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Abundance, structure and function of zooplankton-associated bacterial communities within the York River, VA

Samantha L. Bickel
College of William and Mary - Virginia Institute of Marine Science

ABUNDANCE, STRUCTURE AND FUNCTION OF ZOOPLANKTON-ASSOCIATED BACTERIAL COMMUNITIES WITHIN THE YORK RIVER, VA

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Samantha Lynn Bickel
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APPROVAL SHEET

This dissertation is submitted in partial fulfillment of
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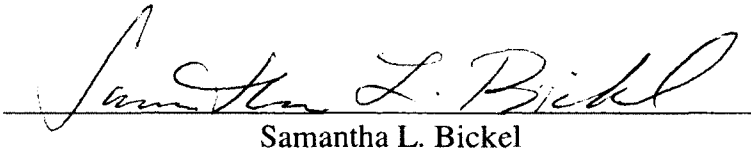

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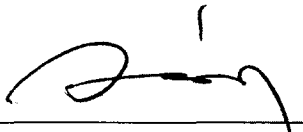
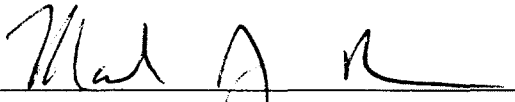
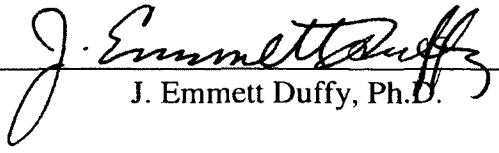


Kam W. Tang, Ph.D.
Committee Chairman/ Advisor



Mark J. Brush, Ph.D.



J. Emmett Duffy, Ph.D.

Kimberly S. Reece, Ph.D.

Fred C. Dobbs, Ph.D.
Old Dominion University
Norfolk, VA

TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Mesozooplankton function as microbial microhabitats and can support concentrations of bacteria orders of magnitude higher than in the surrounding water. These zooplankton-associated bacteria can have much higher production rates than their free-living counterparts. Portions of the zooplankton microhabitat may also be anoxic and provide refuge for anaerobic bacteria and their associated processes within the oxygenated water column. Despite their common presence in the marine environment, zooplankton-associated bacteria are largely ignored by microbial ecologists and zooplankton ecologists alike. Consequently, factors which influence zooplankton-associated bacterial abundance, community composition and function, and how zooplankton-associated bacteria compare to free-living bacteria are not well known. The goal of my research was to investigate which environmental parameters and zooplankton-specific characteristics influenced the zooplankton-associated bacterial abundance, community composition and function. During a year-long field study in the York River, VA, free-living bacteria concentration peaked in the summer, while zooplankton-associated bacteria concentration peaked in both summer and winter. There were no relationships between number of bacteria per individual zooplankter and zooplankter size. Ambient ammonium concentration was the one environmental parameter that correlated with all zooplankton-associated bacterial concentrations. In laboratory experiments, copepods raised in high ammonium concentration had high concentrations of loosely attached bacteria, while copepods raised in low ammonium concentration supported fewer, firmly attached bacteria, suggesting greater exchange between free-living and zooplankton-associated bacterial communities in nutrient rich systems. Zooplankton-associated bacterial communities were genetically distinct from free-living bacterial communities and utilized a wider array of carbon substrates. Changes in ambient environmental conditions played a larger role than zooplankton-characteristics in shaping zooplankton-associated bacterial community composition and function. Additionally, the potential importance of zooplankton guts as anoxic microhabitats was evaluated by comparing carbon substrate usage by the total bacterial (epibiotic + gut) and gut bacterial communities of the calanoid copepod *Acartia tonsa* under aerobic and anaerobic conditions. Gut bacteria were responsible for a large portion of the microbial activity associated with the copepod under both aerobic and anaerobic conditions. A larger variety of substrate subsets were used by zooplankton-associated bacteria than free-living bacteria under anaerobic conditions, suggesting that each zooplankton group selects for a specific combination of bacteria. In fact, some zooplankton-associated bacteria were not detected in the surrounding water and utilized substrates not used by free-living bacteria. These results highlight that zooplankton act as microbial hotspots and zooplankton-associated bacteria are an important part of the total bacterial abundance, diversity and functionality in aquatic systems.

AUTHOR'S NOTE

The original research chapters of this dissertation (Chapters 2-4) were written in the format of the journal for which each manuscript has been submitted. The citations for the chapters are as follows:

Chapter 2
Bickel, S.L., Tang, K.W. (In Review) Zooplankton-associated and free-living bacteria in the York River, Chesapeake Bay: Comparison of seasonal variations and controlling factors. ***Hydrobiologia***

Chapter 3
Bickel, S.L. Tang, K.W., Grossart, H.P. (In Review) Structure and function of zooplankton-associated bacterial communities in a temperate estuary change more with time than zooplankton species. ***Aquatic Microbial Ecology***

Chapter 4
Bickel, S.L., Tang, K.W., (In Review) Carbon substrate usage by aerobic and facultative anaerobic bacteria associated with estuarine zooplankton. ***Aquatic Biology***

ABUNDANCE, STRUCTURE AND FUNCTION OF ZOOPLANKTON-ASSOCIATED BACTERIAL COMMUNITIES WITHIN THE YORK RIVER, VA

CHAPTER 1

Introduction to the Dissertation

INTRODUCTION

Within the aquatic ecosystem, free-living bacteria and mesozooplankton can interact in multiple ways. Mesozooplankton can directly stimulate growth of free-living bacteria through the release of significant amounts of dissolved organic matter (DOM) to the surrounding water through excretions and sloppy feeding (e.g. Møller 2005, Titelman et al. 2008). Free-living bacteria can then utilize this DOM, incorporate it into biomass and reintroduce it to the aquatic food web. The bacterial biomass is grazed mainly by microzooplankton, which are in turn consumed by mesozooplankton (Azam et al. 1983). Bacteria can also contribute directly to the growth of higher trophic levels through ingestion and assimilation by mesozooplankton. Some mesozooplankton can ingest and assimilate the bacterial cells directly (Gophen et al. 1974) or indirectly when bacteria are attached to algal particles (Lawrence et al. 1993). Although the occurrence of mesozooplankton-associated bacteria has long been documented (e.g. Boyle & Mitchell 1978), mesozooplankton and bacteria are still commonly perceived as two separate functional groups within the microbial loop with rare, weak or indirect interactions (Azam & Malfatti 2007). Consequently, the direct relationship between mesozooplankton and bacteria is largely ignored by zooplankton and microbial ecologists alike. The majority of ecological studies only considers free-living bacteria and consequently may grossly underestimate bacterial abundance, production and all associated microbial processes.

The relationships between bacteria and mesozooplankton extend far beyond trophic interactions. A number of studies have detected bacteria in direct association with live mesozooplankton through the colonization of the zooplankter's external

surfaces or gut (reviewed in Tang et al. 2010), highlighting the importance of mesozooplankton as microbial microhabitats. Mesozooplankton are unique microhabitats in the respect that they provide a consistent, nutrient-rich environment through constant feeding and excretion. Gut bacteria may benefit from a concentrated food source and externally attached bacteria may exploit excretions and sloppy feeding for a consistent, immediate source of DOM.

Due to the implications for human and aquatic animal health, a large portion of earlier research on mesozooplankton-associated bacteria focused on disease-causing organisms. The presence of live copepods is essential for the persistence and dispersal of *Vibrio cholerae*, the causative agent of the disease cholera, in aquatic systems (Huq et al. 1983). While *Vibrio* is the most frequently studied, other pathogenic bacteria such as *Pseudomonas* sp. and *Helicobacter pylori* have also been observed in association with copepods (Sochard et al. 1979, Hansen & Bech 1996, Cellini et al. 2005).

There are many ecological implications for the associations between zooplankton and bacteria aside from the stimulation of bacterial growth. Colonization of external or internal zooplankton surfaces may provide a defense mechanism for some bacteria. It has been shown that association with crustacean zooplankton offers bacteria protection from environmental stressors such as UV radiation, heat and ozone (Tang et al. 2011). Bacteria can repeatedly attach and detach from zooplankton exoskeletons, effectively exploiting mesozooplankton's movement to aid dispersal and overcome physical boundaries in the water column such as the pycnocline (Grossart et al. 2010).

Recently there has been an increase in ecologically-based studies concerning mesozooplankton-associated bacteria. These studies have highlighted that

mesozooplankton-associated bacteria concentrations can be on par with or even exceed free-living bacteria concentrations (Tang et al. 2010), and mesozooplankton-associated bacteria can account for 0.4 – 40% of the total bacteria within a system (Heidelberg et al. 2002). The mesozooplankton microenvironment may also support anaerobic microbial processes within the aerobic water column (Bianchi et al. 1992, de Angelis & Lee 1994, Proctor 1997), which may have implication for marine biogeochemical cycles.

The limited data available indicates there are large differences in bacterial abundances and bacterial community compositions (BCC) associated with different mesozooplankton species from the same system, and between the same mesozooplankton species from different systems (e.g. Niswati et al. 2005, Grossart et al. 2009, Brandt et al. 2010). The stability of these mesozooplankton-associated bacterial communities through time, as well as the factors that regulate mesozooplankton-associated bacterial abundance and community composition remain uncertain. Copepods and cladocerans collected from the same location at the same time exhibited very different bacterial communities, suggesting some yet-to-be determined zooplankton-specific characteristics that shape the mesozooplankton-associated BCC (Grossart et al. 2009). It has been suggested that the number of bacteria on a mesozooplankter may be a function of habitat size, i.e. larger mesozooplankters can support more bacteria (Brandt et al. 2010) and BCC could change with molt status (Caro et al. 2012). Other potentially important, yet unexplored influential factors include ambient environmental conditions.

Studies that investigated potential controlling factors of free-living bacterial communities found correlations to environmental conditions such as temperature, phytoplankton biomass, as well as dissolved nitrogen and phosphorus concentrations

(Muylaert et al. 2002, Crump & Hobbie 2005, Fuhrman et al. 2006, Longmuir et al. 2007). Given the extent to which environmental parameters may influence the free-living bacterial community structure and the potential for dynamic exchanges between the mesozooplankton-associated and free-living bacterial communities (Møller et al. 2007, Grossart et al. 2009, Grossart et al. 2010), it is plausible that mesozooplankton-associated communities are impacted by environmental conditions in the same manner as free-living bacteria.

The aforementioned studies have started to bridge the gap between zooplankton ecology and microbial ecology, and have elucidated the potential for complex and ecologically significant interactions between the free-living and mesozooplankton-associated bacterial communities.

STRUCTURE OF DISSERTATION

The general term "zooplankton" encompasses a wide range of zooplankton size classes, which can exhibit very different interactions with bacteria. To simplify the descriptive process in this dissertation, the term "zooplankton" will refer to mesozooplankton (200-2000 μm) from this point forward, unless otherwise noted. Despite the recent advances and greater amount of attention that has been drawn to the subject, very basic information about zooplankton-associated bacterial communities is lacking. The overall goal of my dissertation was to address some of these shortcomings and fill in these gaps in knowledge. In particular, I sought to assess which zooplankton-specific characteristics and environmental parameters may regulate zooplankton-associated bacterial abundance, and their genetic and functional compositions.

Additionally, I studied the relative importance of zooplankton guts vs. exoskeletons as microhabitats for supporting aerobic and anaerobic microbial processes.

This dissertation is divided into three main chapters, with Chapters 2 through 4 discussing the results of a year-long field study and complementary laboratory experiments performed to expand upon the findings of the field study. Chapter 2 describes temporal changes in the abundance of bacteria associated with the calanoid copepod *Acartia* sp. and the barnacle nauplius *Balanus* sp., which were present nearly year-round in the lower York River, a tributary of Chesapeake Bay. Bacterial abundances associated with other periodically dominant zooplankters and free-living bacterial abundances are also reported. Data from additional laboratory experiments conducted to assess the effects of ambient ammonium concentration on zooplankton-associated bacterial abundances are also described. Relationships between zooplankton-associated bacterial abundances and measures of zooplankton size and environmental conditions are discussed.

Chapter 3 describes the differences among the bacteria communities associated with different zooplankton groups and the free-living bacterial community. Temporal changes in the composition of all bacterial communities are also addressed. Bacterial community fingerprint analyses obtained via denaturing gradient gel electrophoresis (DGGE) and carbon substrate utilization patterns of the bacterial communities are reported for the calanoid copepod *Acartia* sp., barnacle nauplius *Balanus* sp. and other prevalent zooplankton groups within each month. The presence of specific DGGE bands and usage of certain carbon substrates are analyzed in relation to ambient environmental

parameters. Differences in the functional and genetic diversity of the different bacterial communities are discussed.

Chapter 4 describes the laboratory experiments and field study conducted to examine zooplankton guts as potential anoxic microhabitats for anaerobic bacteria and their associated processes within the larger oxygenated water column. Carbon substrate utilization patterns are reported for all *Acartia*-associated bacteria and *Acartia* gut bacteria incubated in aerobic and anaerobic conditions. Aerobic and anaerobic substrate usage by bacteria associated with six common zooplankton groups from the York River is also presented. The relative importance of gut bacterial communities to total substrate usage and diversity of substrate usage by each zooplankton group is discussed.

Chapter 5 presents the overall conclusions from the dissertation. Using these conclusions, I have identified promising avenues of future research further linking the fields of zooplankton and microbial ecologies.

Appendix I contains methods and a brief discussion of fluorescence *in situ* hybridization (FISH) with bacterial group-specific probes to identify zooplankton-associated bacteria. FISH was performed to supplement the DGGE data to examine changes in bacterial community composition in Chapter 3. However, due to unexpected difficulty with the application of all FISH probes, these data were not used. I discuss reasons for the unsuccessful application of the FISH probes. Appendix II contains alternative acceptable models as determined by AIC which compare environmental predictor variables to *Acartia*-associated, *Balanus*-associated and free-living bacterial concentrations. Appendix III contains information on zooplankton community composition within the York River.

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CHAPTER 2

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

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 bacterial concentration peaked in the summer while zooplankton-associated bacterial concentration 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, chlorophyll and all term interactions. *Balanus*-associated bacterial concentration was positively correlated with ammonium and phosphate. Ammonium was the one factor which influenced all bacterial communities. In laboratory experiments, copepods raised under high ammonium concentration had higher bacterial concentrations ($2.76x10^{10}$ bacteria ml^{-1} body volume) than those raised under low ammonium condition ($1.23x10^{10}$). 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.

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 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 surface area 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 (Felip et al., 1996, Kirchman, 1994). Other important environmental factors include salinity (Amon & Benner, 1998, Revilla et al., 2000) and primary production (Amon & Benner, 1998, Hoch & Kirchman, 1993) , which is the primary source of labile dissolved organic carbon for free-living bacteria (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 and Wiebe (2001) highlighted the fact that excess nutrients may override temperature limitations on bacterial growth. To our knowledge, the impact of individual or interactions of multiple 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 free-living bacteria, 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 free-living bacteria 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, surface area and volume. Additionally, 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 & METHODS

Field Sampling

Environmental conditions and free-living bacteria

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, 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. Chlorophyll 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 0.2μm pore size filters and stained with DAPI nucleic acid stain to enumerate free-living bacteria (Porter & Feig, 1980). Ten fields of view were counted within each replicate under 1000X 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 one liter, and split into 4 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.

Each zooplankton fraction for bacterial abundance determination was gently concentrated onto a sterile, 200-μm mesh sieve and rinsed 4 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 artificial seawater and narcotized with a small amount of sodium bicarbonate. Preliminary experiments indicated that use of sodium bicarbonate did not significantly affect 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 surface area (SA) of each zooplankter was approximated from length and width measurements with the respective equations for a cylinder with closed ends. Surface area 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:

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

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

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

After being photographed, each individual was transferred to a microcentrifuge tube containing 600 µl of sterile sea water. To account for any free-living bacteria 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 (4W output power, six rounds of five seconds on, five seconds off) to release the attached bacteria (Tang, 2005). After sonication, the probe was rinsed with 600 µl of sterile seawater into the same microcentrifuge tube with the sample. 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 600X total magnification. SYBR-gold stain displayed greater contrast between bacterial cells and zooplankton detritus than DAPI. Preliminary experiments indicated the counts with the two staining methods were comparable. Cell counts were normalized to unit body volume (μm^3) to account for differences in zooplankter sizes throughout the year; body volume 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 which influenced free-living bacteria and bacteria associated with both zooplankton groups, and was the strongest individual predictor for free-living and *Acartia*-associated bacterial concentration. 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 0.2μm filtered artificial seawater: 1) High ammonium (H; ca. 10μM) and 2) low ammonium (L; ca. 2μM). 10μM 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 minutes 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 two weeks.

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 0.2μm filtered ASW of the appropriate ammonium concentration and allowed to clear their guts for 3.5 hours 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, 3 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 hours.

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 free-living bacteria.

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. Simple linear regression was used to test for relationships between log-transformed bacterial concentration and individual environmental parameters or zooplankton-specific characteristics. Pearson correlation coefficients were also calculated between the log-transformed bacterial data and environmental variables. To find the best combination of predictors for each bacterial community, multiple linear regression models were constructed in the format of:

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

where y is the number of bacteria per ml zooplankton BV for attached bacteria, or number of bacteria per ml water for free-living bacteria, and $b_{1,2\ldots k}$ are the coefficients of the predictor variables. $x_1, x_2, \ldots, x_k$ 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 free-living bacteria. 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

Zooplankton Community Composition

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* and the naupliar forms 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.

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). Salinity was slightly less variable and ranged from 17.5 psu in May to 24.5 psu in December (Fig. 1A). A low of 0.39μM ammonium was noted in January and a high of 6.92μM in August, while phosphate was below the detection limit in May and June, and reached a maximum of 0.56μM in December (Fig. 1 B).

Chlorophyll concentration was lowest in December and highest in April (0.03μg L^{-1} and 6.34μg L^{-1}, respectively; Fig. 1C).

In general, free-living bacterial concentration was lowest in the winter and early spring (minimum $0.91x10^6$ cells ml^{-1} in April), increased during summer and peaked in August ($3.90x10^6$ cells ml^{-1}). Zooplankton-associated bacterial abundance changed from month to month. The number of bacteria per individual varied from $0.67x10^5$ to $5.71x10^5$ for *Acartia* and $0.32x10^5$ to $7.41x10^5$ 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 ± 0.28 $x10^5$; mean ±SE), while a second peak of 5.30 ± 0.23 $x10^5$) was observed in December. A similar pattern was noted among *Balanus*-associated bacteria, with a peak in August (7.28 ± 0.31 $x10^5$ cells $individual^{-1}$), and a slightly larger peak in winter (7.41 ± 0.41 $x10^5$ cells $individual^{-1}$in January). On a per volume basis, zooplankton-associated bacteria were 2-6 orders of magnitude more concentrated than free-living bacteria, depending on zooplankton group and month (Fig. 2). The highest and lowest bacterial densities were observed with calanoid copepods: *Pseudodiaptomus* sp. supported 3.58 ± 0.24 $x10^{12}$ cells ml^{-1} body volume in August while *Eurytemora affinis* supported 1.16 ± 0.28 $x10^8$ cells ml^{-1} body volume 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 $1.11x10^9$ and $2.04x10^{10}$ cells ml^{-1} body volume for *Acartia* and $1.69x10^9$ and 5.57 $x10^{10}$ cells ml^{-1} body volume for *Balanus*. The contribution of zooplankton-associated bacteria to total bacterial abundance was estimated from monthly average of *Acartia* densities in the York River (Elliott & Tang, 2011), the monthly average 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

Based on one-factor regressions and correlation analyses, free-living bacterial concentration was strongly positively correlated to ambient water temperature, whereas *Acartia*-associated bacterial concentration was weakly positively correlated (Table 1, $p<0.0001$ for both groups). *Balanus*-associated bacterial concentration was not related to water temperature ($p=0.79$) (Table 1, Figs. 3A and 3B). Free-living bacterial concentration ($p<0.0001$), *Acartia*-associated and *Balanus*-associated concentrations ($p<0.0001$) were all positively correlated with ammonium (Figs. 3E and 3F). Both zooplankton-associated bacterial concentrations were positively related salinity (*Acartia* $p= 0.004$; *Balanus* $p<0.001$; Fig. 3C). *Balanus*-associated bacterial concentration was positively related to phosphate ($p<0.0001$; Fig. 3G) and negatively related to chlorophyll ($p<0.0001$; Fig. 3I). Linear regressions showed no relationship between zooplankton-associated bacterial concentration and free-living bacterial concentration. There were no significant relationships between *Acartia* and *Balanus*-associated bacterial abundance and zooplankter length, width, surface area or body volume (Fig. 4).

The multiple linear regression model with the lowest AIC value and therefore highest weighted probability for each bacterial group is presented in Table 2. Free-living bacterial concentration was best predicted by the model which included temperature, salinity, ammonium, chlorophyll *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 ($R^2 = 0.5969$, $p<0.0001$), while *Balanus*-associated concentration was best described only by ammonium, phosphate and the interaction between the two terms ($R^2 = 0.7067$, $p<0.0001$). All models within 3 AIC units of the model with the lowest AIC value are presented in Appendix II.

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 hours. Low ammonium (L) cultures decreased from 2.19 ± 0.06μM to 0.78 ± 0.02μM (mean ± S.E.) and high ammonium (H) cultures decreased from 10.86 ± 0.07μM to 1.48 ± 0.17μM. After the two-week acclimation period, copepods with full guts in H culture supported significantly higher (one-way ANOVA, $p=0.002$) bacterial concentrations (2.76±0.20 $x10^{10}$ cells ml^{-1} BV; mean ± S.E.) than those in L culture (1.23±0.03 $x10^{10}$; Fig. 5). After gut clearance, bacterial concentration for H culture remained higher (2.38±0.14 $x10^{10}$ cells ml^{-1} body volume) than the L culture (1.28±0.05 $x10^{10}$), although the difference was not significant based on comparison of 95% confidence intervals. The concentration of bacteria associated with copepods in the L culture was nearly the same before and after gut clearance (Fig. 5A), whereas in H culture it showed a small but insignificant decrease (Fig. 5B).

In the transplant experiments, copepod-associated bacterial concentrations in L-L (1.34±0.04 $x10^{10}$ cells ml^{-1} BV) and L-H (1.35±0.13 $x10^{10}$) treatments were not different from the initial values (copepods in L culture after gut clearance; Fig. 5A). For copepods

raised in high ammonium cultures, the associated bacterial concentration decreased insignificantly from 2.38±0.14 $x10^{10}$ cells ml^{-1} BV to 1.35±0.09 $x10^{10}$ in H-L treatments and 1.99±0.24 $x10^{10}$ in H-H treatments (Fig. 5B).

