubated at 19°C for approximately 24 h.

After the incubation, all three copepods from each replicate well were gently removed with a pipette, photographed for biovolume estimation, combined into one microcentrifuge tube and processed for copepod-associated bacteria as described previously. In a few instances one of the copepods within a replicate died during incubation and was removed before processing. The total volume of ambient water from each replicate was collected in a sterile 15-ml centrifuge tube and preserved with formaldehyde (4% final concentration). The entire volume of each sample was stained with DAPI for the enumeration of FLB.

Statistical analyses

Bacterial abundance and concentration (cells ml^{-1} body volume) data were tested for normality with the Kolmolgorov–Smirnov test and homogeneity of variance with Levene's test, and subsequently log-transformed to normalize the data. To find the best combination of environmental predictors for each bacterial community, multiple linear regression models were constructed in the format of:

$$\log_{10}(y) = b_0 + b_1x_1 + b_2x_2 + \cdots + b_kx_k,$$

where y is the number of bacteria per ml zooplankton BV for attached bacteria, or number of bacteria per ml water for FLB, and $b_{1,2\ldots,k}$ are the coefficients of the predictor variables. x_1, x_2,…,x_k represent the predictor variables and the interactions among the predictor variables. All possible combinations of environmental predictors were tested and ranged from single factor models to multiple factor models (up to six predictors) including interaction terms between every two factors. A total of 120 models were tested for zooplankton-associated bacteria and 57 models were tested for FLB. Model fit was assessed using Akaike's Information Criterion (AIC) with correction for sample size (Anderson, 2008) and the weighted probability of each model was calculated. The model with the highest weighted probability was determined to be the best predictor.

For the laboratory experiments, data were tested for normality and homogeneity of variance. A one-way ANOVA with post-hoc Tukey pairwise comparisons of 95% confidence intervals were performed for both the free-living and zooplankton-associated bacterial abundances across the different treatments.

Results

Field study

Environmental conditions and bacterial abundances

Water temperature ranged from a minimum of 3.5°C (January) to a maximum of 30.5°C (July; Fig. 1a).

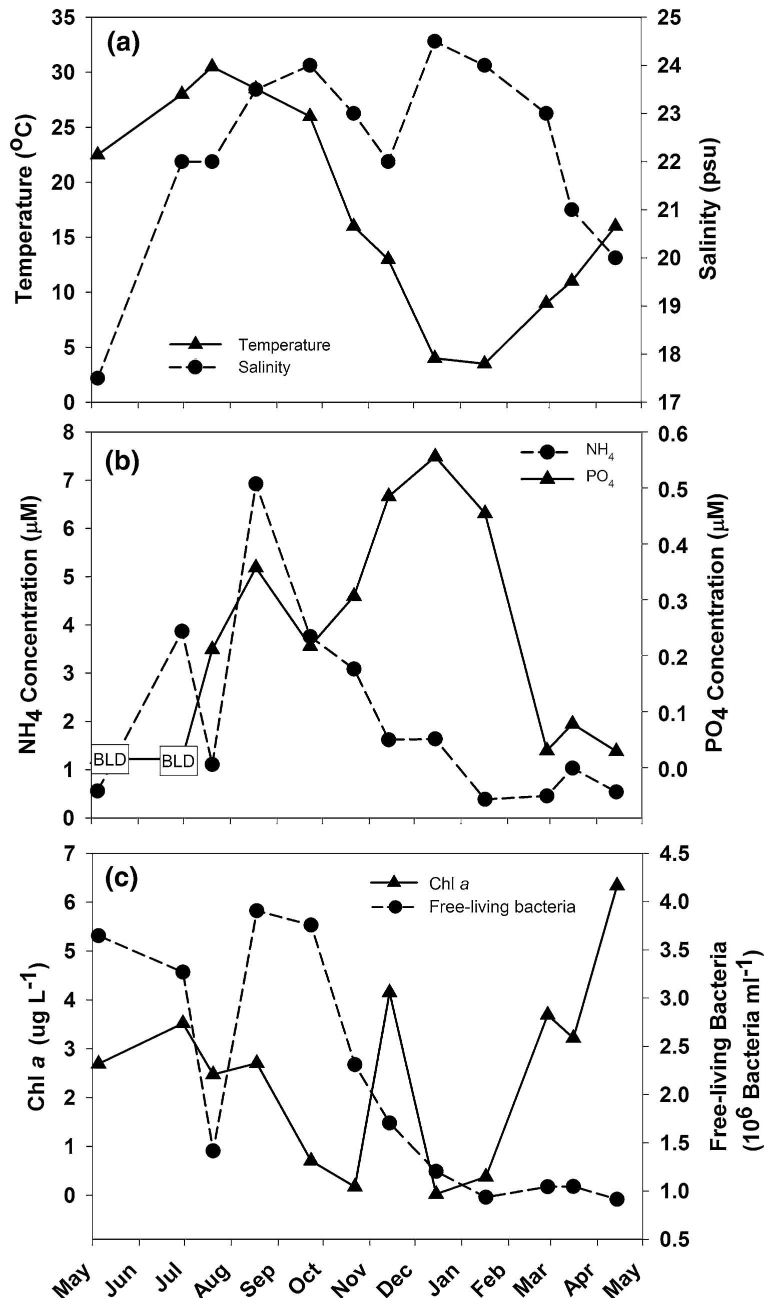

Fig. 1 Monthly values of environmental parameters in the York River, VA between May 2010 and April 2011. Parameters measured include temperature and salinity (**a**), ammonium and phosphate (**b**), and chl *a* and FLB (**c**). *BLD* below level of detection

Salinity was slightly less variable and ranged from 17.5 psu in May to 24.5 psu in December (Fig. 1a). A low of 0.39 μM ammonium was noted in January and a high of 6.92 μM in August, while phosphate was below the detection limit in May and June, and reached a maximum of 0.56 μM in December (Fig. 1b). Chl *a* concentration was lowest in December and highest in April (0.03 and 6.34 μg l^{-1}, respectively; Fig. 1c).

In general, free-living bacterial concentration was lowest in the winter and early spring (minimum 0.91×10^6 cells ml^{-1} in April), increased during summer and peaked in August (3.90×10^6 cells ml^{-1}). Zooplankton-associated bacterial abundance changed from month to month. The number of bacteria per individual varied from 0.67×10^5 to 5.71×10^5 for *Acartia* and 0.32×10^5 to 7.41×10^5 cells for *Balanus*

nauplii. Two peaks were observed with *Acartia*-associated bacterial abundance: the highest average abundance per individual was noted in August ($5.71 \pm 0.28 \times 10^5$; mean ± SE), while a second peak of $5.30 \pm 0.23 \times 10^5$ was observed in December. A similar pattern was noted among *Balanus*-associated bacteria, with a peak in August ($7.28 \pm 0.31 \times 10^5$ cells individual^{-1}), and a slightly larger peak in winter ($7.41 \pm 0.41 \times 10^5$ cells individual^{-1}in January). On a per BV basis, zooplankton-associated bacteria were 2–6 orders of magnitude more concentrated than FLB, depending on zooplankton group and month (Fig. 2). The highest and lowest bacterial densities were observed with calanoid copepods: *Pseudodiaptomus* sp. supported $3.58 \pm 0.24 \times 10^{12}$ cells ml^{-1} BV in August, while *Eurytemora affinis* supported $1.16 \pm 0.28 \times 10^8$ cells ml^{-1} BV in January. *Acartia* and *Balanus*-associated bacterial concentrations exhibited the same temporal pattern as bacterial abundances, with peaks in August and December/January, with variations between 1.11×10^9 and 2.04×10^{10} cells ml^{-1} BV for *Acartia* and 1.69×10^9 and 5.57×10^{10} cells ml^{-1} BV for *Balanus*. The contribution of zooplankton-associated bacteria to total bacterial abundance was estimated from average monthly *Acartia* densities in the York River (Elliott & Tang, 2011), the average monthly number of bacteria per *Acartia* and fraction of total zooplankton comprised by *Acartia* in this study. Throughout the year zooplankton-associated bacteria accounted for less than 0.1% of the total water column bacteria in the York River.