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 (NIST, 2012) and was removed from subsequent analyses. Starting bacterial concentrations were comparable in the low ammonium (1,841 ± 211 cells ml^{-1}; mean ± SE) and high ammonium waters (1,488 ± 93 cells ml^{-1}). There was no significant change in free-living bacteria from the starting concentration in the L-H copepod transplant, while all other transplants showed a significant increase in free-living bacteria (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

The high variability of bacterial abundance found in association with *Acartia* and *Balanus* of different sizes (Fig. 4) suggests that 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 and 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 6 naupliar, 5 copepodite and one adult stage, molting between each stage. Barnacles are meroplanktonic, remaining in the water column for 6 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 showed a lower bacterial diversity than males, which stop molting at senescence and thus can accumulate a diverse bacterial community over time (Caro et al. 2012).

Field observations

The temporal pattern of free-living bacterial abundance within this study was similar to that observed in the Chesapeake Bay main stem, where abundance was lowest in the winter, increased to a maximum in the summer (June-August) and decreased during the fall (Shiah & Ducklow, 1994). In this study, free-living bacterial abundance showed a strong positive relationship with temperature (R^2=0.689). Temperature alone was the dominant controlling factor of bacterial abundance within Chesapeake Bay surface waters over a span of 2 years (Shiah & Ducklow, 1994). Likewise, temperature was noted as an important factor controlling free-living bacterial abundance and production in the York River estuary (Schultz et al., 2003), Delaware estuary (Hoch & Kirchman, 1993) and Neuse River estuary (Peierls & Paerl, 2010), which are all temperate estuaries on the east coast of the United States.

The concentrations (per ml body volume) of bacteria associated with all examined zooplankton groups were two to six orders of magnitude higher than free-living bacteria (per ml). 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} body volume 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 (Tang et al., 2010, Grossart & Tang, 2010).

Acartia- and *Balanus*-associated bacterial concentrations exhibited different temporal patterns than those of free-living bacteria (Fig. 2). Considering that free-living bacterial concentration was strongly related to temperature, it is interesting that *Acartia*-associated and *Balanus*-associated bacterial concentrations showed only weak or no relation to with temperature. The limited relationship with temperature was due to the large spike in zooplankton-associated bacterial concentration in December for *Acartia* and December/January for *Balanus* (Figure 2). Pomeroy and 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 free-living bacteria, thereby moderating their responses to environmental temperature.

The field data indicated that both zooplankton-associated bacteria and free-living bacteria were strongly influenced by nutrients, ammonium in particular (Table 1, Figs. 3 E&F). Ammonium is the preferred nitrogen source for many heterotrophic bacteria in

aquatic systems (Kirchman, 1994) and has been noted as a controlling factor of free-living bacteria in the temperate Urdaibai estuary in Spain (Revilla et al., 2000). 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. Both free-living bacteria and zooplankton-associated 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. 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. For these attached bacteria, 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.

Effects of ammonium treatments in laboratory experiments

Consistent with the field observations, copepods raised in high ammonium (H) condition supported higher bacterial concentrations. Since incubations were conducted in 0.2μm filtered water, and both initial and final free-living bacterial concentrations were very low, we attribute any changes in zooplankton-associated bacterial concentrations to growth of attached bacteria rather than colonization by free-living bacteria. The results suggest that while attachment to zooplankton surfaces allows bacteria to directly exploit nutrient-rich excreta, ambient nutrients 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. 5A), indicating firm attachment of bacteria selected for by the low ammonium environment in the L culture. This is consistent with an earlier report that free-living bacteria 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. 5B) 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 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 free-living bacteria may be more likely to occur in eutrophic systems than in oligotrophic systems.

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, chlorophyll *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 2). The coefficient for chlorophyll *a* was positive for free-living bacteria 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 free-living bacteria (Goosen et al., 1997, Pomeroy et al., 1991, Amon & Benner, 1998), but zooplankton excretions and sloppy feeding also produce large amounts of high quality, labile dissolved organic carbon (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 environmental factors. *Balanus*-associated bacteria were solely impacted by ammonium and phosphate, while *Acartia*-associated bacteria were sensitive to more environmental conditions (Table 2). These results suggest that individual zooplankton groups are able to buffer the impacts of

environmental conditions on their associated bacterial communities, perhaps through the creation of microhabitats specific to each zooplankter group.

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 30.2fg C $cell^{-1}$ and 5.8fg N $cell^{-1}$ (Fukuda et al., 1998), and each copepod contains 0.83 – 2.80μg C $individual^{-1}$ (copepodite – adult) and 0.14 – 0.45μg N $individual^{-1}$(Jones et al.,2002). Based on the results of this study, bacteria could account for 0.001 – 4.19% of measured copepod carbon and 0.41 – 2.61% of measured copepod nitrogen throughout the year. Using the energy flow network constructed by Baird and Ulanowicz (1989), bay anchovy could therefore directly consume a maximum of 8.16 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. The fact that these zooplankton-associated bacteria were influenced by environmental factors differently than free-living bacteria suggests that the two bacterial

communities have very different ecological roles. Tang (2005) estimated that copepod-associated bacteria grow at a rate 3-18 times higher than free-living bacteria. 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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Table 1 Pearson correlation coefficients for relationships between bacterial communities and measured environmental parameters. 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.

	Free-Living bacteria (log_{10} cells ml^{-1})	***Acartia*-associated bacteria (log_{10} cells ml^{-1} body volume)**	***Balanus*-associated bacteria (log_{10} cells ml^{-1} body volume)**
Temp	0.689***	0.318***	-0.028
Sal	-0.089	0.261**	0.625***
NH_4	0.692***	0.431***	0.505***
PO_4	-0.091	0.213	0.759***
Chl *a*	-0.189	0.034	-0.596***
FLB	NA	0.132	0.168

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

Table 2 The best-fitting model produced for each bacterial community as assessed by AIC. 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.

	Free-Living bacteria (log_{10} cells ml^{-1})	***Acartia*-associated bacteria** (log_{10} cells ml^{-1} body volume)	***Balanus*-associated bacteria** (log_{10} cells ml^{-1} body volume)
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 x sal	-0.021***	0.016*	-----
temp x NH_4	-0.0003	-0.034***	-----
temp x PO_4	-----	------	-----
temp x chl a	-0.0322***	-0.043***	-----
temp x FLB	NA	-----	-----
Sal x NH_4	-0.052**	0.255***	-----
Sal x PO_4	-----	-----	-----
Sal x Chl a	-0.064***	0.099**	-----
Sal x FLB	NA	-----	-----
NH_4 x PO_4	------	-----	-0.245
NH_4 x Chl a	0.07**	-0.041	-----
NH_4 x FLB	NA	-----	-----
PO_4 x Chl a	-----	-----	-----
PO_4 x FLB	-----	-----	-----
Chl a x FLB	-----	-----	-----
R^2	0.913	0.597	0.707
p value	<0.001	<0.001	<0.001
AICc	-50.983	123.147	73.454
weighted probability	0.475	0.359	0.248

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

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 Chlorophyll and Free-living bacteria (c). BLD = Below level of detection.

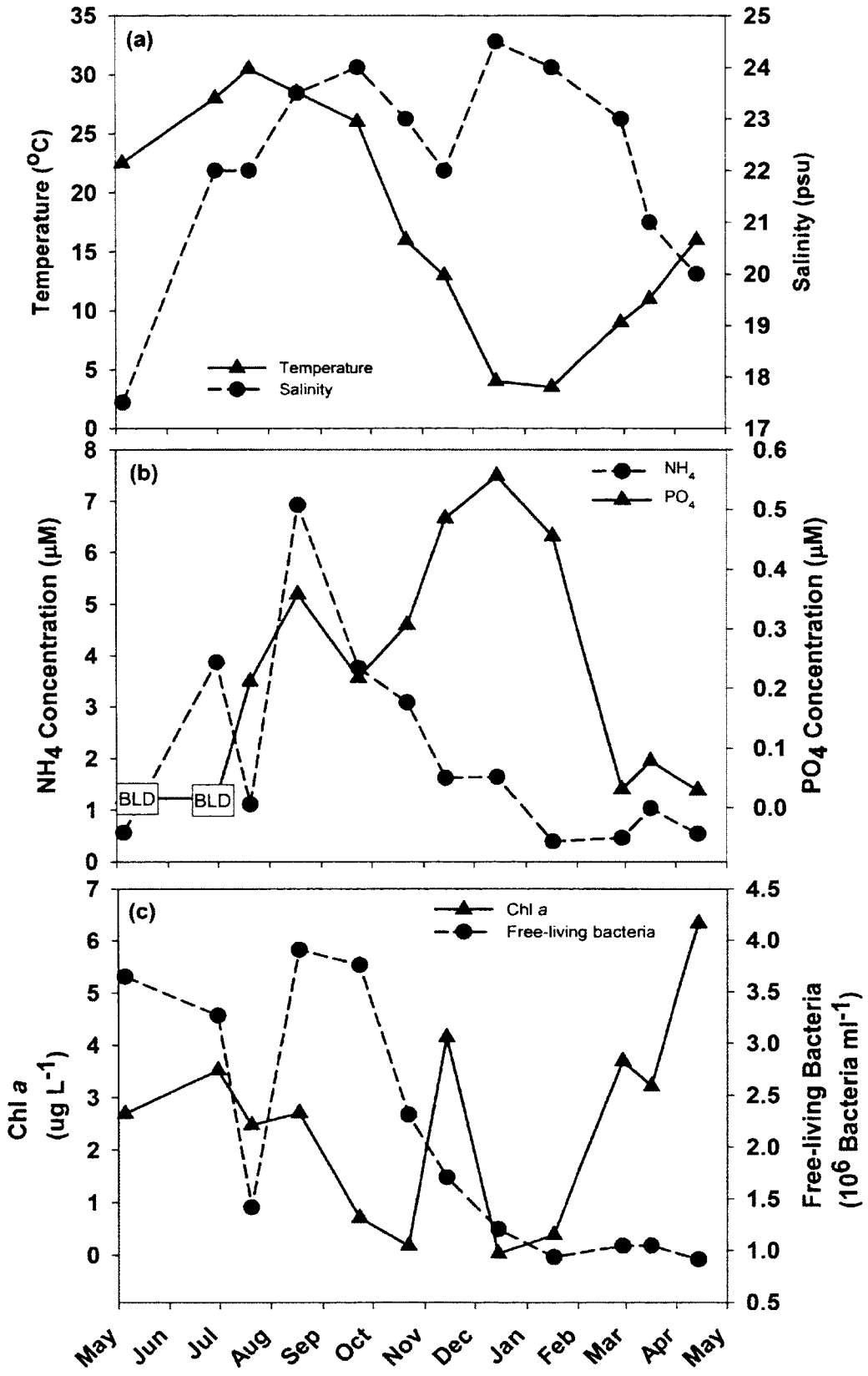

(a)
Temperature (^{o}C)
Salinity (psu)
Temperature
Salinity
(b)
NH_4 Concentration (µM)
PO_4 Concentration (µM)
NH_4
PO_4
BLD
BLD
(c)
Chl *a* (ug L^{-1})
Free-living Bacteria (10^6 Bacteria ml^{-1})
Chl *a*
Free-living bacteria
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May

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 body volume)

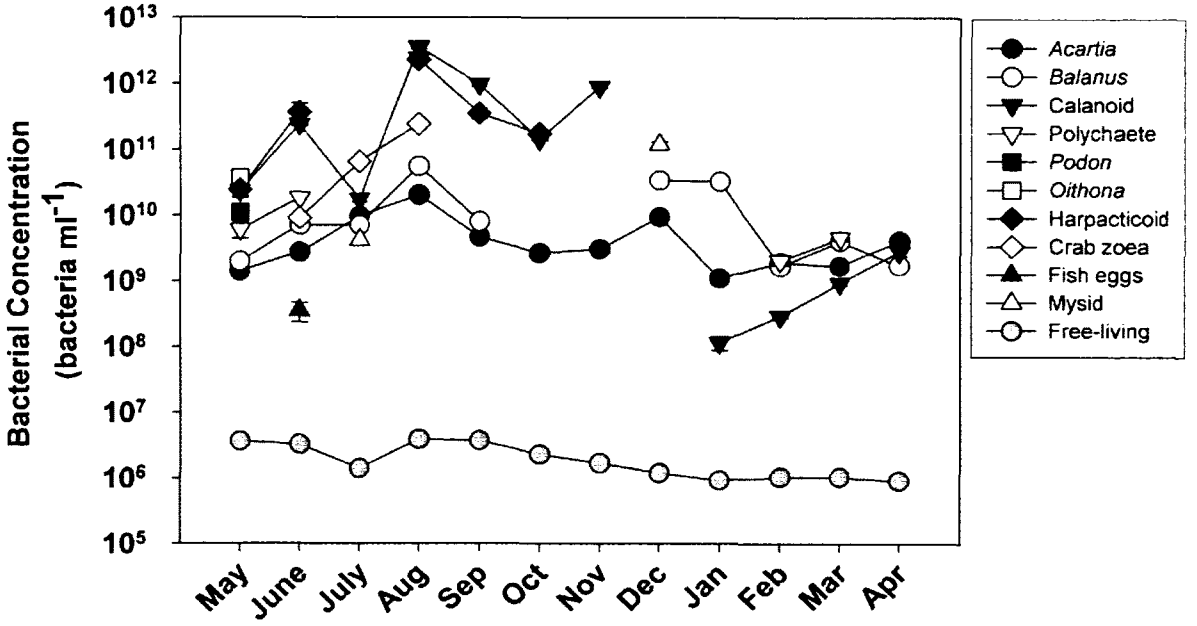

Bacterial Concentration (bacteria ml^{-1})
10^{13}
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
May
June
July
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
Acartia
Balanus
Calanoid
Polychaete
Podon
Oithona
Harpacticoid
Crab zoea
Fish eggs
Mysid
Free-living

Fig. 3 Linear relationships between environmental parameters and zooplankton-associated bacteria (left panels) and free-living bacteria (right panels). Symbols for zooplankton-associated bacteria are the same for all panels. Environmental parameters include temperature (a & b), salinity (c & d), ammonium (e & f), phosphate (g & h) and chlorophyll *a* (i & j)

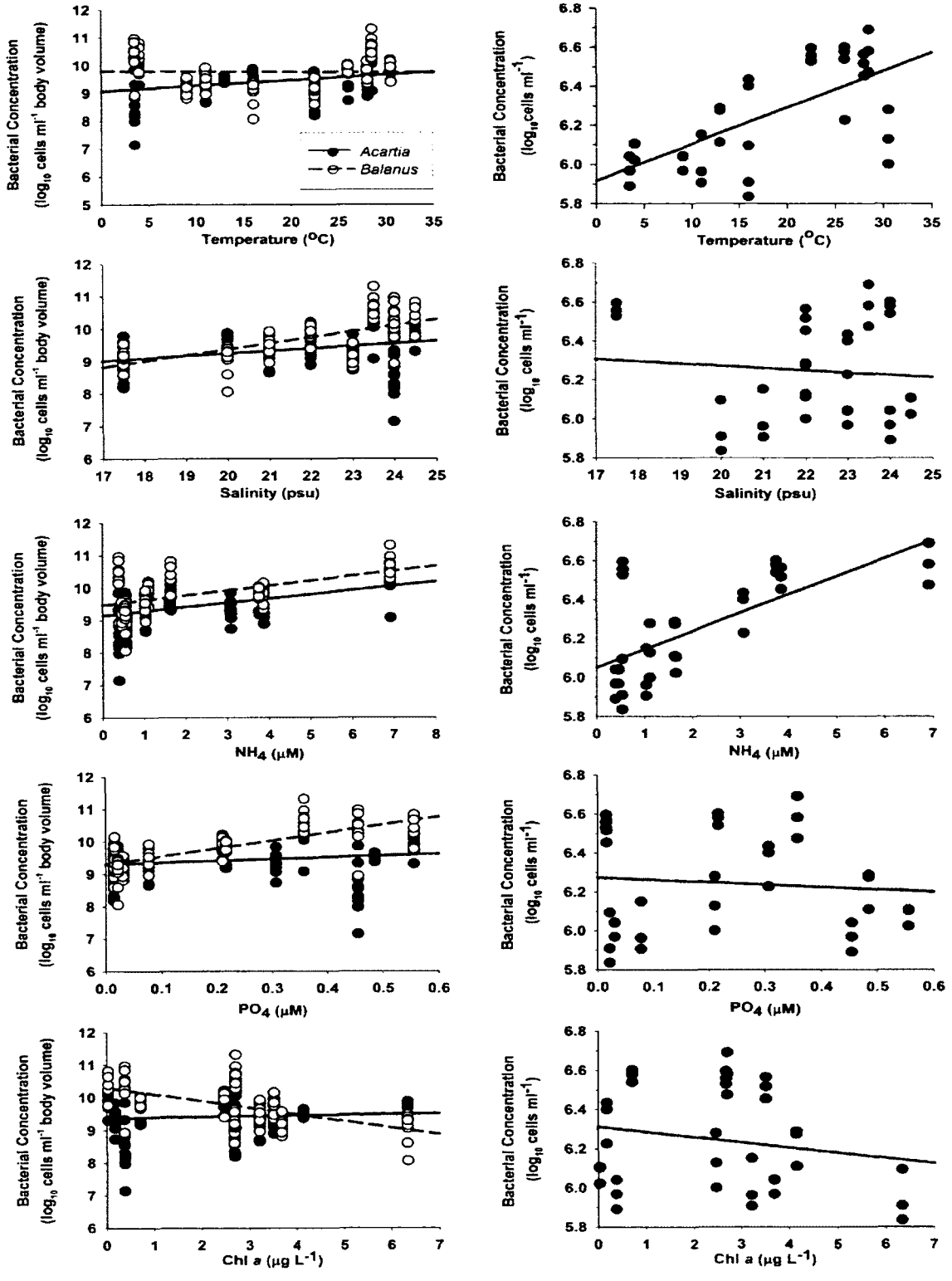

Bacterial Concentration (log10 cells ml-1 body volume)
Bacterial Concentration (log10 cells ml-1)
Acartia
Balanus
Temperature (°C)
Salinity (psu)
NH4 (µM)
PO4 (µM)
Chl a (µg L-1)

Fig. 4 Number of bacteria per individual zooplankter as a function of zooplankton body length (a), width (b), surface area (c), and body volume (d). Filled circles and solid lines represent *Acartia*-associated bacteria, open circles and dotted lines represent *Balanus*-associated bacteria

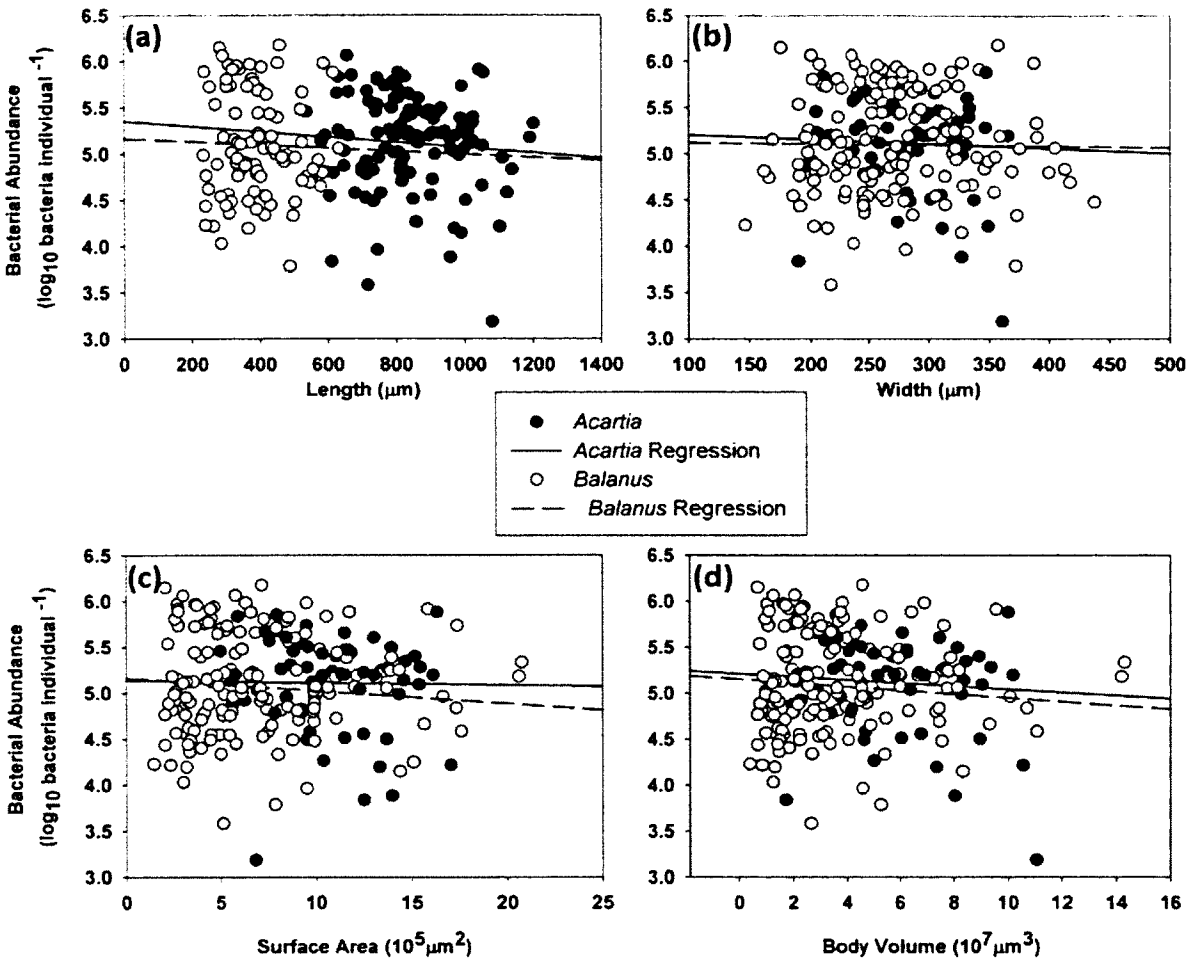
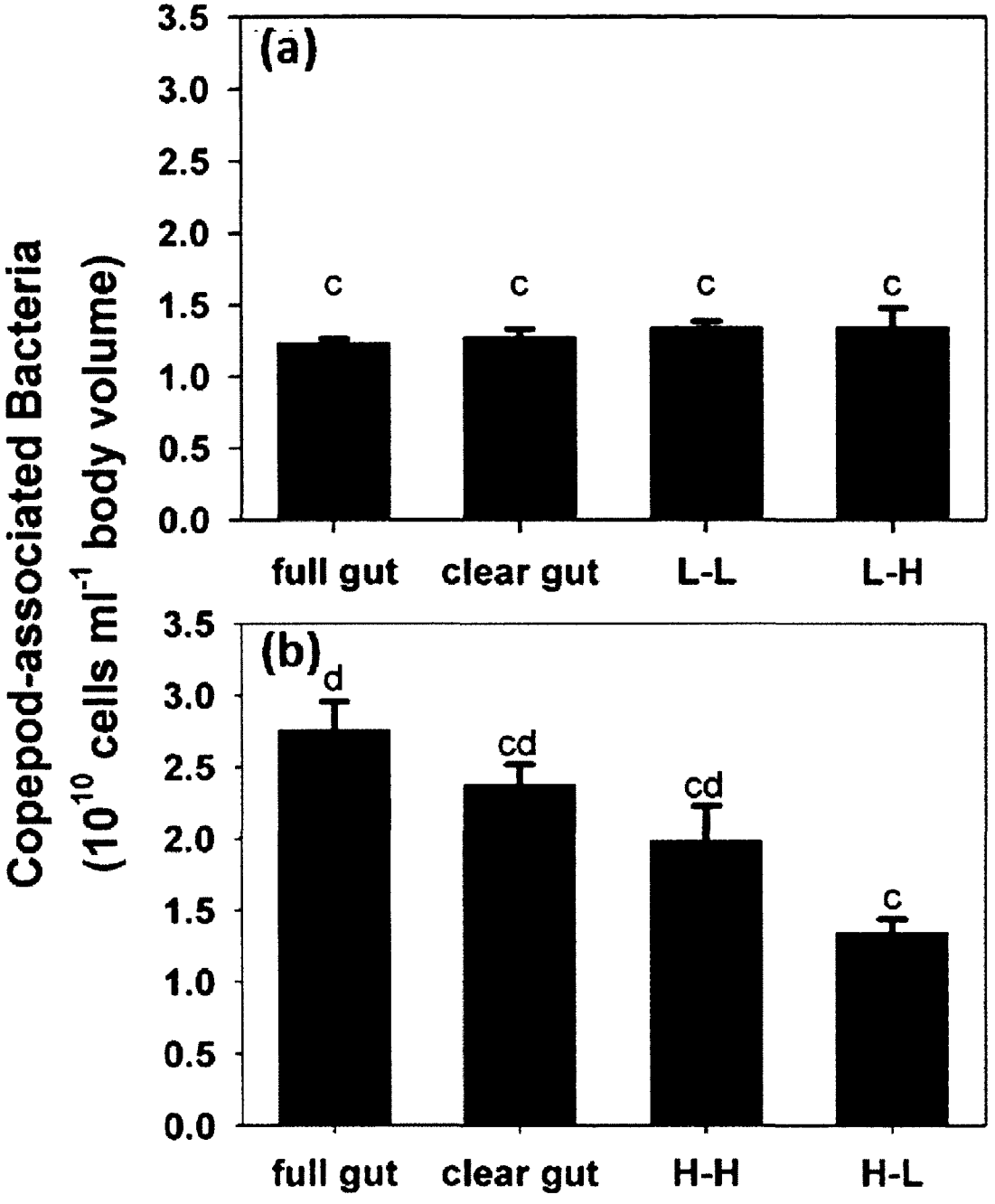