Predictors of bacterial abundance

The multiple linear regression model with the lowest AIC value and highest weighted probability for each bacterial group is presented in Table 1. Free-living bacterial concentration was best predicted by the model which included temperature, salinity, ammonium, chl *a*, and all possible interactions of these variables ($R^2 = 0.9131$, $P < 0.0001$). The same model had the highest weighted probability for *Acartia*-associated bacterial concentration ($R^2 = 0.5969$, $P < 0.0001$); however, many of the environmental conditions had different effects on *Acartia*-associated bacterial communities. For example, the coefficients for both ammonium and Chl *a* were positive in the model for FLB, but negative in the model for *Acartia*-associated bacteria (Table 1). *Balanus*-associated concentration was best described only by ammonium, phosphate, and the interaction between the two terms ($R^2 = 0.7067$, $P < 0.0001$). There were no significant relationships between *Acartia* or *Balanus*-associated bacterial abundance and zooplankter length, width, SA, or BV (Fig. 3).

Copepod transplant experiment

Even with daily water changes in an attempt to maintain steady ammonium concentrations, a significant drawdown of ammonium (paired *t* test, $P < 0.0001$) was still observed after 24 h. Low ammonium (L) cultures decreased from 2.19 ± 0.06 to 0.78 ± 0.02 μM (mean ± SE) and high ammonium (H) cultures decreased from 10.86 ± 0.07 to 1.48 ± 0.17 μM. After the 2-week acclimation period, copepods with full guts in H culture supported significantly higher (one-way ANOVA, $P = 0.002$) bacterial concentrations ($2.76 \pm 0.20 \times 10^{10}$ cells ml^{-1} BV; mean ± SE) than those in L culture ($1.23 \pm 0.03 \times 10^{10}$; Fig. 4). After gut clearance, bacterial concentration for H culture ($2.38 \pm 0.14 \times 10^{10}$ cells ml^{-1} BV) was not significantly different from the L culture ($1.28 \pm 0.05 \times 10^{10}$), based on comparison of 95% confidence intervals. The concentrations of bacteria associated with copepods in the L (Fig. 4a) and H (Fig. 4b) cultures were nearly the same before and after gut clearance.

In the transplant experiments, copepod-associated bacterial concentrations in L–L ($1.34 \pm 0.04 \times 10^{10}$ cells ml^{-1} BV) and L–H ($1.35 \pm 0.13 \times 10^{10}$) treatments were not different from the initial values (copepods in L culture after gut clearance; Fig. 4a). For copepods raised in high ammonium cultures, the associated bacterial concentration remained unchanged in H–L treatments and in H–H treatments (Fig. 4b).

The final free-living bacterial concentrations in the L and H controls for the copepod transplant experiments were subtracted from the respective free-living bacterial concentrations in the copepod treatments to account for bacterial growth due to contamination. In both the L–L and H–H transplants, one of the four replicates for free-living bacterial concentration was determined to be a statistical outlier by Grubbs' test and was removed from subsequent analyses. Starting bacterial concentrations were comparable in the low ammonium ($1,841 \pm 211$ cells ml^{-1}; mean ± SE) and high ammonium waters ($1,488 \pm 93$ cells ml^{-1}). There was no significant change in free-living bacteria from the starting concentration in the L–H copepod

Fig. 2 Monthly values (mean ± SE) of free-living bacterial concentration (bacteria per ml of water) and zooplankton-associated concentrations (bacteria per ml of zooplankton BV)

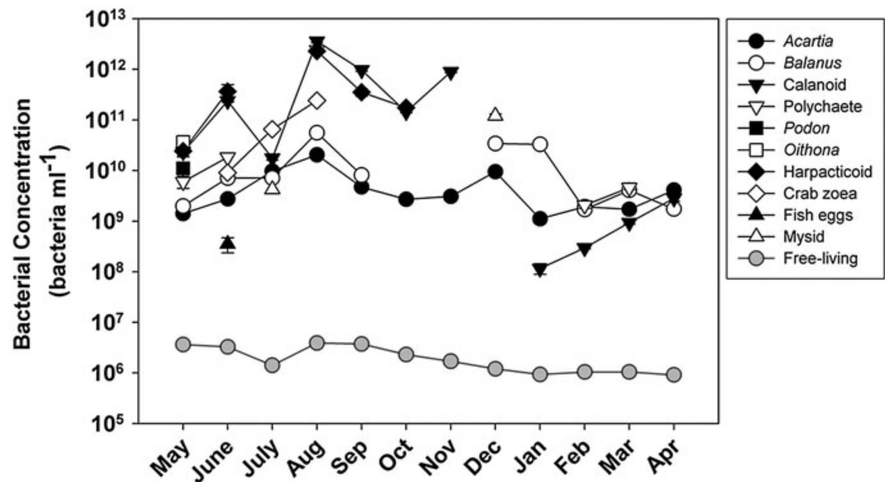

Fig. 3 Number of bacteria per individual zooplankter as a function of zooplankton body length (**a**), width (**b**), SA (**c**), and BV (**d**). *Filled circles* and *solid lines* represent *Acartia*-associated bacteria, *open circles* and *dotted lines* represent *Balanus*-associated bacteria

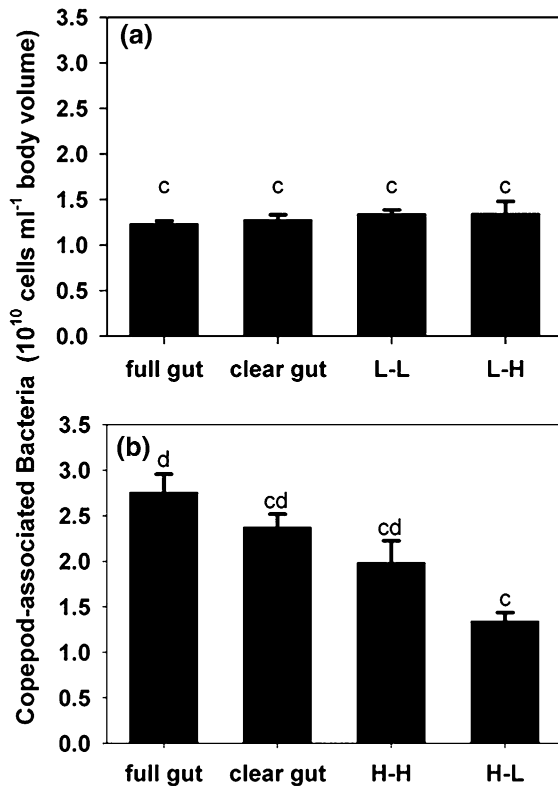

Fig. 4 Bacterial concentrations (mean ± SE; $N = 4$) associated with copepods originally raised under low (**a**) or high (**b**) ammonium condition. Bacterial concentrations were measured before and after gut clearance, and after transplantation to different ammonium treatments. *full gut*: copepods with full guts, *clear gut*: copepods after gut clearance, *L–L*: copepods raised in low culture, maintained in low culture, *L–H*: raised in low, transplanted to high, *H–H*: raised and kept in high, *H–L*: raised in high, transplanted to low. Letters above the error bars indicate statistical differences and are applicable within and between panels

transplant, while all other transplants showed a significant increase in FLB (one-way ANOVA, $P < 0.0001$) from initial values. The H–L and H–H treatments demonstrated the largest average increases of 21,660 and 20,727 cells ml^{-1}, respectively. Both values were significantly higher than the L–L treatment, which showed an average increase of 13,242 cells ml^{-1}.