(a)
(b)
(c)
(d)
Bacterial Abundance (log10 bacteria individual-1)
Length (μm)
Width (μm)
Surface Area (10^5 μm^2)
Body Volume (10^7 μm^3)
Acartia
Acartia Regression
Balanus
Balanus Regression

Fig. 5 Bacterial concentrations (mean ± SE; n = 4) associated with copepods originally raised under low (panel a) or high (panel 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


Copepod-associated Bacteria
(10^10 cells ml^-1 body volume)
(a)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
c
c
c
c
full gut
clear gut
L-L
L-H
(b)
d
cd
cd
c
full gut
clear gut
H-H
H-L

CHAPTER 3

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

ABSTRACT

Zooplankton support distinct bacterial communities in high concentrations relative to the surrounding water, but little is known about how the genetic and functional diversities 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 DGGE of amplified 16S rDNA and by Biolog EcoPlates, respectively. Zooplankton-associated communities were genetically distinct from free-living bacterial communities and utilized a wider 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 (28-30% similarity), which suggests the importance of ambient environmental conditions in shaping resident zooplankton-associated bacterial communities. During winter months, cold temperatures and high ambient phosphate concentrations were linked to the appearance of a single DGGE band and the usage of amino acids as carbon substrates among zooplankton-associated bacterial communities. Monthly shifts in carbon substrate utilization were less extreme for zooplankton-associated bacteria than for free-living bacteria, suggesting that the zooplankton microhabitat is more consistent than the surrounding water and thus supports specific bacterial groups in the otherwise unfavorable conditions in the water column.

INTRODUCTION

Zooplankton represent dynamic microhabitats for bacteria within aquatic systems, often supporting bacterial concentrations 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 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. 2009, Freese & Schink 2011, Homonnay et al. 2012). The few studies that have investigated co-occurring zooplankton found each zooplankton group supported 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 shifts. 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 chlorophyll *a* concentration (Kan et al. 2006) and river discharge (Crump & Hobbie 2005). In their long-term study in lakes, Roesel et al. (2012) found consistent differences in re-occurring patterns of free-living and particle-associated bacteria. In particular, particle-associated bacteria were much more variable over time and often directly related to phytoplankton and zooplankton dynamics. Moreover, Fuhrman and colleagues (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, Grossart et al. 2010), zooplankton-associated bacterial communities may be directly or indirectly shaped by environmental conditions as well.

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, Tang et al. 2009), 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 also 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 genetic and functional diversities of bacterial communities associated with co-occurring 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 shifts. 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 & METHODS

Zooplankton Collection

Zooplankton were collected on a monthly basis from May 2010 to April 2011 at a fixed station in the York River, VA (37°14'50.36"N, 76°29' 58.03W), with a 0.5m mouth diameter, 200μm mesh net. All samples were collected at high or near high tide during daylight hours, and transported immediately back to the laboratory. In the lab, the sample was split into 4 equal fractions. Each fraction was gently concentrated onto a 200μm mesh sieve and transferred to sterile filtered Instant Ocean® artificial seawater (ASW) and 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, 3) zooplankton-associated bacterial abundance, and 4) zooplankton community composition. Subsamples 1, 2 and 4 will be discussed in this manuscript. Zooplankton-associated bacterial abundance and its influencing factors will be reported in detail elsewhere.

The sub-sample for zooplankton community composition was filtered onto a 200μm 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 and water temperature, salinity, chlorophyll *a*, ammonium, phosphate and free-living bacterial abundance, as well community composition, were determined. Salinity was measured with a refractometer and temperature was measured with a thermometer. Chlorophyll *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; Koroleff 1983). Phosphate concentrations were run in duplicate on a Lachat QuikChem 8500 autoanalyzer (detection limit 0.05 µmol/L; Parsons et al. 1984). Free-living bacterial abundance was counted in triplicate by DAPI direct counts (Porter & Feig 1980).

DNA extraction and DGGE

After gut clearance, zooplankton were gently concentrated onto a sterile 200µm mesh sieve and rinsed three 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 three 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. To assess the genetic composition of free-living bacteria, approximately 60ml of 5µm pre-filtered York River water was filtered on to a 0.2µm pore size polycarbonate membrane filter and stored at -40°C until

analysis. Zooplankton samples were centrifuged for two minutes at room temperature at 17,000 RCF 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. DNA was extracted using the phenol - chloroform - isoamylalcohol method with smoldered zirconia beads (Zhou et al. 1996). Extracted DNA was PCR amplified for DGGE using eubacterial primers 341f-gc with a 5' GC clamp (5' CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACG GGAGGCAGCAG 3') and 907r (5' CCGTCAATTCMTTTGAGTTT 3') (Muyzer & Ramsing 1995). Each 50µl PCR reaction contained 5µl 10X PCR buffer, 2.5µl 50mM $MgCl_2$, 5µl of 2.5mM dNTP, 10pmol of each primer, 0.5µl BSA, 0.5 µl red-Taq DNA polymerase (Bioline), 2-3µl of template DNA 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 Muyzer (1993). An average of 540 ng PCR product was loaded into each well of a 7% acrylamide gel with 40-70% denaturing gradients (formamide and urea). Gels were run at 100V for 18h then stained with 1X SYBR-gold for 30 min, destained with Milli-Q water and imaged on a UV light table. Due to the limited number of samples that can fit on one gel, only one replicate of each sample was run on the analyzed gel. Preliminary analyses indicated a high degree of similarity among replicate samples (average of 90.5% similarity), with the exception of all samples from June.

Carbon Substrate Utilization

Biolog EcoPlates were used to assess each bacterial community's ability to utilize a variety of carbon substrates. EcoPlates contain triplicate wells of 31 carbon substrates, and control wells with no substrate addition. 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). The carbon sources can be grouped into the larger biochemical categories of polymers, carbohydrates, carboxylic acids, amino acids, amines and phenolic compounds (Table 3, Choi & Dobbs 1999).

After they cleared their guts, zooplankton were concentrated onto a sterile sieve, rinsed and back-rinsed into a sterile petri dish as described previously, however, zooplankton were not narcotized. Twenty five to 35 individuals of each of the most abundant zooplankton groups were picked and transferred to sterile 15mL centrifuge tubes with 5mL of sterile filtered, autoclaved York River water. To assess the free-living bacterial community, 5ml of 5μm filtered York River water was added to a sterile 15-mL centrifuge tube. Each sample was sonicated for 40 second 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). Samples were brought to 15mL final volume with sterile filtered, autoclaved York River water and centrifuged for 10 minutes at 102 RCF to precipitate any zooplankton debris. The supernatant was gently pipetted into a sterile loading chamber and 150μl 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=590nm) of each well was measured

immediately with a BioTek EXL800 plate reader and after a 7-day incubation at 19°C in the dark. Final OD measurements were adjusted by subtracting control values and initial OD for each well. The average adjusted OD for each substrate (n=3) was compared to an arbitrary threshold of 0.250: A value greater than 0.250 indicated the substrate was used, and a value less than 0.250 indicated the substrate was not used (Lyons et al. 2010). 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 10^5 bacteria (Chapter 2) which led to an initial inoculum density of 10^5 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 and Lind (2006) demonstrated that inoculum density had no impact on average well color development after 72 hours of incubation.

Statistical Analyses

Cluster analysis of DGGE banding patterns was performed with GELCOMPARE 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 PRIMER 6 (PRIMER-E Ltd.), also using the unweighted pair group method with arithmetic averages. Pairwise similarity matrices were calculated for both DGGE banding patterns and carbon substrate utilization patterns using the Dice similarity index (Dice, 1945). Multidimensional Scaling (MDS) was performed in PRIMER 6 using the 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. 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 and free-living bacterial abundance. All CCA analyses were performed with the statistical software R.

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 PSU, with the exception of May 2010, when it was only 17.5 PSU (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 (0.56μmol 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).

Zooplankton community composition

The calanoid copepod *Acartia* sp. was present year-round and composed 23-99% of the zooplankton community (Table 2). The barnacle nauplius *Balanus* sp. was the second most common zooplankter, present in 10 of 12 months and composing up to 5-55% 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).

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, free-living bacteria supported a slightly more genetically diverse bacterial community with 5.27 DGGE bands per month while *Acartia*- and *Balanus*-associated bacteria 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. Within the free-living bacteria there were two main groups: a winter/spring group (December through April) and a summer/fall group (May through November). The

within-month similarities among zooplankton-associated bacterial communities ranged from 22% in June to 100% in February (Figure 2A), with an average monthly similarity of 65.4%. Lower similarities among BCC of zooplankton groups occurred when more DGGE bands were present, while identical BCCs occurred when only one DGGE band was present. There were no distinct seasonal groupings among the zooplankton-associated bacteria.

Bacterial Substrate Usage

Substrate usage was highest in November for free-living and *Acartia*-associated bacteria (12 and 20 substrates, respectively), in December for *Balanus*-associated bacteria (22 substrates) and in February for bacteria associated with polychaete larvae (21 substrates; Fig. 3). *Acartia*-associated bacteria used as few as 2 substrates in April; polychaete larvae-associated bacteria used 3 substrates in October and *Balanus*-associated bacteria used 5 substrates in October and February. Free-living bacteria showed the lowest substrate usage of all samples, utilizing only the carbohydrate D-mannitol in March (Fig. 3). Within each month, zooplankton-associated bacteria used more substrates than the free-living bacteria (Fig. 3). On an annual average, *Acartia*-associated bacteria used 9.3 substrates, *Balanus*-associated bacteria used 11.8 substrates, polychaete larvae-associated bacteria utilized 12.6 substrates and free-living bacteria only used 3.3 substrates. Free-living bacteria predominantly utilized carbohydrates and occasionally carboxylic acids and the polymer glycogen. In contrast, zooplankton-associated bacteria regularly utilized substrates from all of the biochemical categories except phenolic compounds (Table 3). The most commonly used substrates among all

samples were the polymer glycogen and the carbohydrates N-acetyl-D-glucosamine and D-cellobiose (Table 3). Carbon substrate utilization patterns of free-living bacteria from January, February and March were dissimilar from all other samples (<20% similar, Fig. 4A &B). Substrate usage by free-living and zooplankton-associated bacteria was 65% similar in November and identical between *Acartia*-associated and free-living bacteria in April. Substrate usage profiles for bacteria associated with different zooplankton groups were 30% to 80% similar within each month (Fig. 4). When both DGGE and substrate usage patterns were considered, both *Acartia*- and *Balanus*-associated bacteria had fewer DGGE bands but used a higher number of substrates than free-living bacteria.

Relationship with Environmental Factors

Environmental parameters accounted for 33.9% of genetic variability in all zooplankton-associated bacteria (Fig. 5A). The first two canonical axes accounted for 10.3% and 9.4 % of the variability, respectively. Three DGGE bands (18, 21 and 26) were related to high salinity, high phosphate and low temperatures. Many of the bands were not related to measured environmental parameters. A higher proportion of the variation (56.3%) among *Acartia*-associated bacteria (Fig. 5B) was explained by environmental conditions. Again, bands 21 and 26 were linked to high salinity, high phosphate and low temperatures, and a number of bands were not explained by any environmental variables. Among the free-living bacteria (Fig. 5C), environmental parameters accounted for 64.2% of the variability in the bacterial community composition, with the first and second axes contributing 25.9% and 12.7%, respectively. Five DGGE bands (bands 12-14, 21 and 23) were linked to high salinity and free-living

bacterial abundance. Another group of bacteria (bands 4, 5, 20 and 36) was linked to high chl *a* concentrations.

Environmental parameters explained only 31.7% of variability in substrate usage among all zooplankton-associated bacteria (Fig. 6A). The first two canonical axes accounted for 8.4% and 6.7% respectively. Use of the carbohydrate D-xylose and the amine phenylethylamine were linked to high chlorophyll *a* concentrations, while use of the carboxylic acid D-galacturonic acid was linked to high phosphate levels. When only *Acartia*-associated bacteria were examined (Fig. 6B), 77.4% of variation in substrate usage was explained by environmental conditions, with the first two axes accounting for 29.2% and 18.8% respectively. Usage of the amino acid, L-asparagine was tied to high chlorophyll concentrations, while utilization of i-erythritol, D-L-α glycerol phosphate and putrescine were related to high temperatures and free-living bacterial abundances. Use of amino acids L-arginine, L-serine, L-threonine, as well as the carboxylic acid itaconic acid, was linked to higher phosphate concentrations and to a lesser extent ammonium concentrations. Environmental conditions explained all variations (100%) among substrate usage by free-living bacteria (Fig. 6C). Usage of N-acetyl-D-glucosamine was linked to higher salinity, while the usage of the glycogen and D-cellobiose was linked to higher temperatures.

DISCUSSION

Comparison of zooplankton-associated bacterial communities

The genetic similarity of BCCs associated with co-occurring zooplankton groups ranged from 22% to 100% within each month (Fig. 2A), with an annual average similarity of 65.4%, indicating more similarities than differences among the BCCs of different zooplankton groups. Previous DGGE analyses of zooplankton-associated bacteria have shown distinct banding patterns by the bacterial communities associated with the calanoid copepods *Acartia* sp. and *Temora* sp. collected from the North Sea (Brandt et al. 2010). Likewise, copepods and cladocerans from the same freshwater lake supported different bacterial communities (Grossart et al. 2009), as did five different crustaceans from a rice paddy field (Niswati et al. 2005). However, none of these studies quantified the level of similarity or dissimilarity among bacterial communities. These differences in BCC of co-occurring zooplankton suggest that zooplankton-specific characteristics may still influence the associated bacterial community composition. Although the initial source of zooplankton-associated bacteria is not known, it is likely that zooplankton exoskeletons are colonized by free-living bacteria (Grossart et al. 2010), whereas food-associated bacteria may be selectively retained within the gut (Harris 1993). Thus, during the exchange between free-living and zooplankton associated communities (Grossart & Tang 2010), 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. Within this study, free-living and zooplankton-associated bacterial communities were less than 10% similar within each month, which suggests that food-associated bacteria may be more important

for seeding the zooplankton-associated bacterial community. A previous study showed the bacterial community composition of *Acartia* with full guts is influenced by type of food source and whether or not the food carries bacteria (Tang et al. 2009). *Acartia* collected directly from the North Sea supported a different bacterial community than those collected from the field and cultured in the lab on a fixed diet (Brandt et al. 2010). The types of phytoplankton available for consumption by zooplankton in the York River vary throughout the year (Sin et al. 2000), which in turn may elicit changes in the zooplankton-associated bacterial communities via selective retention of food-associated bacteria. It remains to be investigated if differences in the BCC of *Acartia*, *Balanus* and other co-occurring zooplankters are attributable to their different dietary preferences.

Temporal shifts in zooplankton-associated bacteria

To our knowledge, this is the first study to document changes in the zooplankton-associated bacterial community over a period longer than 3 months. Because there were large monthly changes in the BCC of each zooplankton group, bacterial communities of different zooplankters were more similar within each month than BCC of one zooplankton group over time. For example, the average genetic similarity among BCCs of different zooplankton groups within each month was 65.4%, but across all months the genetic similarity of *Acartia*-associated BCCs was only 30.8% and *Balanus*-associated BCCs was only 28.4%. The large monthly shifts in composition and functionality 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 shifts in bacterial community composition were accompanied by shifts in the bacterial carbon substrate usage. The month to month changes in substrate utilization patterns were less extreme than metrics of genetic change, with 51, 53 and 49% similarity among months for *Acartia* and *Balanus* and polychaete larvae-associated bacteria, respectively (Fig. 4A). In contrast, substrate usage profiles by free-living bacterial communities were only 21% similar from month to month (Fig. 4A). Free-living bacteria in the York River previously showed distinct shifts in the carbon substrate usage patterns between winter/spring and summer/fall bacterial communities (Schultz & Ducklow 2000). Seasonal changes in substrate utilization by free-living bacteria were much less pronounced in eutrophic Mediterranean harbors with stable nutrient and dissolved organic carbon (DOC) inputs than in oligotrophic regions (Sala et al. 2006). While environmental conditions within the York River varied throughout the study period (Table 1), nutrient and DOC availability within the zooplankton microenvironment were likely much more consistent due to constant supply of nutrients and DOC via ingestion, excretion and sloppy feeding (Møller 2005, Møller et al. 2007).

The most commonly used substrates were responsible for the similarities in substrate usage through time. Glycogen was utilized in 92% of all zooplankton-associated bacteria samples and is commonly used by bacteria for carbon storage which allows for energy production and biosynthesis during long periods in the absence of nutrients. D-cellobiose was consumed in 85% of all zooplankton-associated samples. D-cellobiose is an intermediate product during the breakdown of cellulose, one of the earth's most

abundant biopolymers. McCallister and colleagues (2004) noted that marsh-derived organic matter supported up to 29% of bacterial biomass production in the mesohaline York River. Additionally, bacteria can preferentially utilize cellulose from the saltmarsh plant *Spartina alterniflora* (Coffin et al. 1990), which is prevalent in the York River near our sampling site (Perry & Atkinson 2008). The carbohydrate N-acetyl-D-glucosamine was also used in 85% of zooplankton-associated samples. N-acetyl-D-glucosamine is the structural monomer of chitin and is used in the formation of peptidoglycan in bacterial cell walls. In aquatic systems amino sugars can be present on the same order of magnitude as amino acids (Nedoma et al. 1994) and autoradiography indicated that N-acetyl-glucosamine was used by bacteria in all freshwater systems studied (Nedoma et al. 1994). Additionally, 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, especially *Vibrionaceae* (Riemann & Azam 2002), which can comprise up to 27% of all bacteria associated with bulk zooplankton (Heidelberg et al. 2002).

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 (Siedler 1991), which is then quantified spectrophotometrically. Therefore, unless the substrate is broken down to fuel cellular respiration, a color change will not occur.

Zooplankton-associated bacteria utilized more of the available amino acids in November (75% of available amino acids), December (44%) and January (38%) than in all other months (17-33% utilization). This increase was largely due to the utilization of L-phenylalanine, L-serine and L-threonine. Amino acids can be an important source of carbon and nitrogen for heterotrophic bacteria (e.g. Wheeler & Kirchman 1986), in fact, uptake of dissolved free amino acids accounted for 28% and 80.6% of bacterial carbon and nitrogen production during February in Chesapeake Bay, respectively (Fuhrman 1990). It is likely that bacteria associated with the surface of the zooplankton are more tightly linked to zooplankton-produced substrates in the winter when ammonium concentration in the surrounding water is low (Table 1).

Environmental influence on bacterial communities

Temporal differences in BCC associated with a particular zooplankter were greater than differences in BCC of co-occurring zooplankton groups (Fig. 2), suggesting that ambient environmental conditions are a stronger selective force on zooplankton-associated bacteria than zooplankton-specific selective forces. Likewise, Kan and colleagues (2007) noted a similar bacterial composition throughout Chesapeake Bay at any given point in time. However, large seasonal changes in the bacterial community indicated that environmental conditions with strong seasonality played a larger role in shaping the microbial community than any regional dynamics. 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, Kan et al. 2006, Fuhrman et al. 2006, Roesel 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, Roesel et al. 2012) and even grazing pressure (Muylaert et al. 2002). Environmental parameters may act directly on the zooplankton-associated communities, shaping them in a comparable manner as 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.

Certain environmental parameters were associated with specific DGGE bands and substrate usage, most notably DGGE band 26, present only in zooplankton samples collected in November – February, and a major component of the genetic composition during these months. Despite the low numbers of DGGE bands during these winter months, bacterial abundances associated with zooplankton were as high as in summer (unpubl. data). CCA results indicated that Band 26 was linked to phosphate concentrations (Fig. 5A&B), which were highest from November - January (Table 1). A decreased diversity of attached bacteria was previously noted in replete phosphorus conditions. For example, the genetic diversity of bacteria associated with the green alga *Scenedesmus obliquus* was lower when grown in high phosphorus conditions than when nitrogen and phosphorus were limiting (Leflaive et al. 2008). In aquatic systems, low species richness is commonly observed when resources are in excess, especially among phytoplankton, zooplankton and macrophytes (Dodson et al. 2000). Therefore, the decreased species richness associated with excess resources is likely due to a strong response by one species which out-competes others (Mittelbach et al. 2001).