Discussion

Zooplankton-specific characteristics

A recent study of bacteria attached to abiotic plastic surfaces indicated that bacteria may take advantage of any space available for colonization (Zettler et al., 2013). The high variability of bacterial abundance found in association with *Acartia* and *Balanus* of similar sizes (Fig. 3) suggests that zooplankton surface are different than abiotic surfaces, and bacterial colonization was not a simple function of host's body size. Bacterial distribution on zooplankton body surface can be patchy, with the formation of clumps and chains allowing bacteria to reach high abundances without using all available surfaces (Carman & Dobbs, 1997; Caro et al., 2012). Using electron microscopy, Carman & Dobbs (1997) observed that bacteria concentrated around the mouthparts and anal region of copepod exoskeletons, presumably where nutrient release would be the highest. By primarily colonizing these high nutrient areas, the amount of suitable habitat available for bacteria would be greatly reduced and less dependent on the overall size of the zooplankter. *Acartia tonsa* is a holoplanktonic copepod which progresses through six naupliar, five copepodite, and one adult stage, molting between each stage. Barnacles are meroplanktonic, remaining in the water column for six naupliar stages before metamorphosing to a cyprid form and settling onto a permanent surface (Qiu et al., 1997). It is conceivable that all external bacteria are lost during molting and the exoskeleton must be recolonized by new bacteria. This idea was suggested for female marine isopods, which molt throughout their lives forcing bacteria to continually recolonize the exoskeleton. Female isopods, which showed a lower bacterial diversity than males, stop molting at senescence and thus can accumulate a diverse bacterial community over time (Caro et al., 2012).

Field observations

The concentrations (per ml BV) of bacteria associated with all examined zooplankton groups were two to six orders of magnitude higher than FLB (per ml, Fig. 2). These high bacterial concentrations are similar to those associated with the marine copepod *Calanus* spp., which were three orders of magnitude higher than the surrounding North Sea water (Møller et al., 2007). Bacterial concentrations between 10^7 and 10^{11} cells ml^{-1} BV have been reported for other individual calanoid copepods, *Artemia*, and freshwater cladocerans (reviewed in Tang et al., 2010). *Acartia* were the dominant copepod in the York River and present

year-round, which provided the opportunity for comparison of zooplankton-associated and free-living bacterial abundance. *Acartia*-associated bacterial abundances and concentrations were among the lowest observed for all zooplankton groups (Fig. 2) which yielded a conservative estimate that zooplankton-associated bacteria accounted for less than 0.1% of total water column bacteria within the York River. Although zooplankton-associated bacteria were not numerically dominant, the high bacterial concentrations associated with individual zooplankters support the idea that zooplankton function as microbial hotspots. In addition to creating localized areas of elevated bacterial abundance and production (Carman, 1994; Møller et al., 2007), zooplankton can support distinct bacterial communities and play an important role in shaping the overall microbial diversity and functions through the creation of distinct microhabitats (Grossart & Tang, 2010; Tang et al., 2010).

Interactions of multiple controlling factors

Bacterial abundance is rarely controlled by only one environmental factor. It is therefore important to consider the effects of interactions among multiple environmental factors (Pomeroy & Wiebe, 2001). The multiple regression model that included temperature, salinity, ammonium, chl *a*, and all possible interactions among the variables accounted for 91.31% of the variability associated with free-living bacterial concentrations and 59.69% of variability within *Acartia*-associated bacterial concentrations. These environmental factors affected the two bacterial communities in different manners (Table 1).

Temperature, a significant predictor of free-living bacterial concentration ($P < 0.001$, Table 1), was an important controlling factor of free-living bacterial abundance and production in Chesapeake Bay surface waters (Shiah & Ducklow, 1994) and other temperate estuaries along the east coast of the United States (Hoch & Kirchman, 1993; Schultz et al., 2003; Peierls & Paerl, 2010). Temperature exhibited a limited relationship with zooplankton-associated bacterial concentration (Table 1) due to the large spike in zooplankton-associated bacterial concentration in December for *Acartia* and December/January for *Balanus* (Fig. 2). Pomeroy & Wiebe (2001) highlighted that substrate availability can be as important as, or more important than, temperature in regulating heterotrophic microbial processes. Association with zooplankton may give attached bacteria access to resources not available to FLB, thereby moderating their responses to environmental temperature.

Multivariate regressions underscored that the interactive effects of available resources can lead to unexpected results. For example, zooplankton-associated bacteria decreased, while FLB increased with ammonium (Table 1), which is the preferred nitrogen source for many heterotrophic bacteria in aquatic systems (Kirchman, 1994). The presence of excess nutrients may override limitations of bacterial growth in low temperatures (Pomeroy & Wiebe, 2001), a phenomenon observed in experimental and natural systems. Incubations of bacteria from Conception Bay at 2°C exhibited a three-fold increase in bacterial respiration when substrates were supplemented (Pomeroy et al., 1991). Likewise, mid-winter bacterial production rates in Lake Michigan were comparable to mid-summer rates after a large storm resuspended nutrient-rich sediments (Cotner et al., 2000). Within the present study, zooplankton-associated bacterial concentration peaked during the coldest months of the year to values comparable to summer peaks. Free-living bacteria in the York River may be limited by cold temperatures in winter (Schultz et al., 2003). However, gut flora will benefit from nutrients taken in by the host zooplankter, and excretions by the zooplankter also provide an excess of nutrients for externally attached bacteria, such that zooplankton-associated bacteria may be able to overcome temperature limitation.

The coefficient for chl *a* in the multiple regression model was positive for FLB but negative for *Acartia*-associated bacteria, suggesting an increase in *Acartia*-associated bacterial concentration with decreasing chl *a*. Phytoplankton is traditionally the primary source of DOC for FLB (Pomeroy et al., 1991; Goosen et al., 1997; Amon & Benner, 1998), but zooplankton excretions and sloppy feeding also produce large amounts of high quality, labile DOC (Møller, 2005; Møller et al., 2007), phosphorus (Titelman et al., 2008), and nitrogen (Carman, 1994), which can enhance both free-living and attached bacterial production. Zooplankton-associated bacteria are therefore unlikely to rely on phytoplankton as the primary source of carbon substrates. Although phytoplankton comprises a large portion of copepod diet, *Acartia* can switch to motile microzooplankton prey when phytoplankton concentrations are low (Kiørboe et al., 1996). Microzooplankton tend to contain more protein than

phytoplankton (Kleppel, 1993), and consumption of high protein prey would lead to higher nitrogen excretion in copepods (Conover & Mayzaud, 1975). The ability of *Acartia* to feed omnivorously would allow them to maintain or even increase excretion rates as chl *a* concentrations decrease. DOC concentrations in Chesapeake Bay are typically high but only a small fraction of the bulk DOC pool is labile (Raymond & Bauer, 2001). Ambient DOC concentrations were not directly measured in this study. Given that zooplankton produce high quality labile DOC, future studies are needed to determine the importance of ambient DOC for zooplankton-associated bacterial growth.

Despite the fact that *Acartia* and *Balanus* were collected from the same location, multiple regression analyses indicated that the bacterial concentrations associated with these zooplankton taxa were influenced by different combinations of environmental factors. *Balanus*-associated bacteria were solely impacted by ammonium and phosphate, while *Acartia*-associated bacteria were sensitive to more environmental conditions (Table 1). These results suggest that individual zooplankton groups are able to buffer the impacts of environmental conditions on their associated bacterial communities to varying degrees, perhaps through the creation of microhabitats specific to each zooplankter group. The *Balanus* microenvironment may be more buffered from environmental conditions than the *Acartia* microenvironment.

Effects of ammonium treatments in laboratory experiments

Carman (1994) demonstrated in laboratory incubations that copepod-attached bacteria accounted for almost 20% of all bacterial production and suggested that attached bacteria can directly exploit the copepod excretions. The relative importance of zooplankton-derived nutrients versus those available in the water column is unknown, and it is possible that the nutrient status of the system may impact the zooplankton–bacteria association. This issue was addressed in the laboratory experiments.

Copepods raised in high ammonium (H) condition supported higher bacterial concentrations. Since incubations were conducted in 0.2 μm filtered water, and both initial and final free-living bacterial concentrations were very low, we attributed any changes in zooplankton-associated bacterial concentrations to growth of attached bacteria rather than colonization by FLB. The results suggest that while attachment to zooplankton surfaces allows bacteria to directly exploit nutrient-rich excreta, ambient nutrients may also stimulate growth of copepod-associated bacteria, leading to more abundant zooplankton-associated bacteria.

Bacterial concentrations associated with copepods raised in low ammonium (L) culture remained rather constant after gut clearance and in both L–L and L–H transplant experiments (Fig. 4a), indicating firm attachment of bacteria selected for by the low ammonium environment in the L culture. This is consistent with an earlier report that FLB in oligotrophic lakes had little or no attachment webs, whereas attached bacteria had very large fibrillar networks allowing for secure attachment (Paerl, 1980).