As with DGGE Band 26, amino acids L-arginine, L-serine and L-threonine were also linked to elevated phosphate levels; they were used predominantly during the months when Band 26 was present. Zooplankton excreta and sloppy feeding produce dissolved amino acids which were important bacterial substrates during late winter in Lake Constance (Rosenstock & Simon 2001) and in Chesapeake Bay (Fuhrman 1990). As ambient ammonium concentrations reached their absolute lowest levels during January (Table 1), the attached bacteria may have utilized amino acids as their primary nitrogen source (Middelboe et al. 1995). This suggests that DGGE band 26 represents a zooplankton specific, cold-adapted bacterium that thrives under higher phosphate conditions and potentially uses amino acids as the primary nitrogen and carbon source.

Environmental parameters accounted for 33.9% and 56.3% of the variation in bacterial community composition associated with all zooplankton and *Acartia*, respectively, which are within the range of previous studies of free-living bacteria. Environmental parameters explained 12.7 – 27.5% of variation in free-living bacterial community structure in 4 shallow, eutrophic lakes (Muylaert et al. 2002), 32% of bacterial community variation in 31 British Columbian lakes (Longmuir et al. 2007) and 100% of variation in 5 Swedish lakes of varying eutrophic status (Lindström 2000).

Zooplankton-associated vs. free-living bacteria

Multidimensional scaling of DGGE results showed a large difference between zooplankton-associated and free-living bacterial communities (Fig. 2), supporting the idea that zooplankton create a unique microhabitat supporting a bacterial community different from that in the surrounding water (Grossart and 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).

Month to month substrate usage was more stable among zooplankton-associated bacteria (49-53% similarity over 12 months, Fig. 4) than for free-living bacteria (21% similarity over time). In the Mediterranean Sea, oligotrophic regions that received sporadic inputs of dissolved organic carbon (DOC) having variable composition exhibited larger seasonal shifts in substrate usage than eutrophic harbors that received a consistent supply of DOC of relatively stable composition (Sala et al. 2006). The authors hypothesized that the stable DOC supply allowed a stable bacterial community to establish. 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 (Grossart & Tang 2010).

The lower number of DGGE bands and higher 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 macroalgae *Ulva australis* was

described by the competitive lottery model (Burke et al. 2011), where a number of bacterial species with the same functional capacity were present within a source community. The specific niches in the *Ulva* ecosystem were assigned randomly, filled by whichever species from the source community colonized the *Ulva* first (Burke et al. 2011). Viewing zooplankton in the same manner, functional niches on zooplankton would be filled by a subset of a more genetically diverse free-living bacterial community. Within the York River samples, 15 substrates were utilized by zooplankton-associated but not by free-living bacteria (Table 3). 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 or 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 unique microenvironments within their guts and on their external surfaces which may allow certain bacterial groups to flourish, increasing their overall presence and importance within an aquatic system. It is known that zooplankton-associated bacteria have higher production rates than free-living bacteria (Carman 1994, Møller et al. 2007), and our results indicate that zooplankton-associated bacteria may utilize a wider variety of substrates than free-living ones. 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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Table 1. Water quality measurements and free-living bacterial abundances for the York River during the field study. BLD = below level of detection

Month	Water Temperature (°C)	Salinity (PSU)	Ammonium ($\mu mol\ L^{-1}$)	Phosphate ($\mu mol\ L^{-1}$)	Chl *a* ($\mu g\ L^{-1}$)	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

Table 2. Zooplankton community composition during the field study.

		May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr
% total zooplankton	***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
	Harpacticoid	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

Table 3. Monthly carbon substrate utilization by zooplankton-associated and free-living bacteria of the York River, Chesapeake Bay. Black squares indicate the substrate was used. Carbon substrates are grouped according to their biochemical category. Substrate numbers correspond to numbers used in CCA analyses of the EcoPlate data (Figs. 7 & 8). Not all zooplankton groups were present in all month. Within substrates: P.C. = phenolic compounds. For sample names: A = *Acartia*, B = *Balanus*, Po= Polychaete larvae, Ps = *Pseudodiaptomus*, C = Crab zoea, M = Mysid, FL = Free Living. AUG = August, SEP = September, OCT = October, NOV = November, DEC = December, JAN = January, FEB = February, MAR = March, APR = April.

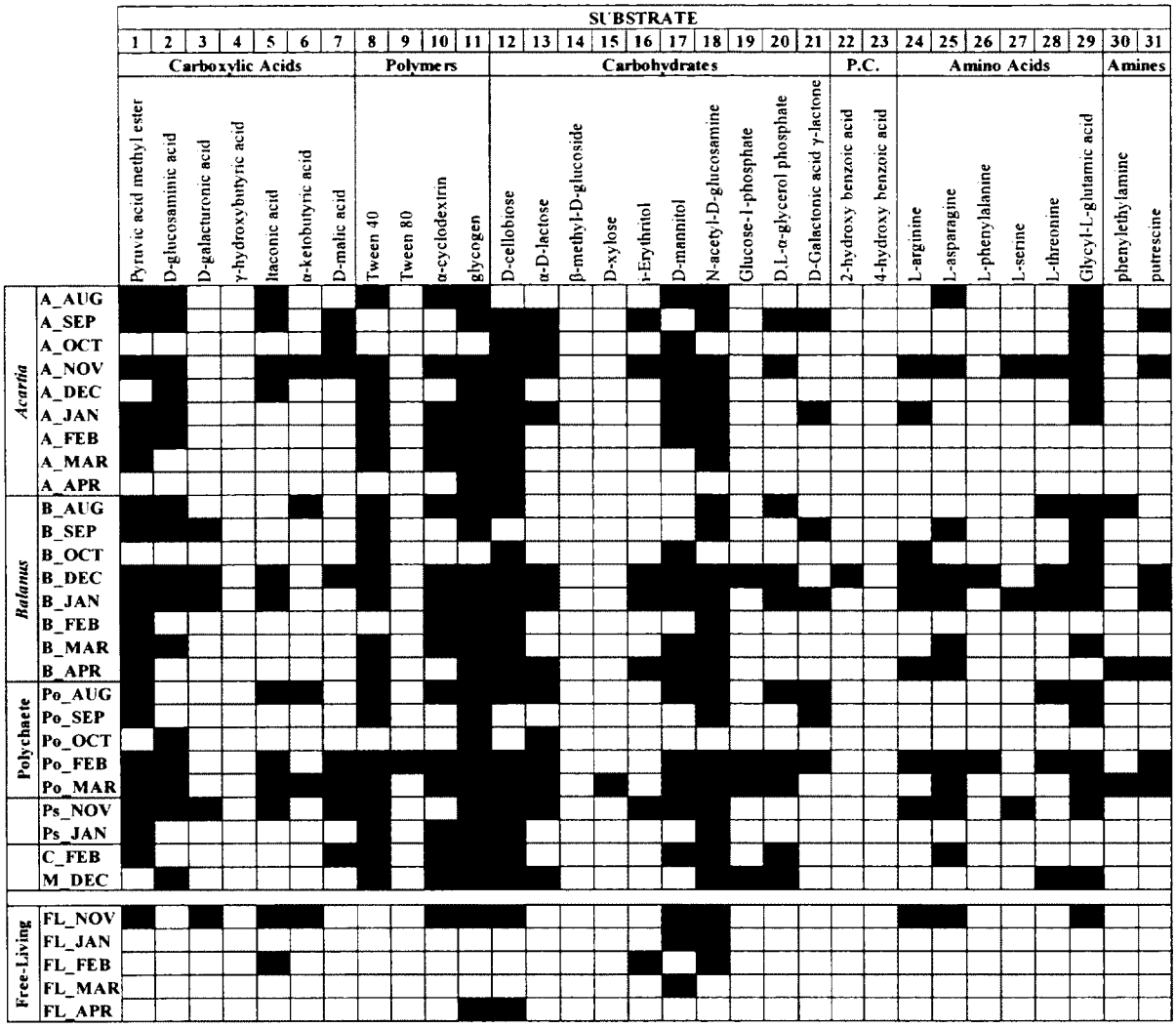

SUBSTRATE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
Carboxylic Acids
Polymers
Carbohydrates
P.C.
Amino Acids
Amines
Pyruvic acid methyl ester
D-glucosaminic acid
D-galacturonic acid
γ-hydroxybutyric acid
Itaconic acid
α-ketobutyric acid
D-malic acid
Tween 40
Tween 80
α-cyclodextrin
glycogen
D-cellobiose
α-D-lactose
β-methyl-D-glucoside
D-xylose
i-Erythritol
D-mannitol
N-acetyl-D-glucosamine
Glucose-1-phosphate
D,L-α-glycerol phosphate
D-Galactonic acid γ-lactone
2-hydroxy benzoic acid
4-hydroxy benzoic acid
L-arginine
L-asparagine
L-phenylalanine
L-serine
L-threonine
Glycyl-L-glutamic acid
phenylethylamine
putrescine
Acartia
A_AUG
A_SEP
A_OCT
A_NOV
A_DEC
A_JAN
A_FEB
A_MAR
A_APR
Balanus
B_AUG
B_SEP
B_OCT
B_DEC
B_JAN
B_FEB
B_MAR
B_APR
Polychaete
Po_AUG
Po_SEP
Po_OCT
Po_FEB
Po_MAR
Ps_NOV
Ps_JAN
C_FEB
M_DEC
Free-Living
FL_NOV
FL_JAN
FL_FEB
FL_MAR
FL_APR

Fig. 1. Total number of DGGE bands present in monthly samples from zooplankton-associated and free-living bacterial communities. Figure key: Filled circles = *Acartia*; open circles = *Balanus*; Filled triangles = Free-living bacteria.

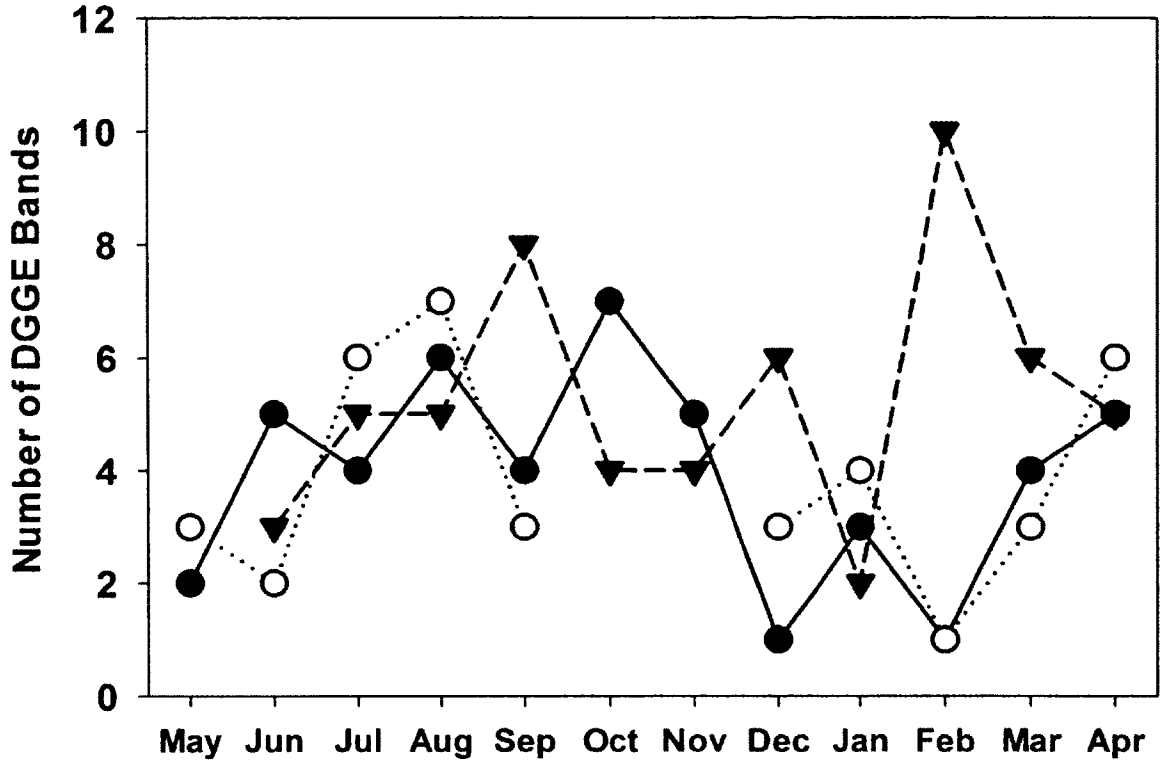

Number of DGGE Bands
12
10
8
6
4
2
0
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr

Fig. 2. UPGMA dendrograms of similarities among DGGE banding patterns for zooplankton-associated and free-living bacterial communities (A). MDS plot for DGGE banding patterns of zooplankton-associated and free-living bacteria (B). Stress = 0.1. Figure key: Filled circles = *Acartia*; open circles = *Balanus*; Filled triangles = free-living bacteria; open triangles = Crab zoea; filled squares = *Pseudodiaptomus*; open squares = Mysid

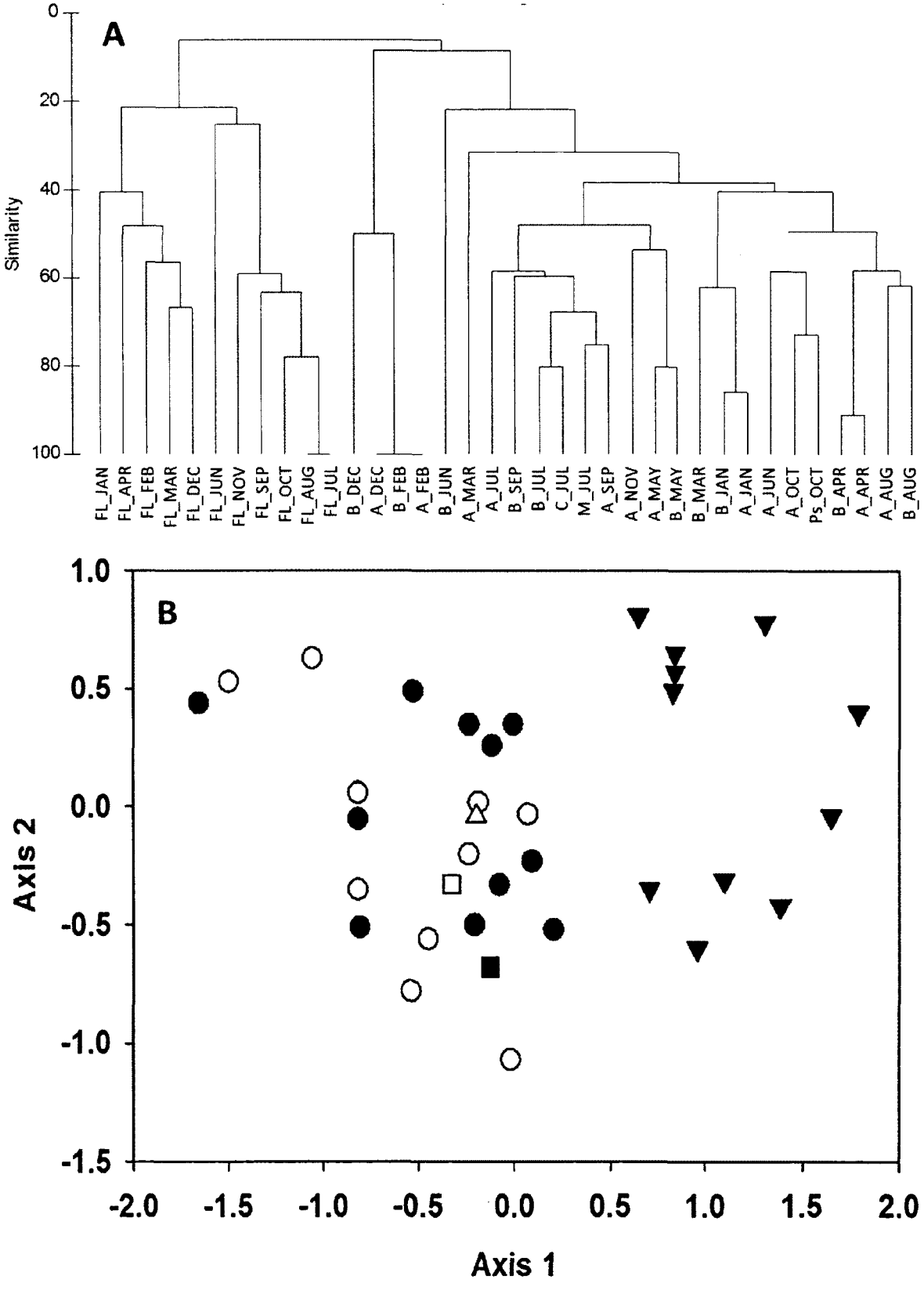

A
Similarity
0
20
40
60
80
100
FL_JAN
FL_APR
FL_FEB
FL_MAR
FL_DEC
FL_JUN
FL_NOV
FL_SEP
FL_OCT
FL_AUG
FL_JUL
B_DEC
A_DEC
B_FEB
A_FEB
B_JUN
A_MAR
A_JUL
B_SEP
B_JUL
C_JUL
M_JUL
A_SEP
A_NOV
A_MAY
B_MAY
B_MAR
B_JAN
A_JAN
A_JUN
A_OCT
Ps_OCT
B_APR
A_APR
A_AUG
B_AUG
B
Axis 2
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
Axis 1

Fig. 3. Total number of carbon substrates utilized by zooplankton-associated and free-living bacteria communities. No samples were collected in May, June or July. Figure key: Filled circles = *Acartia*; open circles = *Balanus*; Filled triangles = Free-living bacteria; filled squares = *Pseudodiaptomus*; filled diamond = polychaete larvae

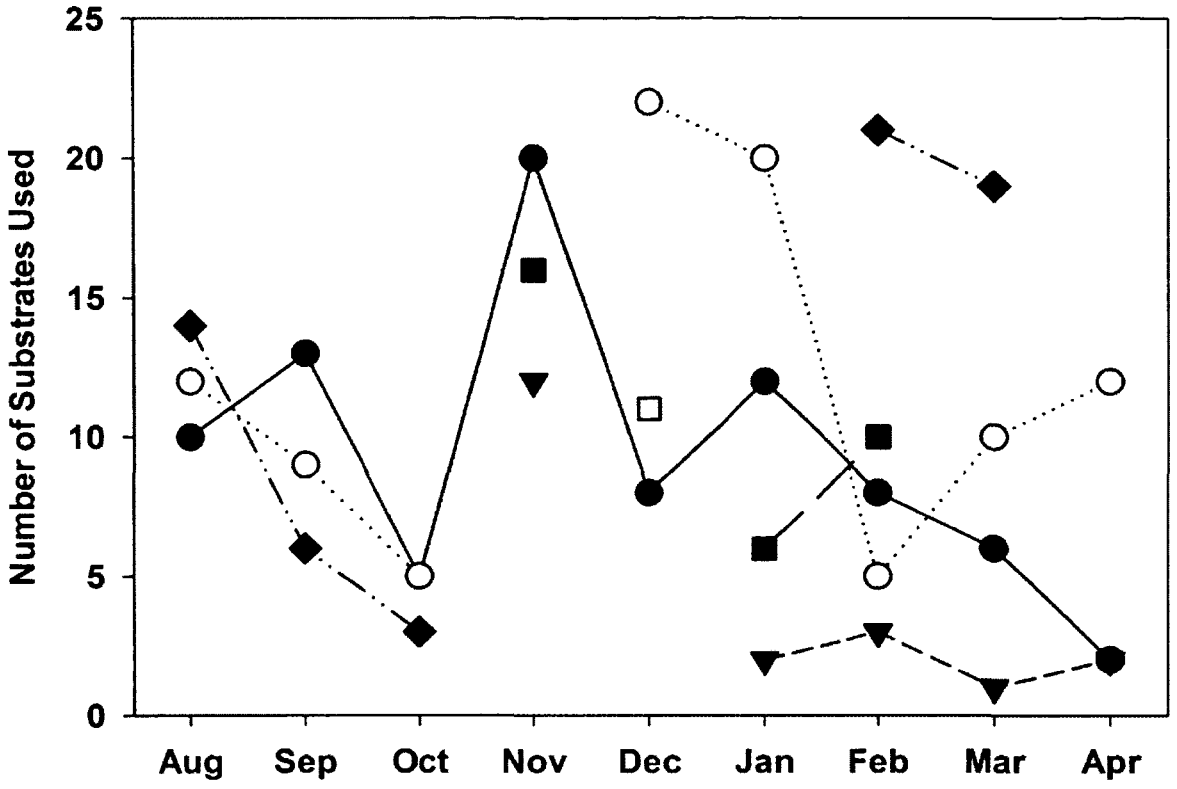
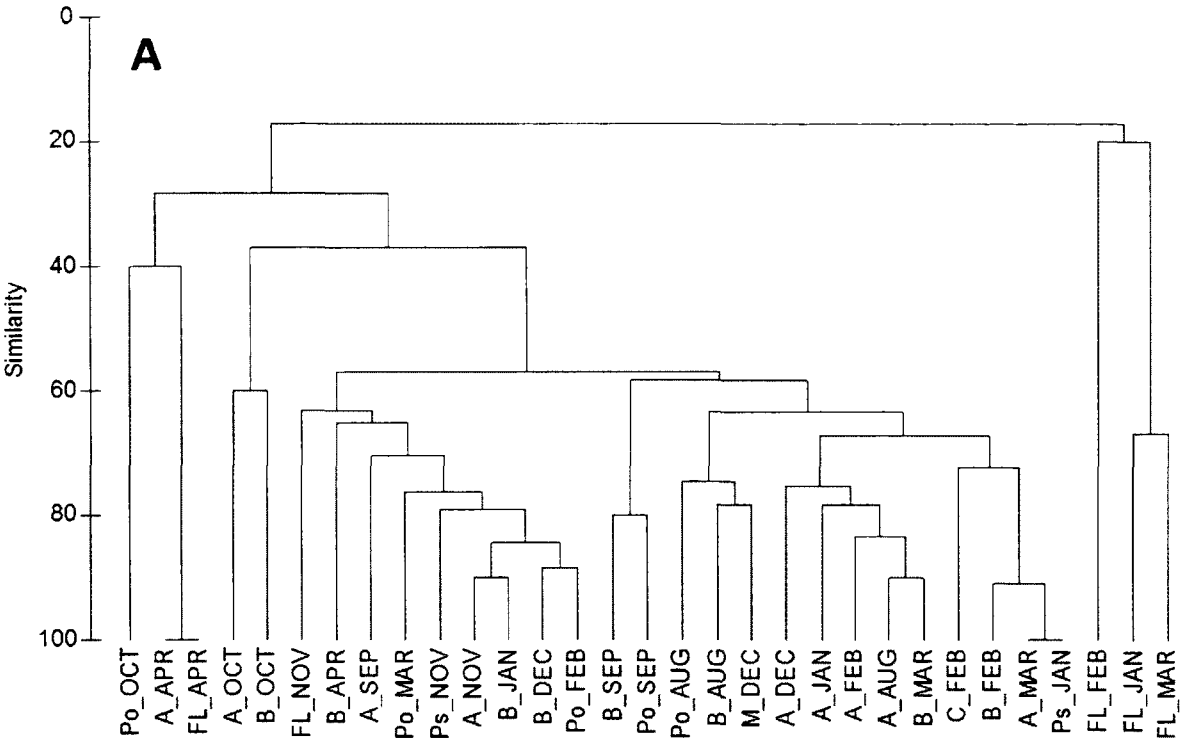

Number of Substrates Used
25
20
15
10
5
0
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr

Fig. 4. UPGMA dendrograms of carbon substrate usage profiles for zooplankton-associated and free-living bacterial communities (A). MDS plot of carbon substrate usage profiles for and zooplankton-associated and free-living bacteria collected from the York River (B). Stress = 0.14. Filled circles = *Acartia*; Unfilled circles = *Balanus*; Filled triangles = Free-living; Unfilled triangles = crab zoea; filled squares = *Pseudodiaptomus*; open squares = Mysid; Filled diamonds = polychaete larvae


A
Similarity
0
20
40
60
80
100
Po_OCT
A_APR
FL_APR
A_OCT
B_OCT
FL_NOV
B_APR
A_SEP
Po_MAR
Ps_NOV
A_NOV
B_JAN
B_DEC
Po_FEB
B_SEP
Po_SEP
Po_AUG
B_AUG
M_DEC
A_DEC
A_JAN
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A_MAR
Ps_JAN
FL_FEB
FL_JAN
FL_MAR

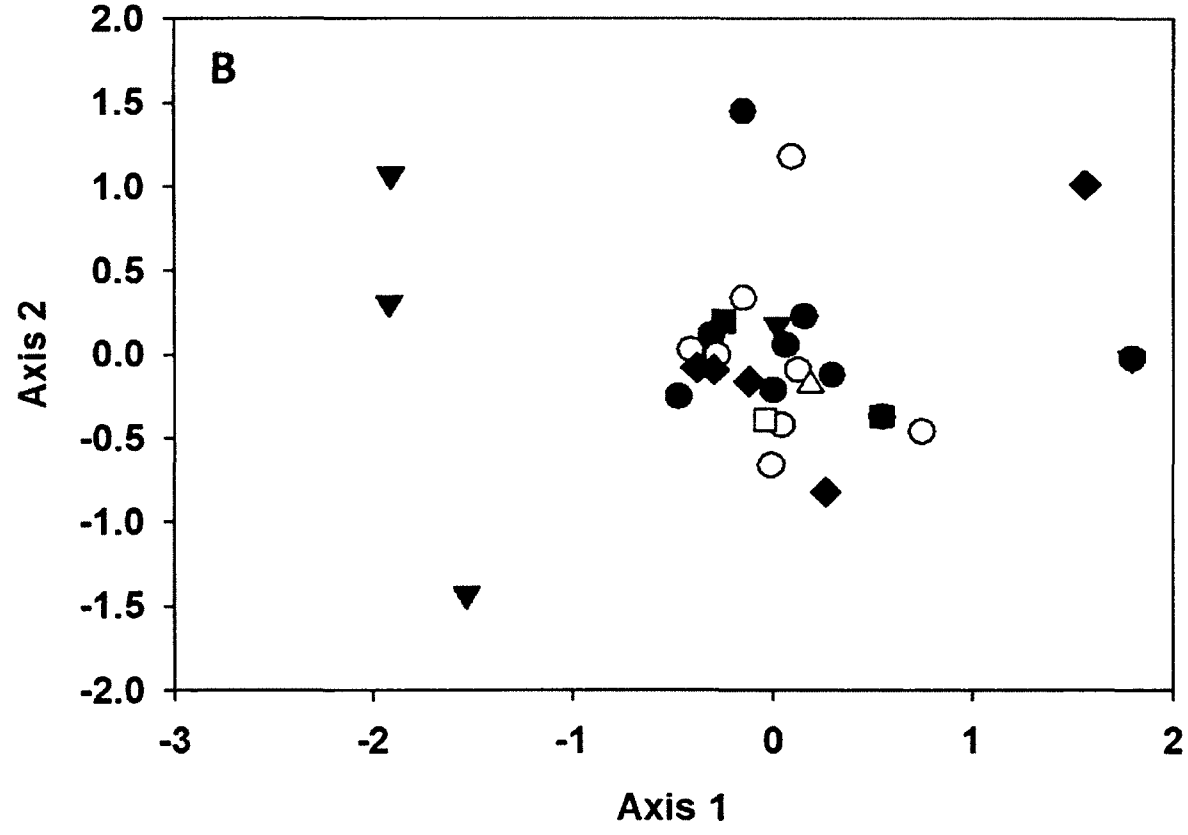

B
Axis 2
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-3
-2
-1
0
1
2
Axis 1

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, Sal = Salinity, Chl = Chlorophyll *a*, PO4 = Phosphate, NH4 = Ammonium, Temp = Temperature.

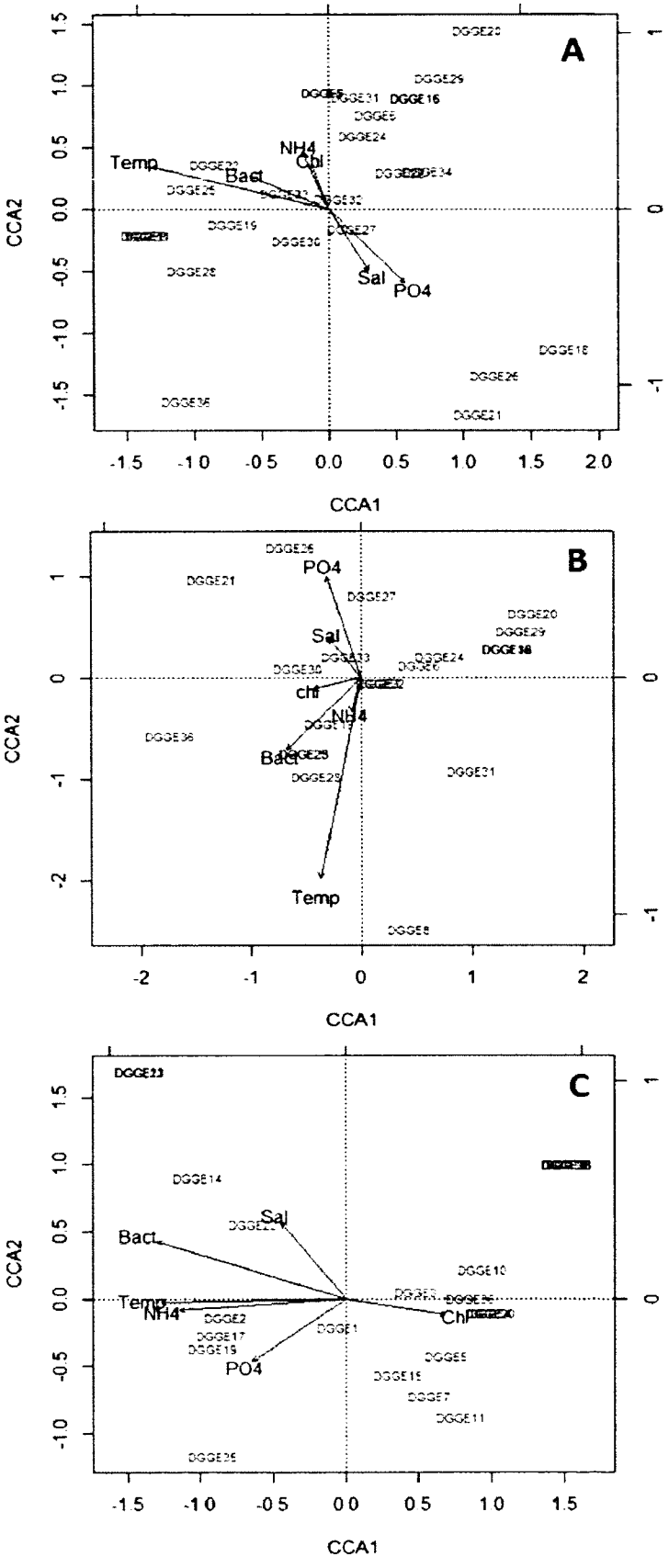
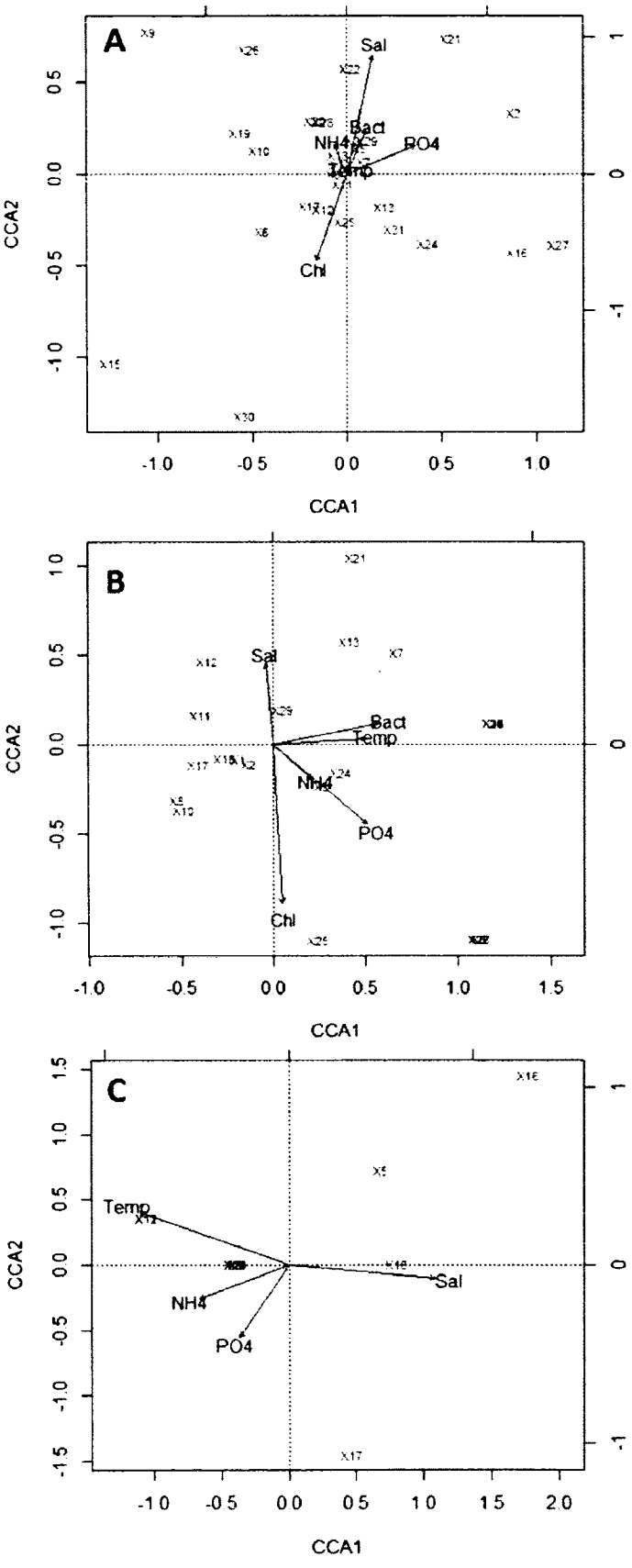

A
Temp
Bact
NH4
Chl
Sal
PO4
CCA1
CCA2
B
PO4
Sal
chl
NH4
Bact
Temp
CCA1
CCA2
C
Sal
Bact
Temp
NH4
Chl
PO4
CCA1
CCA2

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 X1 through X31 as defined in Table 3. Bact = Free-living bacterial abundance, Sal = Salinity, Chl = Chlorophyll *a*, PO4 = Phosphate, NH4 = Ammonium, Temp = Temperature.


A
Sal
Bact
NH4
PO4
Temp
Chl
CCA1
CCA2
B
Sal
Bact
Temp
NH4
PO4
Chl
CCA1
CCA2
C
Temp
Sal
NH4
PO4
CCA1
CCA2

CHAPTER 4

Carbon substrate usage by aerobic and facultative anaerobic bacteria associated with estuarine zooplankton

ABSTRACT

Previous studies which documented the occurrence of anaerobic bacteria in association with zooplankton assumed the anaerobic bacteria reside within the anoxic zooplankton guts. In this study we examined the carbon substrate usage patterns of the entire bacterial community and gut bacterial community of the calanoid copepod *Acartia tonsa* under aerobic and anaerobic conditions. The gut microbial community utilized the same number of substrates as the entire microbial community under aerobic (11 substrates) and anaerobic (13 substrates) conditions. Different substrate subsets were utilized under aerobic and anaerobic conditions, but gut bacteria and the total bacteria utilized very similar substrate subsets in each aerobic condition, suggesting that gut bacteria were responsible for a large portion of the heterotrophic microbial activity associated with the copepod. Aerobic and anaerobic carbon substrate usage patterns were also assessed for bacterial communities associated with six common zooplankton groups and free-living bacteria within the York River Estuary. Free-living bacteria used more substrates than each zooplankton-associated bacterial community under both aerobic and anaerobic conditions. More combinations of substrate subsets were used by the cumulative zooplankton-associated bacteria under anaerobic conditions. These results suggest each zooplankton group selects for a specific combination of bacteria such that the number and type of substrates used by the entire zooplankton-associated bacterial community are regulated by the composition of the zooplankton community.

INTRODUCTION

Zooplankton provide a nutrient rich microhabitat for associated bacteria which can be orders of magnitude more concentrated than in the surrounding water (Tang et al. 2010). In addition to colonizing a zooplankter's external surfaces, aquatic bacteria can utilize zooplankton guts as microhabitats. Zooplankton guts may provide even more concentrated nutrients and different pH and oxic conditions than external surfaces and the surrounding water column (Tang et al. 2011). These strong selective forces may play an important role in shaping the enteric microbial composition and function.

While a number of studies have detailed the presence of both external and gut bacteria (e.g. Huq et al. 1983, Nagasawa 1992) and shown that zooplankton-associated bacteria are more metabolically active than free-living bacteria (Carman 1994, Møller et al. 2007), differentiation between epibiotic bacteria and gut bacteria is difficult. Consequently very few studies have addressed the differences in the functional roles between external and gut microbial communities. Donachie & Zdanowski (1998) suggested that bacteria provide a digestive function in the gut of the euphausiid *Euphausia superba*. Other studies indicate that gut bacteria may play an important role in nutrient cycling, especially with regard to anaerobic processes (Bianchi et al. 1992, Marty 1993, de Angelis & Lee 1994, Stief et al. 2009). Although zooplankton typically reside in the aerobic water column, multiple studies have demonstrated the occurrence of anaerobic microbial processes in association with zooplankton materials. Incubations of fecal pellets and copepods with empty guts collected from the Mediterranean Sea produced low but measurable rates of methanogenesis (Bianchi et al. 1992, Marty 1993). Also, methane production was noted in association with live copepods fed radiolabeled

phytoplankton at levels up to 20.4 pmol d^{-1} $copepod^{-1}$ (de Angelis & Lee 1994), demonstrating the potential for copepods to support anaerobic methanogenesis in an oxygenated water column. Nitrogen-fixing purple sulfur bacteria were found in anaerobic incubations of copepods with full or empty guts, but not with fecal pellets; concurrent observations of acetylene reduction confirmed the occurrence of nitrogen fixation only in the incubations with copepods (Proctor 1997). This suggests that the N-fixing bacteria were resident gut bacteria and were not packaged into fecal pellets and egested (Proctor 1997). The presence of other anaerobes such as sulfate reducers and fermentative bacteria in association with copepods has also been mentioned (Proctor 1997). In all previous studies it was assumed that the detected anaerobes resided within the presumably anoxic guts of zooplankton or fecal pellets. Microelectrodes have since been used to demonstrate that the gut of the large Arctic/Subarctic copepod, *Calanus* sp. was, in fact suboxic and anoxic (Tang et al. 2011). We hypothesized that all zooplankton can support metabolically active epibiotic and gut bacterial communities and that all facultative anaerobic bacterial substrate usage will be due to activities of gut bacteria. We also hypothesized that zooplankton-associated bacteria will utilize more carbon substrates than free-living bacteria under anaerobic conditions.

Zooplankton-associated bacterial communities may be influenced by the type of food consumed as well as whether or not the food source carries a bacterial community (Tang et al. 2009). Yet, the relative importance of biochemical composition of the food source and selective retention of food-associated bacteria by zooplankton has not been explored. Axenic and non-axenic cultures of the same phytoplankton strain can be utilized to assess these impacts. Bacterial colonization of the gut microenvironment is

highly variable among species. Scanning electron microscopy (SEM) showed that marine, wood-boring isopods had densely colonized exoskeletons, but their guts were completely devoid of bacteria (Boyle & Mitchell 1978). Similarly, SEM illustrated that both the guts and fecal pellets of the copepod *Pseudocalanus newmani* and the amphipod *Themisto japonica* were lacking bacteria (Nagasawa 1992). In contrast, the guts of multiple other copepod species such as *Calanus plumchrus* (Nagasawa 1992), *Acartia* sp., *Pleuromamma* sp., *Pontellis regalis*, *Labidocera aestiva* and *Centropages furcatus* (Sochard et al. 1979) support dense bacterial communities. Given the large variability in bacterial colonization, it is likely that bacteria associated with different co-occurring zooplankton groups may serve different functional roles. Therefore, we hypothesized that the bacteria associated with each zooplankton group from the same environment will utilize a unique subset of carbon substrates.

Biolog EcoPlates™ provide an efficient way to examine the functionality of bacterial communities by assessing the usage of 31 carbon substrates commonly used by environmental bacteria (e.g. Garland & Mills 1991, Choi & Dobbs 1999, Sala et al. 2005, Lyons & Dobbs 2012). Twenty-one of the 31 substrates present on the EcoPlate are known to be utilized by anaerobic bacteria, allowing for an adequate comparison of aerobic and anaerobic carbon substrate utilization by bacterial communities (Christian & Lind 2006).

To address our hypotheses we performed a laboratory experiment using EcoPlates to assess carbon substrate usage by the gut bacteria and total bacteria (epibiotic + gut) associated with the calanoid copepod *Acartia tonsa* under both aerobic and anaerobic conditions. Additionally, to investigate the variability among different host zooplankton

species, we compared the substrate usage of aerobic and facultative anaerobic bacteria associated with six common zooplankton groups from the York River tributary of the Chesapeake Bay.

MATERIALS AND METHODS

Laboratory experiments for total bacteria vs. gut bacteria

A full factorial design experiment was established to determine the impact of axenic and non-axenic food sources on the carbon substrate usage patterns of food-associated bacteria as well the entire copepod-associated bacterial community and copepod gut bacterial communities under both aerobic and anaerobic conditions. In a biosafety hood, 200 ml of axenic or non-axenic *Dunaliella tertiolecta,* respectively, were added to sterile 1 L glass bottles. An additional 800 ml of 20 psu Artificial Sea Water (ASW) were filtered twice through 0.2 μm filters and then added into each bottle. Prior to addition, a subsample of the axenic phytoplankton culture was collected and checked for the presence/absence of bacterial contamination with DAPI staining (Porter & Feig 1980). After the phytoplankton and water were added to each bottle, a 5 ml aliquot was taken to determine final phytoplankton density. The final concentrations of axenic and non-axenic *D. tertiolecta* were 2.5×10^5 cells ml^{-1} and 2.1×10^5 cells ml^{-1} respectively.

Acartia tonsa copepodites and adults from laboratory cultures were concentrated onto a 200 μm mesh sieve and transferred to each of the two incubations bottles containing phytoplankton. Incubation bottles were closed with sterile foam plugs, gently aerated and copepods were allowed to feed overnight at 25°C. After feeding, the copepods from each incubation bottle were gently collected onto 200 μm mesh sieves, transferred to 500 ml of sterile-filtered ASW and allowed to clear their guts for 4 hours to eliminate any food and food-associated bacteria. The copepods from each respective feeding were again concentrated onto a 200 μm mesh sieve, rinsed gently with sterile-

filtered ASW, back rinsed into a sterile petri dish and narcotized with sodium bicarbonate. Copepods fully recovered from narcotization after transfer to clean water, and preliminary experiments indicated that treatment with sodium bicarbonate did not influence copepod-associated bacterial abundance or function. The narcotized copepods were evenly divided for 2 treatments: one group received no treatment in order to capture the entire (epibiotic + gut) copepod-associated bacterial community. To capture only the gut bacteria, the second group of copepods was placed in a 5% sodium hypochlorite solution for 5 minutes then gently rinsed with sterile-filtered ASW to remove any residual hypochlorite. Hypochlorite is commonly used to remove external and epibiotic bacteria without killing the host while also keeping the gut bacterial community intact (King et al. 1991, Greenstone et al. 2012).

From each of the axenic and non-axenic food treatments, 13-14 individual copepods were picked and transferred to a 15 ml centrifuge tube containing 5 ml of sterile-filtered ASW for aerobic and anaerobic incubations, respectively. Triplicates were established for each incubation type for a total of 24 tubes. Each sample was homogenized on ice with an ultrasonic homogenizer at 4W output power for 40 seconds (modified from Tang 2005), after which the homogenizer probe was rinsed with a small amount of filtered ASW. All samples were centrifuged at 102 RCF for 10 min at room temperature to remove any tissue remains of the copepods. 150 µl of the bacteria containing supernatant was added to each well of an EcoPlate. Each of the triplicate samples was loaded onto one of the 3 replicate wells within each EcoPlate in order to achieve true replication within one plate. The initial optical density (OD) of each well on the plate was measured at 590nm with a BioTek plate reader. Samples for anaerobic

incubation were placed in a gas-tight chamber containing a Mitsubishi Anaeropak to remove all oxygen from the chamber. The anaerobic condition throughout the incubation was verified by a small vial of 0.0002% Rezasurin anaerobic indicator inside the chamber (Karakashev et al. 2003). Both aerobic and anaerobic incubations were performed in the dark at 25° C for 1 week, after which the final OD of each well was measured at 590nm. The procedure of the laboratory experiment is outlined in Fig. 1. Initial inoculum densities were only on the order of 10^5 cells ml^{-1} for all samples, however, the importance of inoculum density diminishes after 72 hours (Christian & Lind 2006).

To assess food-associated bacteria, triplicate 15 ml aliquots of both axenic and non-axenic phytoplankton cultures were added to sterile centrifuge tubes and placed at 25 °C for the same duration that copepods were feeding. The samples were centrifuged for 15 minutes at 200 RCF to concentrate the phytoplankton cells and allow removal of excess growth media. Microscopic inspection verified that centrifugation did not damage the phytoplankton cells. Concentrated phytoplankton samples (250 µl, 3.72 $x10^6$ cells for axenic culture; 190 µl, 3.20$x10^6$ cells for non-axenic culture) were divided into 2 portions, one for aerobic incubation, one for anaerobic incubation. The concentrated samples were diluted to 5 ml with sterile-filtered ASW, and were then processed and incubated in the same manner as the copepod samples.