In contrast, high ammonium condition could favor loosely attached bacteria. As copepods are stressed through starvation and transplanted to L conditions, loosely attached bacteria may detach, leaving only those capable of firm attachment, similar to what would be expected in a low nutrient system, where the benefits from attachment would be greater. The observations of more detachment under high nutrients (Fig. 4b) is consistent with an earlier study that followed the changes in copepod-associated bacterial community composition during nutrient shifts: When copepods from a eutrophic lake were incubated in the same eutrophic water, they maintained 78% of their bacterial community composition, while copepods transplanted from the eutrophic lake into an oligotrophic lake retained only 28% of the original bacterial community (Grossart et al., 2009). Identical attachment web structures were observed on free-living and attached bacteria in eutrophic lakes (Paerl, 1980) and identical bacterial phylotypes were found attached to copepods and in the surrounding water of the eutrophic North Sea, suggesting an active exchange between the two bacterial communities (Møller et al., 2007). The continual detachment of bacteria associated with H culture copepods suggests that under high nutrients, the majority of bacteria are only loosely attached to copepods, and exchange between zooplankton-associated and FLB may be more likely to occur in eutrophic systems than in oligotrophic systems.

The laboratory results seem to be in direct contrast to the negative relationship between ammonium and *Acartia*-associated bacterial concentrations indicated

by the multiple linear regression model (Table 1). It should be considered, however, that the laboratory experiments were conducted under constant temperature and salinity conditions, while the multiple regression model incorporated data from a range of temperatures and salinities. When *Acartia*-associated bacterial densities from similar temperatures (within 0.5°C) were examined, increased bacterial densities were found with higher ammonium concentrations at low (3.5–4°C, *t* test, $P < 0.001$) and high temperatures (28–28.5°C, *t* test, $P < 0.001$), but not at an intermediate temperature (16°C, *t* test, $P = 0.274$). The multiple regression analysis of field samples highlights that zooplankton-associated bacterial densities are not a simple function of ammonium concentrations, and the importance of complex interactions between ammonium and other environmental conditions should be considered when examining in situ bacterial communities.

Other ecological implications

Zooplankton-associated bacterial biomass can be directly passed on to higher trophic levels when the zooplankton are eaten by planktivores, such as the bay anchovy, an important component of Chesapeake Bay's food web. To estimate this potential trophic transfer of bacterial biomass, we used the copepod *Acartia tonsa* as the representative zooplankter. We assumed each bacterium contains 30.2 fg C $cell^{-1}$ and 5.8 fg N $cell^{-1}$ (Fukuda et al., 1998), and each adult copepod contains 6.1 μg C $individual^{-1}$ and 1.6 μg N $individual^{-1}$(Durbin et al., 1992). Based on the results of this study, bacteria could account for up to 0.57% of measured copepod carbon and 0.22% of measured copepod nitrogen throughout the year. Using the energy flow network constructed by Baird & Ulanowicz (1989), bay anchovy could therefore directly consume a maximum of 1.12 mg bacterial C m^{-2} during the summer.

Even though zooplankton-associated bacteria within the York River accounted for less than 0.1% of the total water column bacterial abundance, and are not consumed in significant amounts by higher trophic levels, all examined members of the zooplankton community carried bacterial concentrations orders of magnitude higher than those found in the surrounding waters, making them potential hotspots for microbial activities and production. Tang (2005) estimated that copepod-associated bacteria grow at a rate 3–18 times higher than FLB. Zooplankton guts are partially anoxic and can support anaerobic microbial processes that are otherwise not favored in the oxygenated water column (Tang et al., 2011). Anaerobic bacteria have been found in zooplankton guts (Marty, 1993; Proctor, 1997), and methane production by actively grazing zooplankton has been reported (de Angelis & Lee, 1994). On average, 12% of the global primary production passes through zooplankton via grazing alone (Calbet, 2001). Hence, the highly concentrated and active bacterial communities associated with zooplankton could potentially play a significant but previously overlooked role in marine biogeochemical cycles. Further research into the compositions and activities of these bacterial communities is warranted.

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