Aerobic and anaerobic bacteria associated with York River zooplankton

On two occasions, two weeks apart during July 2012, zooplankton were collected during high tide from the York River near Gloucester Point, VA with a 200µm mesh, ½ m mouth diameter net with a solid cod end. During the first sampling, only the

ctenophore *Mnemiopsis leidyi* was present in large enough numbers for analysis. Individual *Mnemiopsis* sustained some bodily damage during collection in the net, however all individuals were still alive with cilia comb rows beating. Six individual *Mnemiopsis*, ranging in size from 2.8-3.7cm (average 3.4cm), were gently transferred to clean beakers with 300 ml of sterile-filtered ASW (20psu) and allowed to clear their guts for approximately 1 hour. Each individual was then gently removed from the beaker and transferred to a sterile 50 ml centrifuge tube, and the volume was brought to 7.5 ml with sterile-filtered ASW.

A more diverse zooplankton sample was collected during the second trial, which included crab zoea, polychaete larvae, harpacticoid copepods and the calanoid copepods *Acartia tonsa* and *Paracalanus* sp. After collection, the mixed zooplankton assemblage was transferred to a clean beaker with 2L of sterile-filtered ASW and allowed to clear their guts for 1 hour. Triplicate samples of each zooplankton taxa were sorted for both aerobic and anaerobic incubations. Depending on their abundances in the sample, 2 to 15 individuals per replicate of each taxon were used for incubation. On both sampling occasions, additional water samples were collected in triplicate to assess the carbon substrate usage of free-living bacteria. From each sample 15 ml of whole water were placed in a 15 ml centrifuge tube. All zooplankton and water samples were homogenized, centrifuged and added to EcoPlates. The EcoPlates were incubated under both aerobic and anaerobic conditions and the initial and final OD were measured in the same manner as in the laboratory experiments.

Statistical analyses

All final OD readings were corrected by subtracting initial and control OD from each well. For analysis purposes, a substrate with a corrected OD greater than zero was denoted as used (Christian & Lind 2006). For comparisons of bacterial communities (free-living vs. zooplankton total vs. zooplankton gut) and incubation conditions (aerobic vs. anaerobic), the mean number of substrates used among all replicates within each treatment was determined. The total number of substrates used in each treatment was not normally distributed and could not be normalized via transformation. The non-parametric Kruskal-Wallace test was therefore used to test for differences in total substrate utilization among the different samples.

The mean OD of each substrate across all replicates within a treatment was calculated to determine if that substrate was used by the bacterial community. The Dice coefficient was used to calculate similarities among the different substrate usage profiles and generate a similarity matrix comparing all samples. Similarities of substrate usage by bacteria in each treatment were compared via multidimensional scaling (MDS).

The N-use index was calculated for all sample types to determine the importance of the usage of nitrogen containing substrates in relation to the total number of substrates consumed (Sala et al. 2006). Ten of the 31 carbon substrates in an EcoPlate contained both carbon and nitrogen (6 amino acids, two amines, one carboxylic acid and 1 carbohydrate). The N-use index was calculated as the percentage of substrates used accounted for by N-containing compounds and can range from 0 to 100. If all substrates on the Ecoplate are utilized, N-use = approximately 32%. If only Nitrogen containing substrates are utilized, N-use = 100%.

RESULTS

Laboratory experiments

No bacteria were noted when axenic phytoplankton cultures were initially examined for contamination via DAPI staining. However, substrate usage was observed among all axenic food samples indicating bacterial contamination. The number of substrates by the two food types were not significantly different (t-test, $p = 0.96$) and visual inspection of the data indicated similar substrates were used by the two groups. Subsequently, data from copepods fed axenic and non-axenic phytoplankton were pooled together before analysis.

The number of substrates used by food-associated bacteria appeared higher than that used by bacteria associated with copepods not treated with hypochlorite (hereafter referred to as epibiotic + gut bacteria) and bacteria associated with hypochlorite-treated copepods (hereafter referred to as gut bacteria), under both aerobic and anaerobic conditions, although the difference was not significant ($p=0.165$, Fig. 2). The number of substrates used in aerobic and anaerobic conditions was not significantly different within any of the bacterial groups ($p= 0.899$). All substrates were categorized into the biochemical groups of carboxylic acids, polymers, carbohydrates, phenolic compounds, amino acids and amines (Choi & Dobbs 1999). Within the aerobic incubations, more carbohydrates were used by the food-associated bacteria than either copepod-associated bacteria group ($p=0.048$, Fig. 3C). There were no significant differences among the aerobic treatments for any other biochemical groups (Fig. 3A,B,D-F). Under anaerobic conditions, food-associated bacteria used significantly more carboxylic acids ($p=0.013$,

Fig. 3A), phenols (p=0.002, Fig. 3D) and amines (p=0.016, Fig. 3F) than both copepod-associated bacterial groups, while gut bacteria in the anaerobic treatment used fewer carbohydrates (p=0.032, Fig. 3C) than the other two groups.

Multidimensional scaling showed that the types of substrates used by food-associated bacteria under aerobic and anaerobic conditions were very similar to each other, and very dissimilar from those used by copepod-associated bacteria regardless of whether or not free oxygen was present (Fig. 4). Among the copepod-associated bacteria, grouping of their substrate usage patterns depended less on where the bacteria were located (epibiotic vs. gut), and more on the oxygen environment (aerobic vs. anaerobic) (Fig. 4).

Nitrogen containing compounds comprised a lower percentage of all substrates used by both the aerobic epibiotic + gut bacteria (N-use = 21%) and aerobic gut bacteria (N-use= 17%) associated with *Acartia tonsa* than the aerobic food-associated bacteria (N-use = 28%; Fig. 5). Differences in N-use were not significant however (ANOVA, p=0.359). The N-use index was very similar (ANOVA, p= 0.875) for all three bacterial groups under anaerobic conditions, with a value of 31, 29 and 27% for food-associated bacteria, epibiotic +gut bacteria and gut bacteria, respectively.

York River Zooplankton-Associated Bacteria

Among the zooplankton taxa, polychaete-associated bacteria utilized the most substrates aerobically (24±6; mean ±S.D.) and crab zoea-associated bacteria utilized the most anaerobically (17±15). There was no significant difference in the total number of substrates used by the different bacterial communities under aerobic (p=0.122, Table 1)

or anaerobic conditions (p=0.117, Fig. 6), nor were any biochemical groups used preferentially under aerobic or anaerobic conditions across all samples.

Except for *Mnemiopsis*-associated and polychaete-associated bacteria, the total number of substrates used was not significantly different under aerobic and anaerobic conditions (p=0.05 for each taxon; Table 1). *Mnemiopsis*-associated bacteria had higher usage of carboxylic acids (p=0.05), carbohydrates (p=0.05), phenolic compounds (p=0.025) and amino acids (p=0.043) under aerobic conditions than anaerobic conditions (Table 1). Aerobic bacteria associated with polychaete larvae used more polymers than anaerobic bacteria. The only instances of higher substrate usage under anaerobic conditions were for carboxylic acids by free-living bacteria and amines by crab zoea-associated bacteria (Table 1).

To compare substrate usage between zooplankton-associated bacteria and free-living bacteria, the ratio of the total number of substrates utilized by each community was calculated for both aerobic and anaerobic incubations (Fig. 6). A ratio of one indicates that the same number of substrates was used by free-living and zooplankton-associated bacteria. A ratio greater than 1 indicates more substrates used by zooplankton-associated bacteria while a ratio less than 1 indicates greater substrate utilization by free-living bacteria. Only aerobic *Mnemiopsis*-associated bacteria showed a ratio greater than 1, although the difference was not significant. The ratio for crab zoea-associated bacteria was significantly less than 1 in aerobic conditions, while the ratios for *Mnemiopsis*, *Acartia*, polychaete and harpacticoid-associated bacteria were all less than one in anaerobic conditions (Fig. 6). When all zooplankton groups were pooled, the ratio was 1 for aerobic condition and 0.84 for anaerobic condition. In anaerobic incubations,

zooplankton-associated bacteria used the substrates 2-hydroxybenzoic acid, L-phenylalanine and putrescine, while free-living bacteria did not.

Multidimensional scaling of substrate usage profiles for all York River samples indicated a higher degree of similarity among the aerobic carbon substrate usage profiles than the corresponding anaerobic ones, among which there were large dissimilarities (Fig. 7). Anaerobic substrate usage profiles of free-living bacteria were similar to their aerobic counterparts. The anaerobic substrate usage profiles of zooplankton-associated bacteria were dissimilar from each other and from the aerobic substrate usage profiles (Fig. 7), suggesting a wide functional diversity of anaerobic bacteria associated with different zooplankton groups.

Mnemiopsis-associated bacteria had a slightly higher N-use index in aerobic conditions (40%) than bacteria within the corresponding whole water samples (36%), although differences were not significant due to high variation among replicates (Fig. 8). Most aerobic bacterial communities collected during the second sampling had an N-use index in the range of 32-35%, with the exceptions of crab zoea (16%), *Paracalanus* (24%) and Harpacticoid (14%) samples (Fig. 8). The anaerobic N-use indices among different zooplankton-associated communities ranged from 17% (*Acartia*) to 36% (Crab zoea).

DISCUSSION

Food-associated vs. copepod-associated bacteria

Before ingestion, food particles could be colonized by the very diverse free-living bacterial community in the water column (Acinas et al. 1999). Ingestion of bacteria-laden food particles can therefore introduce diverse bacterial communities to the copepods (Harris 1993). Upon ingestion, these particle-associated bacteria would pass through the digestive tract where they experience a drastic change in the physical-chemical conditions (Tang et al. 2011) and be subject to digestive enzymes (Mayzaud & Poulet 1978). It is therefore instructive to view copepods as a filter that selects and retains a subset of the food-associated bacteria that are able to establish permanent residence within the copepod bodies. Indeed, an earlier study which examined the impact of food source on copepod-associated bacterial composition noted that *A. tonsa* with full guts showed a larger number of DGGE bands than copepods that had been starved for 24 hours (Tang et al. 2009), suggesting that the copepod-associated bacteria were genetically less diverse than food-associated bacteria.

Consistent with the earlier findings, the number of substrates utilized by *Acartia*-associated bacteria in this study was less than those used by food-associated bacteria under both aerobic and anaerobic conditions (Fig. 2). Thus, the copepod-associated bacteria had a lower functional diversity than the food-associated bacteria. This reduction in genetic diversity (Tang et al. 2009) and functional diversity (this study) during passage through the copepod bodies suggests that some of the food-associated bacteria are most likely digested by the copepods (Lawrence et al. 1993) or repackaged

into fecal material (King et al. 1991, Lawrence et al. 1993) rather than being integrated into the existing copepod gut bacterial community.

Both phytoplankton and zooplankton serve as a source of dissolved organic material for attached bacteria. Within aerobic incubations, the food-associated bacteria used more carbohydrates than the epibiotic + gut bacteria or gut bacteria (Fig. 3C). *Dunaliella tertiolecta* can release up to 20% of its fixed carbon as extracellular glycolate (Leboulanger et al. 1998) and bacteria can take up large amounts of the dissolved organic carbon released by phytoplankton (Malinsky-Rushansky & Legrand 1996). Consequently, food-associated bacteria are likely adapted to exploit carbohydrate released by phytoplankton. In contrast, there is no information to indicate that copepods excrete carbon in the form of carbohydrates. Therefore, copepod-associated bacteria are likely not as well suited to utilize carbohydrate substrates.

Total bacteria vs. gut bacteria

It is difficult to separate epibiotic and gut communities when studying zooplankton-associated bacteria. King & colleagues (1991) found that treatment with a weak hypochlorite solution efficiently removed externally attached bacteria while leaving gut bacteria unharmed. Treatment with weak hypochlorite has also been used to remove external contaminating DNA from arthropod predators when extracting DNA for gut content analysis (Greenstone et al. 2012). Treatment of the copepods with hypochlorite allowed us to assess the functionality of the gut-specific community as well as the entire copepod-associated bacterial community.

The average total number of substrates used by the entire bacterial community and gut community were nearly identical in both aerobic and anaerobic incubations (Fig. 2); multidimensional scaling showed that the types of substrates used by each community were also very similar (Fig. 4). These results indicate that either the entire bacterial community and gut bacterial community were functionally very similar, or the gut bacterial community was responsible for most of the carbon substrate usage. In previous studies, bacteria associated with copepods exhibited higher leucine incorporation (Carman 1994) and higher bacterial production and growth rates (Møller et al. 2007) than free-living bacteria. Carman (1994) acknowledged the potential presence of gut bacteria but attributed all activity to epibiotic bacteria because autoradiography only detected radioactivity on the surface of the copepod. Møller and colleagues (2007) also noted the problematic mixture of epibiotic and gut bacteria but hypothesized that their production measurements were underestimates due to the unequal distribution of labeled compounds inside and outside the copepod. Our results suggest that bacteria residing with zooplankton guts can be an important contributor to the overall bacterial function associated with the zooplankton and should be considered when estimating zooplankton-associated bacterial processes.

A few substrates, such as the carbohydrates i-erythritol and D-mannitol as well as the amino acid L-threonine were used in samples containing epibiotic + gut bacteria but not in samples with only gut bacteria, indicating that externally-attached bacteria were responsible for the consumption of these substrates. These three substrates were consumed by food-associated bacteria in the laboratory experiment and free-living bacteria collected for the field study as well as by estuarine aggregate-associated and the

corresponding free-living bacteria (Lyons et al. 2010, Lyons & Dobbs 2012). The shared substrate usage by externally-attached, food-associated and free-living bacteria supports the idea of active exchange between free-living and attached bacterial communities (Møller et al. 2007, Grossart et al. 2010), and that bacteria capable of using these substrates may not be adapted to the gut environment inside a zooplankter.

Average N-use indices were lower among the whole *Acartia*-associated bacterial community and *Acartia* gut bacterial community than the food-associated community (Fig. 5). During the development of the N-use index for EcoPlates, Sala and colleagues (2006) noted a negative logarithmic relationship between N-use indices and ambient ammonium concentration in natural systems. Additionally, N-use indices decreased substantially when experimental incubations were supplemented with ammonia or amino acids (Sala et al. 2006). The authors concluded that the N-use index could be used as an indicator of the bacterial nitrogen consumption based on the nutritional status of the bacteria (i.e. N-limited bacteria should have a higher N-use index). This interpretation would imply that food-associated bacteria are more nitrogen-limited than *Acartia*-associated bacteria, especially the gut bacteria. Direct observations of externally attached bacteria have shown higher concentrations around mouthparts, intersegmental regions and the anus, where nutrient release would be the largest (Carman & Dobbs 1997). Additionally, ammonium addition had no impact on bacterial production rates when copepods were also present (Carman 1994) indicating that copepods relieved bacteria from nitrogen limitation. Zooplankton guts represent an environment of elevated nutrients and gut bacteria are not expected to encounter nitrogen limitation in this microenvironment, which may explain their lower N-use index.

Aerobic and anaerobic conditions

The majority of bacteria collected from the York River utilized a similar number of substrates under aerobic and anaerobic incubation conditions (Table 1), indicating the presence of a functionally diverse, facultative anaerobic bacterial community both free-living in the water column and associated with zooplankton. With the exception of aerobic bacteria associated with *Mnemiopsis*, all zooplankton-associated bacterial groups used fewer substrates than the corresponding free-living bacteria (Fig. 6). When pooled, zooplankton-associated bacteria used the same number of substrates as the free-living bacteria under aerobic conditions, but still used fewer substrates than free-living bacteria under anaerobic conditions (Fig. 6). The lower anaerobic use of substrates by all zooplankton-associated bacteria was not surprising because not all ingested facultative anaerobic bacteria are expected to survive and adapt to the specific zooplankton gut microenvironments. It should be noted that our incubations were prepared aerobically but incubated anaerobically. Thus obligate anaerobes may not have been represented and any substrate usage would principally be due to facultative anaerobic bacteria. Bacteria sampled from bottom waters at the onset of a hypoxic event in Chesapeake Bay were very genetically similar to bacteria collected at the same time from fully oxygenated surface waters (Crump et al. 2007). It was suggested that many aerobic bacteria within Chesapeake Bay may be facultative anaerobes, persisting under anaerobic conditions for a period of time (Crump et al. 2007).

While EcoPlates have been used to assess bacteria collected from hypoxic or anoxic systems such as mine drainages (Kim et al. 2009) and an anaerobic sludge blanket

reactor (Cardinali-Rezende et al. 2011), few have actually incubated the EcoPlates under anaerobic conditions (Christian & Lind 2006, 2007), thereby truly testing bacterial functional abilities in anaerobic conditions. Christian & Lind (2006) noted that all 31 substrates were used when water collected from anoxic bottom waters of a eutrophic freshwater reservoir were prepared and incubated anaerobically. To our knowledge, however, ours is the first study to anaerobically incubate samples collected from oxygenated systems, thereby measuring potential anaerobic activity within an aerobic system.

Bacteria associated with polychaete larvae used significantly more substrates aerobically than anaerobically. Adult polychaete worms reside in the benthos and play an important role in the bioturbation of sediments and consequently influence many sediment biogeochemical cycles such as the coupling of nitrification and denitrification (e.g. Kristensen et al. 1991). Sulfate reducing bacteria have been found within the tubes of the marine infaunal polychaete *Diopatra cuprea* (Matsui et al. 2004), and the relative abundance of anaerobic bacteria increased toward the posterior of the polychaete *Neanthes glandicincta* gut (Li et al. 2009). To our knowledge, no studies have examined the bacterial community composition associated with polychaete larvae. Further investigation is needed to determine if there are shifts in the associated bacterial community composition as polychaetes transition from their planktonic larval stage to benthic adult stage.

Variability in functional diversity among zooplankton groups

The carbon substrate usage patterns were quite different among the individual zooplankton-associated and free-living bacterial communities sampled. The dissimilarities were especially pronounced in anaerobic incubations (Fig. 7), which are consistent with other studies indicating that each zooplankton group supports a unique bacterial community. Denaturing gradient gel electrophoresis and band sequencing revealed that *Acartia* sp. and *Temora* sp. co-existing in the North Sea supported different bacterial communities (Brandt et al. 2010). Likewise, within a freshwater lake the copepod *Thermocyclops oithonoides* and cladoceran *Bosmina coregoni* supported very different bacterial communities (Grossart et al. 2009). Although the species identities of the zooplankton-associated bacteria were not known in this study, a complementary genetic fingerprinting study found that co-occurring zooplankton groups within the York River Estuary supported distinct bacterial communities (Chapter 3), which in turn demonstrated unique carbon substrate usage profiles. Variability in rates of zooplankton-associated anaerobic processes has also been observed among different zooplankters. DeAngelis & Lee (1994) noted that microbial methane production was not the same among all copepods collected from the Long Island sound; measurable production was found with *Temora longicornis*, while no methane production was noted with *Acartia tonsa*. Likewise, nitrous oxide emissions (presumably due to incomplete denitrification) were highly variable among aquatic macrofauna (Stief et al. 2009). The occurrence and overall importance of anaerobic processes associated with zooplankton is likely dependent on the makeup of the zooplankton community and their corresponding bacterial communities.

Mnemiopsis-associated bacteria had the highest average N-use index of all samples (40%). This value was higher than the largest N-use value observed in the oligotrophic Arenes de Mar in Blanes Bay, Spain (Sala et al. 2006), but similar to N-use of bacteria associated with organic aggregates (Lyons & Dobbs 2012). *Mnemiopsis* exudates are high in carbon relative to nitrogen with a DOC:DON ratio of 29:1 (Condon et al. 2010). As a consequence, bacteria directly associated with *Mnemiopsis* may encounter nitrogen limitation. In comparison, *Acartia*-associated bacteria had an average N-use index of 35% while other zooplankton-associated bacteria had much lower N-use indices (Fig. 8). This is consistent with the observation that *Acartia* excretia had a relatively low C:N ratio at approximately 2:1 (Saba et al. 2009), such that *Acartia*-associated bacteria are less likely to encounter nitrogen limitation.

Importance of the zooplankton microenvironment

The results of the laboratory experiment indicate that copepod gut bacteria were just as functionally diverse and active as the externally attached bacterial community, if not more so. The heterotrophic gut bacterial community was capable of utilizing a variety of substrates under both aerobic and anaerobic conditions and may contribute significantly to zooplankton-associated bacterial production. Consequently, gut bacterial communities should be considered when estimating the contributions of zooplankton-associated bacteria to total bacterial activity.

The subsets of substrates used by the bacteria of an individual zooplankton group were distinct and diverse, especially under anaerobic conditions. These observations suggest that each zooplankton group functioned as a selective filter, retaining only a

small portion of the bacteria they came into contact with in the water column or ingested with food particles. Bacteria selected by the zooplankter may be less common in the water column, but the zooplankton microenvironment may allow this select subset of bacteria to flourish on a localized scale. The presence of many different bacterial filters (i.e. zooplankton groups) may therefore increase the relative abundance of less common bacterial groups and their associated substrate usages within a system. The bacterial communities selected for by the zooplankton microenvironment could complement the functionality of the free-living bacteria, potentially expanding the suite of substrates that can be utilized by the overall estuarine bacterial community. Consequently, the amount and types of biogeochemical processes within a system could be greatly underestimated if only free-living bacteria are considered.

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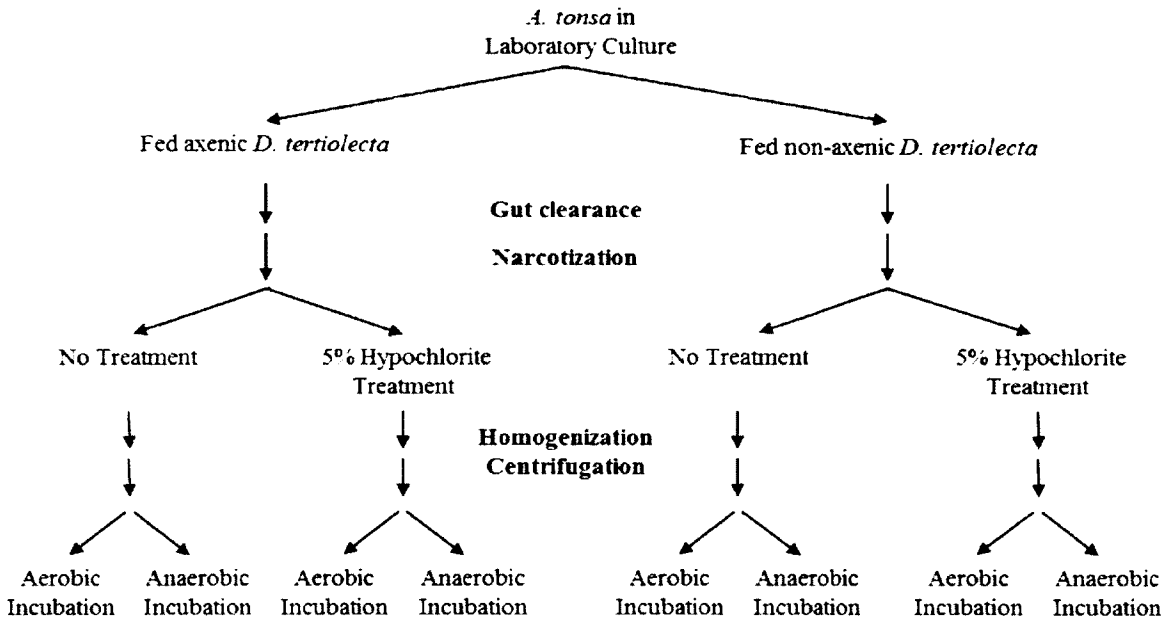
Table 1. Number of substrates (mean ±S.D, n=3) from each biochemical category used by free-living and zooplankton-associated bacteria in the York River. Total number of substrates available within each category is bolded in parentheses, below the category name. Brackets indicate significant differences in the numbers of substrates used between aerobic and anaerobic incubations of the same source communities. Category abbreviations are the same as in Fig. 6. WYRW = whole York River water from sample occasions 1 and 2 respectively.

Sample and Incubation		Carboxylic Acids (7)	Polymers (4)	Carbohydrates (10)	Phenolic Compounds (2)	Amino Acids (6)	Amines (2)	Total (31)
WYRW1	Aerobic	2.7±1.2	1.7±2.1	5.7±2.1	0.3±0.6	2.7±2.1	0.7±1.2	14±9
	Anaerobic	5.3±0.6	4.0±0.0	8.3±1.5	1.0±0.0	4.7±0.6	1.7±0.6	25±2
Mnem	Aerobic	4.0±0.0	1.0±1.0	6.7±1.5	1.0±0.0	3.3±1.2	1.7±0.6	18±3
	Anaerobic	1.7±1.5	1.3±0.6	2.0±2.0	0.0±0.0	0.7±0.6	1.0±1.0	7±6
WYRW2	Aerobic	5.3±1.2	4.0±0.0	8.3±0.6	1.0±1.0	5.3±1.2	1.3±0.6	25±4
	Anaerobic	6.7±0.6	4.0±0.0	8.7±1.5	0.0±0.0	4.7±1.2	1.0±1.0	25±4
Acar	Aerobic	4.3±1.2	2.3±1.2	7.3±2.9	0.3±0.6	4.3±2.1	1.3±1.2	20±9
	Anaerobic	4.0±1.0	2.0±1.0	5.7±2.5	1.3±0.6	1.0±1.0	0.3±0.6	14±4
Crab	Aerobic	2.7±0.6	1.0±0.0	5.7±1.5	0.3±0.6	1.0±0.0	0.0±0.0	11±2
	Anaerobic	3.0±3.6	2.0±1.0	5.7±4.2	0.7±1.2	3.7±3.2	1.7±0.6	17±13
Para	Aerobic	3.3±1.5	0.7±0.6	5.7±3.8	0.3±0.6	2.3±2.5	0.7±0.6	13±9
	Anaerobic	4.0±1.7	1.7±1.2	4.0±3.6	1.0±1.0	1.7±1.5	0.7±1.2	13±9
Poly	Aerobic	4.7±1.5	3.3±0.6	8.7±1.2	1.0±1.0	4.7±1.5	1.7±0.6	24±6
	Anaerobic	2.3±0.6	0.7±0.6	4.3±3.2	0.0±0.0	1.3±1.5	0.3±0.6	9±4
Harp	Aerobic	3.3±1.5	1.3±1.5	5.3±4.5	0.7±1.2	2.0±2.6	0.7±1.2	13±12
	Anaerobic	3.7±1.5	2.0±1.0	6.7±0.6	0.0±0.0	2.7±1.5	1.0±1.0	16±4

Fig. 1. Schematic diagram of steps performed during the laboratory experiment with *Acartia tonsa* to determine the aerobic and anaerobic functionality of the entire copepod-associated bacterial community and gut bacteria community


A. tonsa in
Laboratory Culture
Fed axenic *D. tertiolecta*
Fed non-axenic *D. tertiolecta*
Gut clearance
Narcotization
No Treatment
5% Hypochlorite
Treatment
No Treatment
5% Hypochlorite
Treatment
Homogenization
Centrifugation
Aerobic
Incubation
Anaerobic
Incubation
Aerobic
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Incubation

Fig. 2. Total number of substrates used (mean ±S.D.) by bacteria associated with the food source, *D. tertiolecta*, and those associated with the whole *Acartia tonsa* body (Epibiotic + Gut) or *A. tonsa* gut.

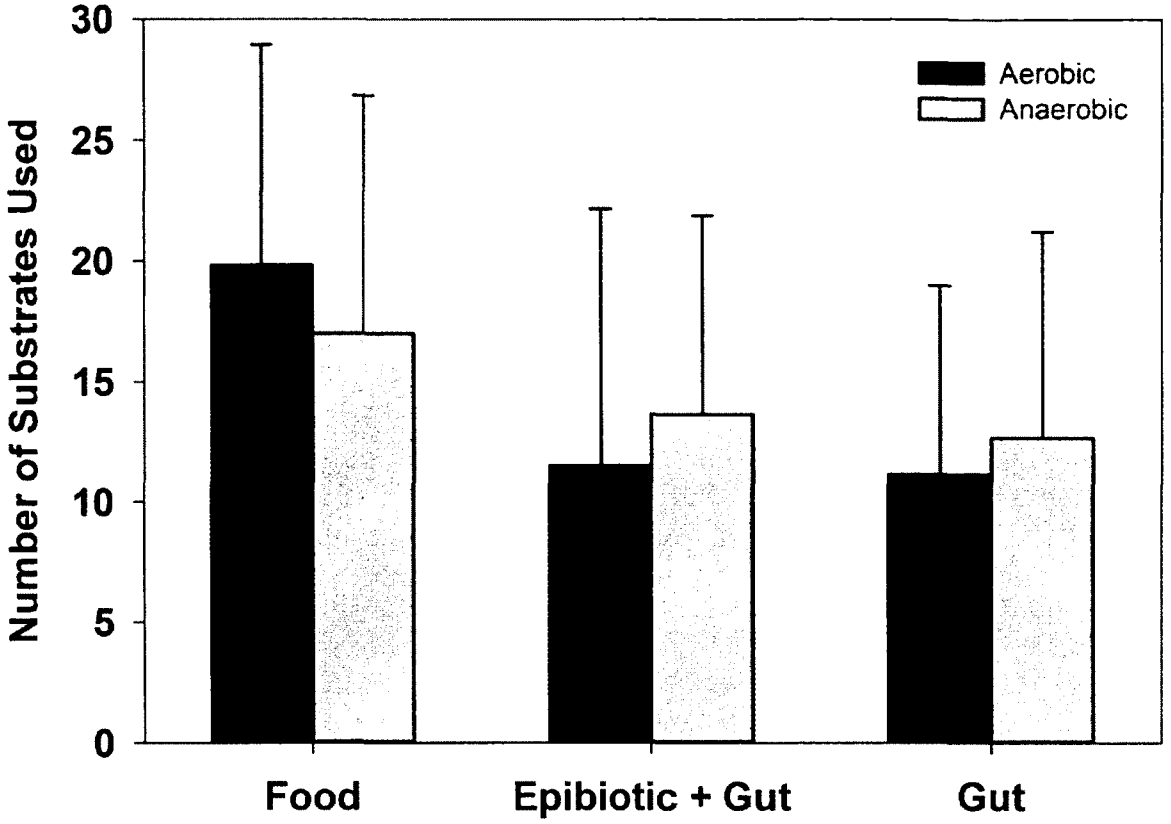

Number of Substrates Used
30
25
20
15
10
5
0
Aerobic
Anaerobic
Food
Epibiotic + Gut
Gut

Fig. 3. Number (mean ± S.D.) of substrates used by food-associated bacteria, and those associated with the whole *Acartia tonsa* body or only *A. tonsa* gut. The dashed line represents the maximum number of each respective biochemical category available to bacteria. Hash marks (#) denote significant differences within aerobic samples. Asterisks (*) denote significant differences within anaerobic samples. Figure legends are the same for all panels.

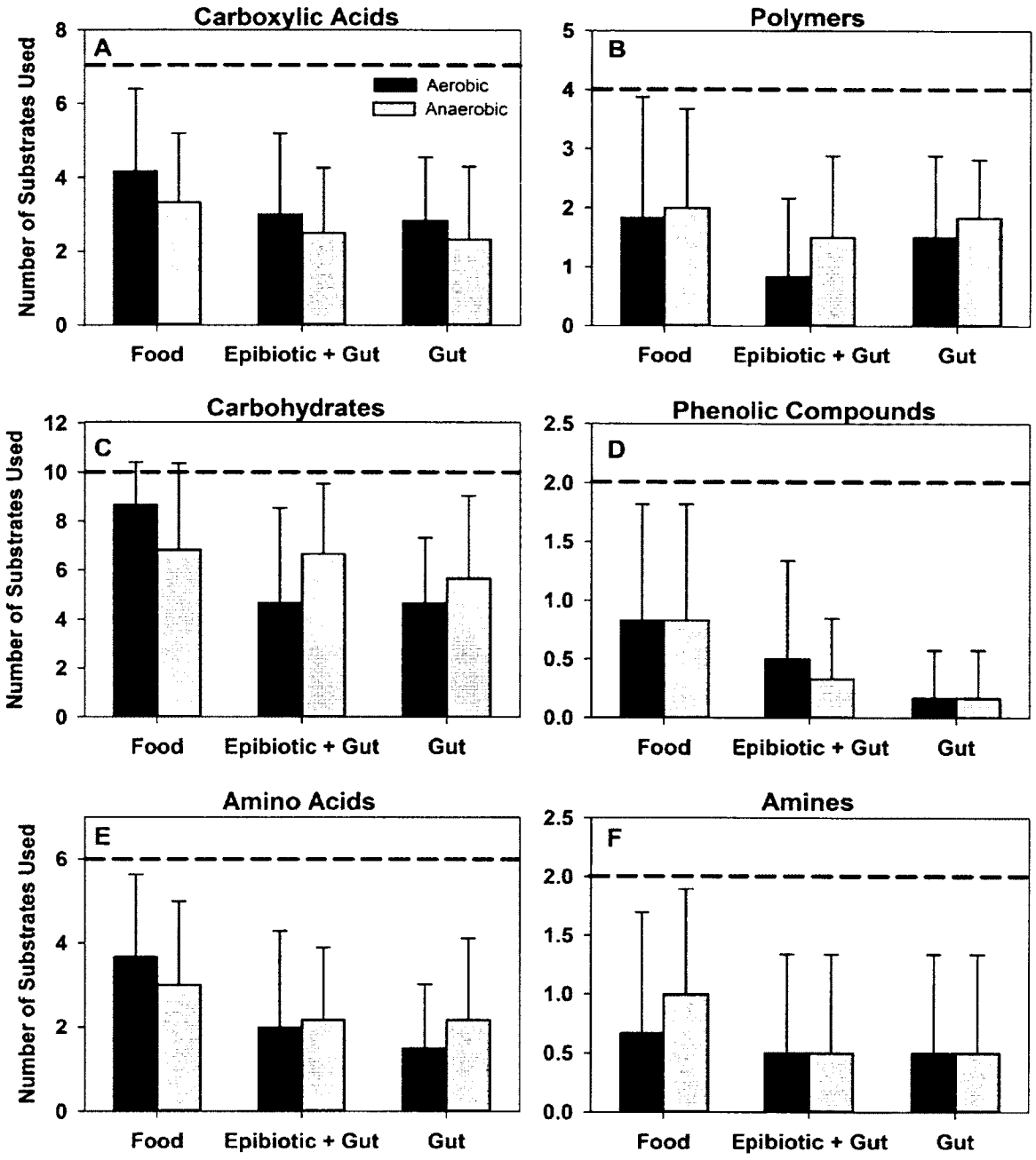

Carboxylic Acids
A
Aerobic
Anaerobic
Number of Substrates Used
0
2
4
6
8
Food
Epibiotic + Gut
Gut
Polymers
B
0
1
2
3
4
5
Food
Epibiotic + Gut
Gut
Carbohydrates
C
Number of Substrates Used
0
2
4
6
8
10
12
Food
Epibiotic + Gut
Gut
Phenolic Compounds
D
0.0
0.5
1.0
1.5
2.0
2.5
Food
Epibiotic + Gut
Gut
Amino Acids
E
Number of Substrates Used
0
2
4
6
Food
Epibiotic + Gut
Gut
Amines
F
0.0
0.5
1.0
1.5
2.0
2.5
Food
Epibiotic + Gut
Gut

Fig. 4. Multidimensional scaling plot of substrate profiles used by food-associated bacteria (triangles), epibiotic + gut bacteria (squares) and gut bacteria (circles) associated with *Acartia tonsa*. Data points located closer together indicate higher similarity between the corresponding bacterial groups.

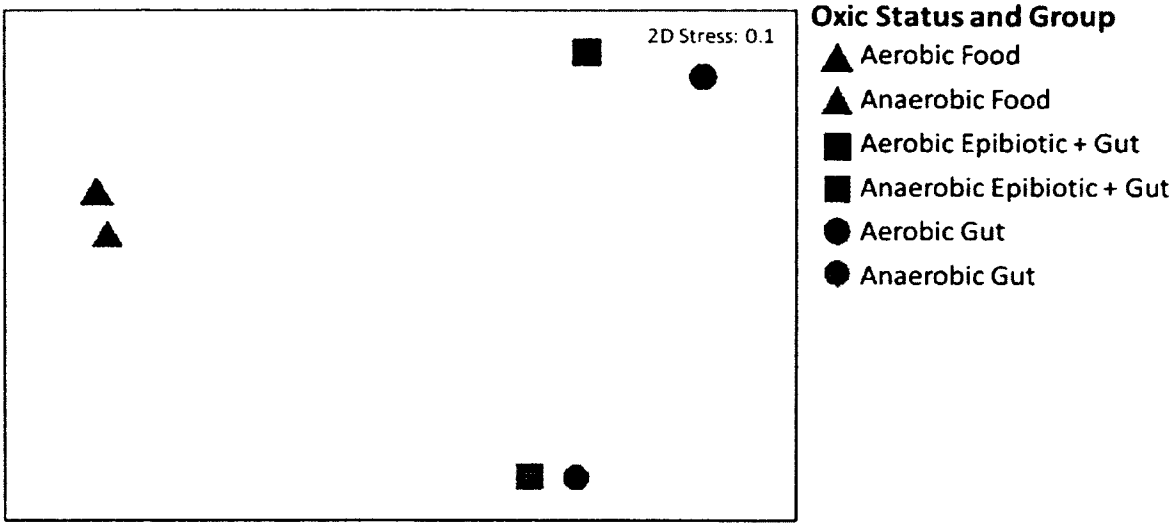

2D Stress: 0.1
Oxic Status and Group
Aerobic Food
Anaerobic Food
Aerobic Epibiotic + Gut
Anaerobic Epibiotic + Gut
Aerobic Gut
Anaerobic Gut

Fig. 5. Mean (±S.D.; n=6) nitrogen use (N-use) index of bacteria associated with food, and those associated with the whole *Acartia tonsa* body (Epibiotic + Gut) or *A. tonsa* gut.

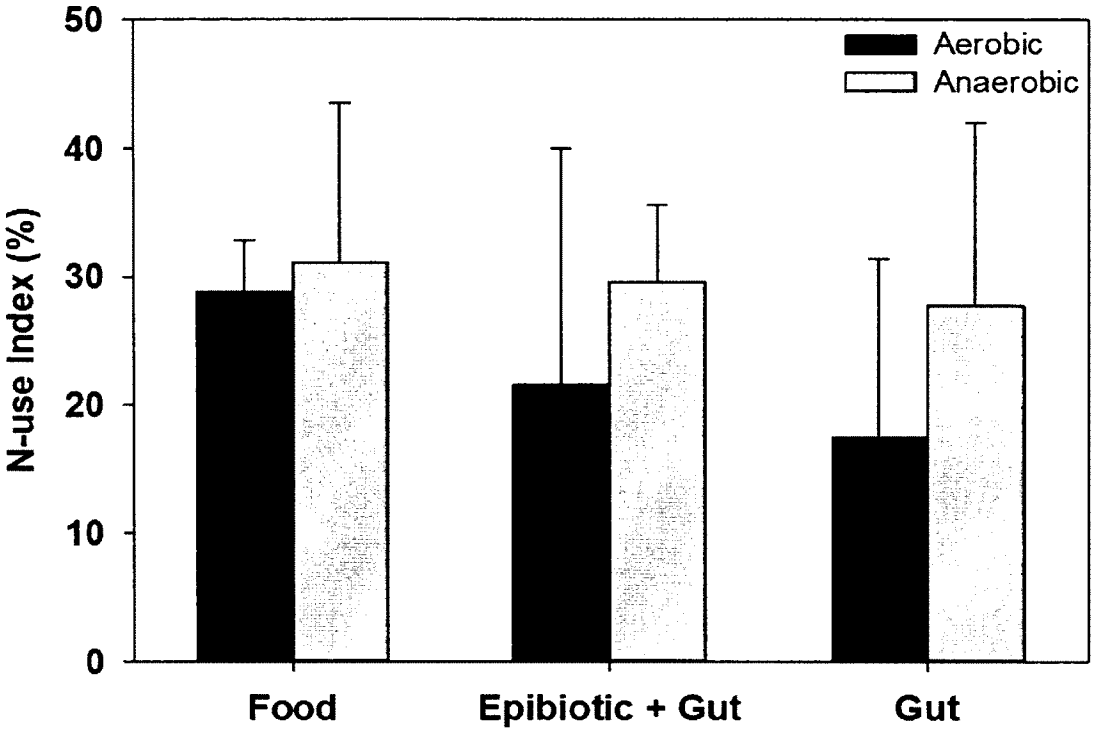

50
40
30
20
10
0
N-use Index (%)
Aerobic
Anaerobic
Food
Epibiotic + Gut
Gut

Fig. 6. Ratio (mean ±S.D.) of the number of substrates used by zooplankton-associated bacteria to bacteria in whole water samples. Asterisk denotes samples significantly different from 1(dotted line). **Mnem** = *Mnemiopsis*, **Acar** = *Acartia tonsa*, **Zoea** = Crab zoea, **Para** = *Paracalanus* sp., **Poly** = Polychaete larvae, **Harp** = Harpacticoid copepods, **All Zoop** = All zooplankton.

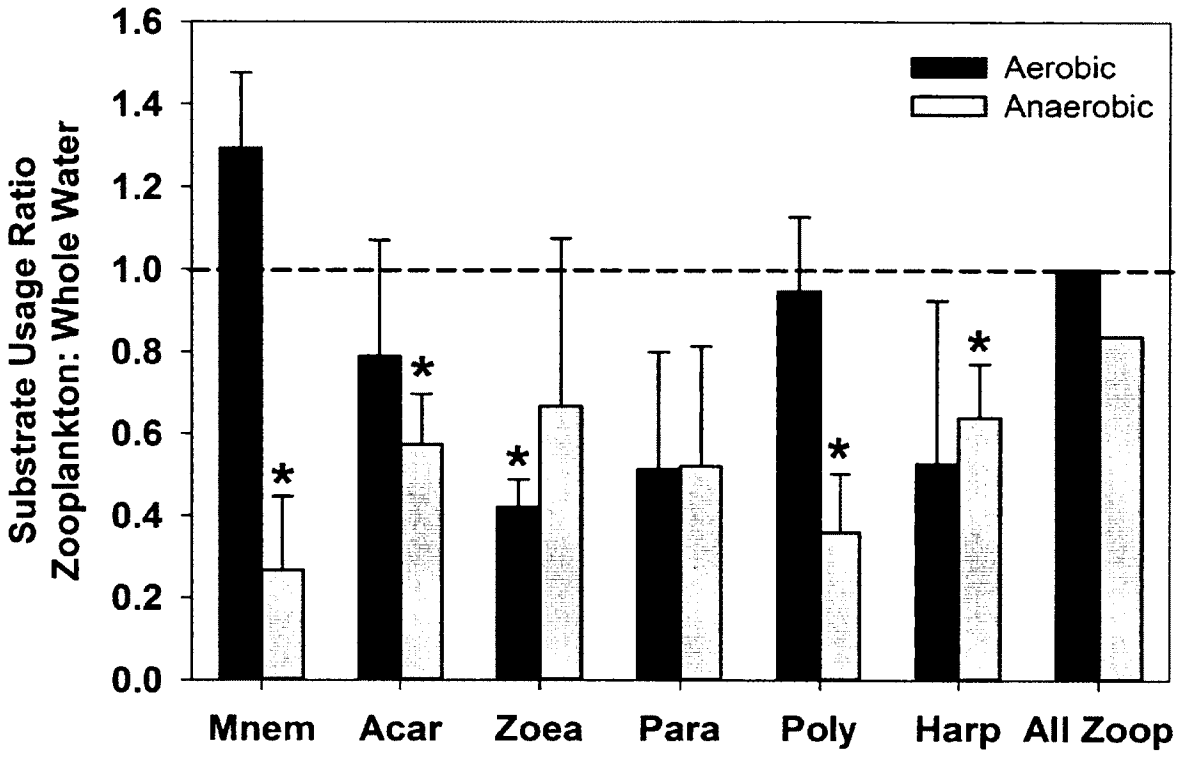

Substrate Usage Ratio
Zooplankton: Whole Water
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Aerobic
Anaerobic
*
*
*
*
*
Mnem
Acar
Zoea
Para
Poly
Harp
All Zoop

Fig. 7. Multidimensional scaling plot of substrate usage profiles for different bacterial communities from the York River incubated under aerobic (black symbols) and anaerobic (grey symbols) conditions. Data points located closer together indicate greater similarity between the corresponding bacterial communities

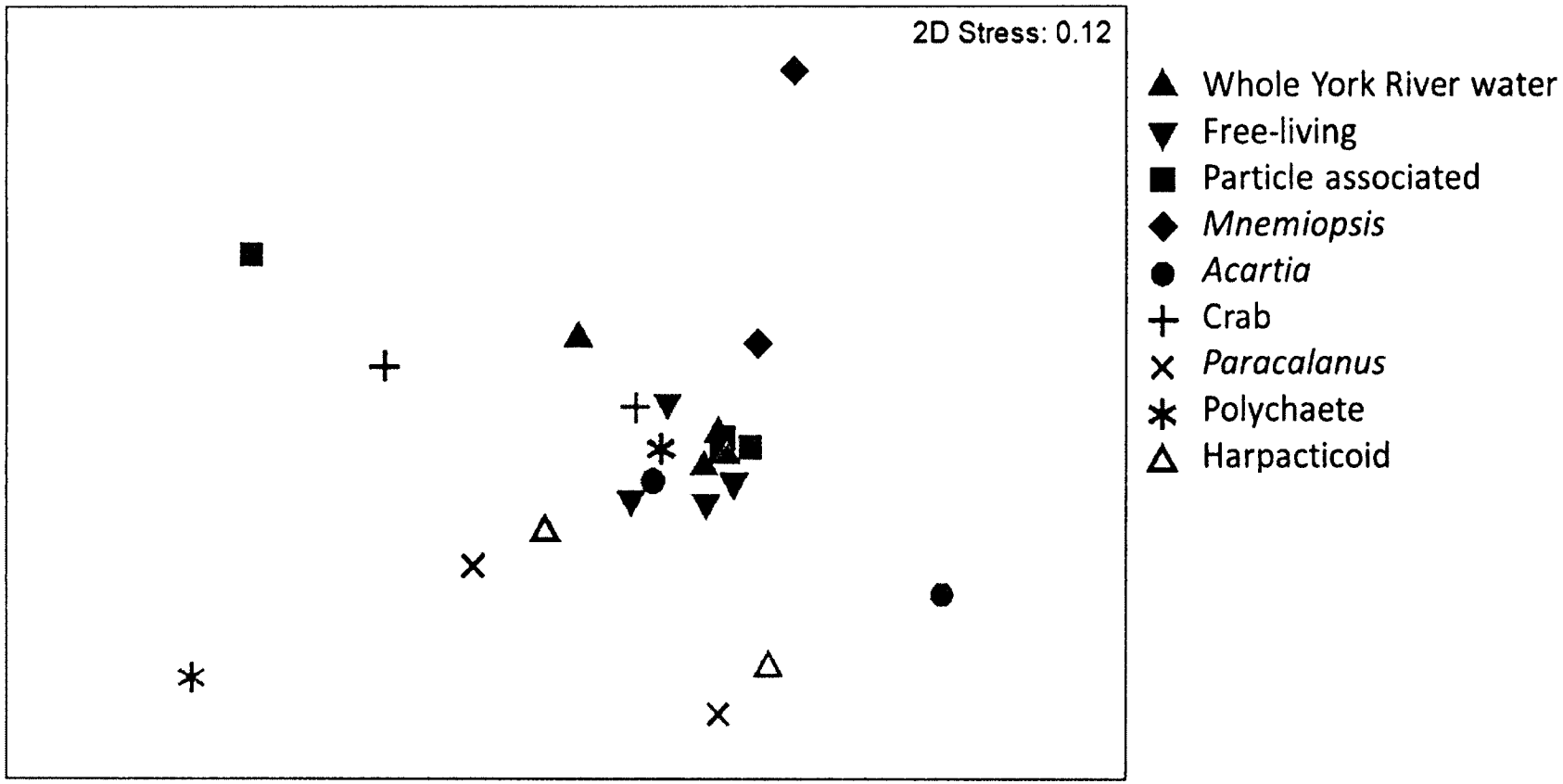
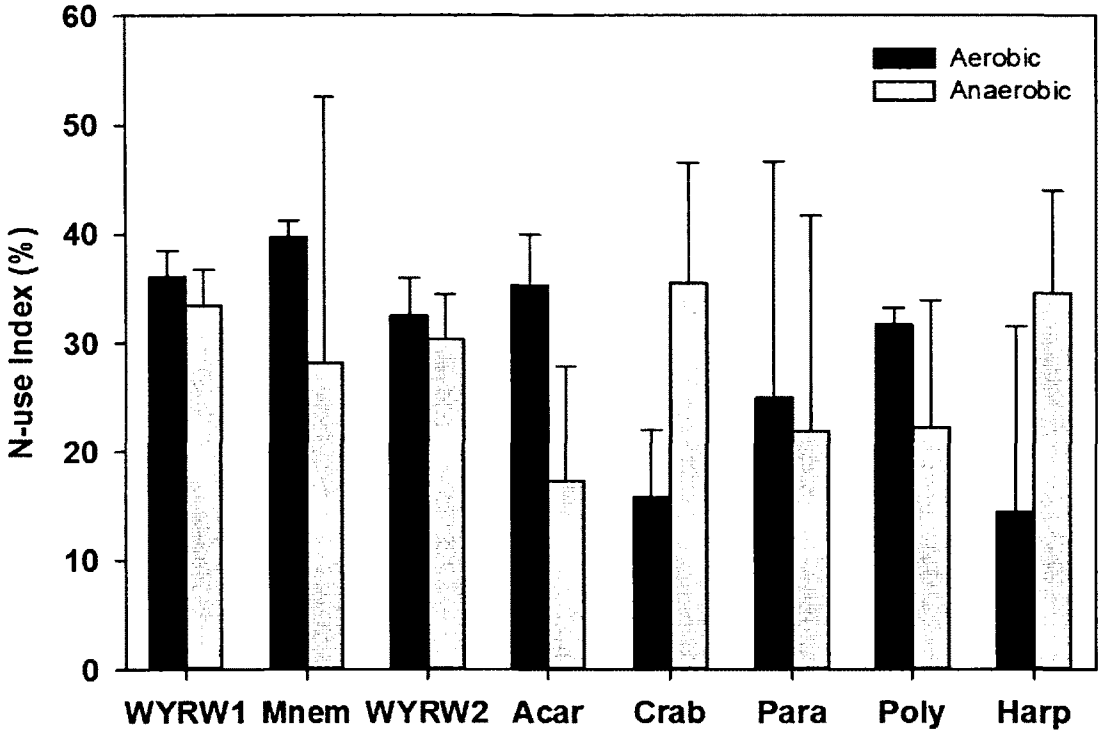

2D Stress: 0.12
Whole York River water
Free-living
Particle associated
Mnemiopsis
Acartia
Crab
Paracalanus
Polychaete
Harpacticoid

Fig. 8. Mean value (±S.D.; n=3) of N-use index for free-living, and zooplankton-associated bacteria under aerobic and anaerobic conditions. Category abbreviations are the same as in Fig. 6.


60
50
40
30
20
10
0
N-use Index (%)
Aerobic
Anaerobic
WYRW1
Mnem
WYRW2
Acar
Crab
Para
Poly
Harp

CHAPTER 5

Conclusions and Future Research

Zooplankton-associated bacteria in Estuarine Systems

Multiple studies have documented bacterial attachment to copepods and other mesozooplankton in freshwater, estuarine and marine systems (reviewed in Tang et al. 2010). Zooplankton microhabitats are microbial hotspots where bacteria are highly concentrated relative to the surrounding water (Tang et al. 2010). Externally attached and gut bacteria benefit from a consistent supply of zooplankton-produced dissolved organic carbon and nutrients (e.g. Gaudy et al. 2000, Møller 2005), and exhibit elevated production (Carman 1994, Møller et al. 2007). The bacterial communities associated with zooplankton can be different than bacterial communities in the surrounding water (Grossart et al. 2009). Additionally, the zooplankton microenvironment may support anaerobic bacteria and their associated processes such as methanogenesis (Bianchi et al. 1992, Marty 1993, de Angelis & Lee 1994), nitrogen fixation (Proctor 1997, Braun et al. 1999) and denitrification (Stief et al. 2009). While the topic of zooplankton-associated bacteria is attracting more attention, factors controlling temporal changes in zooplankton-associated bacterial abundance and community composition are poorly understood. Additionally, little is known about how the bacterial communities of sympatric zooplankton species and free-living bacteria compare (Niswati et al. 2005, Grossart et al. 2009, Brandt et al. 2010). In this dissertation I compared temporal changes in free-living bacterial community composition with bacterial communities associated with common mesozooplankton groups. Additionally I examined factors which potentially control zooplankton-associated bacterial abundance and community composition.

My research illustrated that all sampled mesozooplankton groups supported bacterial concentrations which were orders of magnitude higher than the surrounding water (Chapter 2), thus supporting the concept that living mesozooplankton act as microbial hotspots within the water column (Tang et al. 2010). Bacteria associated with the calanoid copepod *Acartia tonsa* and barnacle nauplius *Balanus* sp. were influenced by different combinations of environmental parameters, despite coming from the same aquatic environment. Both free-living and zooplankton-associated bacterial communities showed a strong positive relationship with ambient ammonium concentration. In laboratory experiments copepods supported more, loosely attached bacteria in high ammonium concentration (Chapter 2). This suggests that exchange between free-living and zooplankton-associated bacterial communities may be greater in nutrient rich systems.

Mesozooplankton also supported a bacterial community that was genetically distinct from the surrounding free-living bacterial community. While the zooplankters *Acartia* and *Balanus* did not support identical bacterial communities each month, there were many similarities between the two bacterial communities within each month. Temporal changes in environmental parameters were more important for the regulation of zooplankton-associated bacterial community composition than any difference between the zooplankters themselves (Chapter 3). Both *Acartia* and *Balanus*-associated bacteria demonstrated an unexpected secondary peak in abundance during the winter (Chapter 2). The genetic diversity of zooplankton-associated bacteria was very low during the winter and dominated by a single DGGE band. That particular DGGE band was linked to high ambient phosphate conditions and low temperatures. An analysis of carbon substrate

utilization patterns of zooplankton-associated bacteria revealed that the use of amino acids was also linked to high phosphate (Chapter 3). Thus, during winter months, one particular type of bacteria is able to efficiently exploit the zooplankton microenvironment and outcompete all other bacteria.

In laboratory experiments, copepod gut microbial communities utilized the same number of substrates as the total (epibiotic + gut) copepod microbial community under both aerobic and anaerobic conditions (Chapter 4). This indicates that gut bacteria may be responsible for most of the zooplankton-associated bacterial activities and functions. The bacterial communities associated with six different zooplankton groups collected from the York River, VA each utilized a unique subset of carbon substrates under aerobic and anaerobic conditions (Chapter 4).

My results indicate that zooplankton create a relatively stable environment which may allow for the persistence of certain bacteria within a system when ambient environmental conditions may not be conducive to their growth. Additionally, each zooplankter acts as a selective filter, retaining only a portion of bacteria it comes into contact with via the attachment of free-living bacteria or consumption of prey-attached bacteria. It is likely that each zooplankton group selects for a specific subset of bacteria and allows them to flourish. The presence of many different filters (i.e. zooplankton groups) can therefore increase the relative abundance and frequencies of less common bacterial groups (and their associated functions) within a system.

The environmental heterogeneity hypothesis predicts that biodiversity within a system would increase with habitat diversification and complexity. This pattern has been observed among freshwater phytoplankton (Richerson et al. 1970), and zooplankton

(Whiteside & Harmsworth 1967). Utilizing advances in molecular methods, this hypothesis has now been extended to free-living aquatic microbial communities (Horner-Devine et al. 2004, Shade et al. 2008). Free-living bacteria congregate around microscale nutrient patches (Blackburn et al. 1998) and it has been suggested that the abundance and connectivity of microscale patches may influence bacterial diversity by increasing habitat heterogeneity (Shade et al. 2008). From a microbial perspective, high zooplankton diversity could also mean increased habitat heterogeneity. Therefore, systems with a diverse zooplankton community would be expected to support a more diverse zooplankton-associated bacterial community than a system dominated by one or two zooplankton groups.

Of the 36 DGGE bands detected in the year-long field study, 13 were only found in association with zooplankton. Likewise, 15 of the 27 carbon substrates utilized in this study were used only by zooplankton-associated bacteria. Thus, ignoring zooplankton-associated bacteria in Chesapeake Bay could lead to an underestimation of bacterial genetic diversity by ~36% and functional diversity by 56%. Due to the ubiquity of copepods and other zooplankton in marine and freshwater systems, neglecting the presence of zooplankton-associated bacteria could lead to a significant underestimation of the overall bacterial abundance, diversity and functionality within aquatic systems.

Future Research Directions

My dissertation research has highlighted the importance of live zooplankton as microhabitats for bacteria within aquatic systems and determined some factors which influence the zooplankton-associated bacterial abundance and community composition, while also providing avenues for future research. My results indicate that the zooplankton

microenvironment may be more stable than the surrounding water in terms of substrate supply (Chapter 3). Thus, the zooplankton microenvironment may act as a buffer to changes in ambient environmental conditions. Future research should investigate if free-living and zooplankton-associated bacterial communities respond in similar manners to long-term environmental changes such as eutrophication and climate change.

Zooplankton serve as dynamic microhabitats and microbial hotspots similar to organic aggregates within aquatic systems; however, there are major differences between the two. Organic aggregates are primarily composed of dead material with a limited supply of substrates for the bacteria and no protection from flagellate grazers, while live zooplankton continually supply bacteria with substrates and may offer protection from bacterivores (Tang et al. 2010). Organic aggregates also support a bacterial community different from the free-living bacteria (Bidle & Fletcher 1995, Crump et al. 1999, Rösel et al. 2012). Given the inherent differences between aggregates and living zooplankton, it is not known if the two microbial hotspots would support similar bacterial communities. Particles can be colonized by free-living bacteria (Simon et al. 2002), which in turn may be consumed by zooplankton. Future studies should incorporate assessments of the free-living, particle-associated and zooplankton-associated bacteria to compare similarities among their compositions and determine if organic aggregates and other particles serve as transitional areas between free-living and zooplankton-associated bacterial communities.

The zooplankton gut microenvironment can be partly acidic and anoxic (Tang et al. 2011a), creating a completely different environment than the surrounding water column. My dissertation work has shown that mesozooplankton gut bacteria utilize a

wide array of carbon substrates under aerobic and anaerobic conditions, and copepod gut bacteria may account for a significant portion of the activity by copepod-associated bacteria (Chapter 4). Recent studies have explored the bacterial community composition in guts of freshwater zooplankton (Peter & Sommaruga 2008, Freese & Schink 2011, Freese & Schink 2011, Homonnay et al. 2012), but the functions of these bacteria are still unknown. Future studies could delve into functional roles of zooplankton-associated bacteria (both epibiotic and gut), possibly using transcriptomics to investigate functional gene expression within the microbial communities and by measuring anaerobic process rates. This could provide an estimation of the importance of zooplankton as sites of microbial-mediated anaerobic biogeochemical processes such as methanogenesis and denitrification.

Previous studies have demonstrated that bacteria receive many benefits from their association with zooplankton: elevated production supported by zooplankton-produced dissolved organic carbon (Møller et al. 2007), ammonium (Carman 1994) and dissolved DNA (Titelman et al. 2008), protection from environmental stresses (Tang et al. 2011b) and even transport across small scale physical boundaries like a pycnocline (Grossart et al. 2010). It is less clear, however, whether the bacteria provide any benefits to the zooplankter in the natural environments. It has been suggested that bacteria in the gut of crustaceans aid in digestion (Fong & Mann 1980, Wainwright & Mann 1982, Dempsey & Kitting 1987, Donachie & Zdanowski 1998). A diverse epibiotic and gut bacterial community may also prevent colonization and infection by pathogenic bacteria, similar to that observed in insects (Dillon & Dillon 2004) and aquaculture species (Rico-Mora & Voltolina 1995, Verschuere et al. 1999, Verschuere et al. 2000). A recent study even

indicated that the natural microbial community may play a crucial role in copepod development: Newly hatched harpacticoid copepod nauplii treated with antibiotics showed lower survivorship and arrested development in the naupliar phase (Edlund et al. 2012). With the increased addition of antibiotics to aquatic systems via municipal wastewater treatment plants and aquaculture and poultry processing effluent (e.g. Chambers & Leiker 2006), the bacterial communities associated with copepods may be altered, and copepod development compromised. Increased mortality in the naupliar stage could potentially impact zooplankton population dynamics and consequently trophic interactions.

It is evident that the relationships between bacteria and zooplankton extend far beyond the microbial loop and further research is needed to fully understand the importance of zooplankton-associated bacterial communities in both zooplankton ecology and biogeochemical cycling within aquatic systems.

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APPENDIX I

Fluorescence *in situ* Hybridization with bacterial group specific probes

MATERIALS & METHODS

Zooplankton Collection

Zooplankton were collected on a monthly basis, with a 0.5 m mouth diameter, 200μm mesh net from a fixed station in the York River Estuary, VA, a tributary of Chesapeake Bay. All samples were collected at high or near high tide during daylight hours. Multiple net tows were performed, combined and transported immediately back to the laboratory. In the lab, the combined zooplankton sample was split into 4 equal fractions. Each fraction was gently concentrated onto a 200μm mesh sieve and transferred to 0.2μm filtered Artificial Seawater (ASW) and zooplankton were allowed to clear their guts overnight. After gut clearance, one of the four fractions was used to assess zooplankton-associated bacterial genetic diversity via denaturing gradient gel electrophoresis (DGGE) and relative composition of different bacterial groups via Fluorescence *in situ* Hybridization. DGGE procedure and results are discussed in Chapter 3.

Application of Fluorescence *in situ* Hybridization

After gut clearance, zooplankton were gently concentrated onto a sterile 200μm mesh sieve and rinsed three times with 0.2μm 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. After narcotization, 5-10 individuals of each of the most prevalent zooplankton taxa were transferred to sterile microcentrifuge tubes and preserved with 300µl 4% paraformaldehyde for 3 hours. 400µl of 95% ethanol was then added to each tube and samples were stored at -40°C until analysis (Peter and Sommaruga 2008).

Each sample was centrifuged at 10°C for 10 minutes at 13000 rpm to pellet out the zooplankton. All but approximately 50µl of the supernatant was carefully pipetted off. Zooplankton were ground with a sterile pestle, and the homogenate was filtered onto a 25mm diameter, 0.2µm pore size, white polycarbonate membrane filter. Filters were stored between 2 pieces of aluminum foil at -20°C until analysis. Each filter was cut into 8 pieces to allow analysis with multiple ribosomal RNA gene probes. Seven different probes were used: the general bacterial probe EUB 338(I-III), the control probe NON338, and group specific probes for α-proteobacteria, β-proteobacteria, γ-proteobacteria, Bacteroidetes and Archaea (Table 1). The eighth filter piece was used to determine total bacterial abundance via direct counts with SYBR-gold nucleic acid stain.

The general protocol of Bouvier & del Giorgio (2002) was used with slight modifications. Briefly, each filter section was placed on a 6 µl drop of hybridization solution (percent formamide dependent upon probe; Table 1) containing 2 ng probe μl^{-1} on a parafilm covered slide. Filter pieces were incubated overnight at 46°C in an equilibrated chamber. After incubation, filters were transferred to a sterile 1.5 ml microcentrifuge tube with 750µl of the appropriate pre-warmed (48°C) wash solution (Table 1) and incubated for 15 minutes at 48°C. Filter pieces were dried on Fisherbrand P5 porosity filter paper then placed on a slide and covered with 6 µl citiflour solution and a cover slip. The entire area of each filter piece was counted on an epifluorescent

microscope with a green light filter. Binding efficiency of the probe was determined by comparing total EUB counts to direct counts made with SYBR-gold nucleic acid stain. Group specific probe counts were corrected for non-specific binding by subtracting NON338 counts and the percentage of each bacterial group was determined by dividing group specific probe counts by the general bacterial EUB counts.

RESULTS & DISCUSSION

The data from all fluorescence in-situ hybridization probes could not be used for further analysis. Positive counts within the NON338 controls were very high, indicating that some type of non-specific binding occurred. To investigate the cause of these high false positives, a series of controlled experiments were performed. First, laboratory culture-raised copepods were autoclaved. Half of the copepods were also bleached after sterilization. All sterilized copepods still exhibited positive fluorescence when mixed with the control probe. Next, an in-situ probe with the same fluorescent tag (Alexa 488), designed for the eukaryotic oyster parasite, *Bonamia*, was used in place of the bacterial probes to investigate the possibility of the interaction between the fluorophore and the sample. Positive fluorescence was observed with this probe as well.

A negative control with no probe added also indicated a substantial amount of autofluorescence by the copepod tissue. Peter and Sommaruga (2008) also used an Alexa 488 labeled probe to investigate gut bacteria in paraffin embedded cross-sections of cladocerans and noted high autofluorescence by the chitinous exoskeleton which could impair identification of probe-labeled cells. While sonication adequately broke up the copepods to release any attached or internal bacteria, the fluorescent exoskeleton was also broken into small pieces that interfered with the counting.

In order to use FISH in future experiments with copepod-associated bacteria, alternative protocols should be investigated. An alternative fluorophore with a maximum excitation outside the range of chitin autofluorescence may be considered. Additionally, sample counterstaining or microscope filter sets may help eliminate background fluorescence.

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Table 1. Targeted groups and sequences of FISH probes. % Formamide = Percentage of formamide used in hybridization solution. IS = Ionic Strength (mM NaCl) of wash solution.

Probe	Target Taxa	Sequence (5'-3')	Fluorophore	% Formamide	IS
EUB 338 (I-III)	Bacteria	GCTGCCTCCCGTAGGAGT	Alexa488	30	102
NON338	non-prokaryotes	ACTCCTACGGGAGGCAGC	Alexa488	30	102
ALF1b	α-*proteobacteria*	GCTTCGYTCTGAGCCAG	Alexa488	40	440
BET42a	*β*-*proteobacteria*	GCCTTCCCACTTCGTTT	Alexa488	30	102
GAM42a	*γ*-*proteobacteria*	GCCTTCCCACATCGTTT	Alexa488	30	102
CF319a	Bacteroidetes	TGGTCCGTGTCTCAGTAC	Alexa488	30	80
ARCH915a	Archaea	GTGCTCCCCCGCCAATTCCT	Alexa488	25	102

APPENDIX II

Alternative Multiple Regression Models Assessed by AIC

Table 1 Potential multivariate models for the prediction of free-living bacteria, assessed by AIC. Models within 2 of the minimum AICc are presented. 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. Asterisks denote significant values: * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

	Model Number		
	78	**80**	90
Intercept	1.221	-6.704*	6.766***
Temp	0.434***	0.548***	-0.007
Sal	0.209**	0.520***	-----
NH_4	-3.422***	1.216**	0.055
PO_4	18.250**	-----	-1.488
Chl *a*	-----	1.771***	-0.288
FLB	NA	NA	NA
temp x sal	-0.020***	-0.021***	-----
temp x NH_4	-0.003	-0.0003	0.008*
temp x PO_4	0.036	-----	-0.154***
temp x chl a	-----	-0.032***	0.013
temp x FLB	NA	NA	NA
Sal x NH_4	0.169***	-0.052**	-----
Sal x PO_4	-0.784**	-----	-----
Sal x Chl a	-----	-0.064***	-----
Sal x FLB	NA	NA	-----
NH_4 x PO_4	-0.869**	------	0.402***
NH_4 x Chl a	-----	0.07**	-0.101*
NH_4 x FLB	NA	NA	NA
PO_4 x Chl a	-----	-----	0.914***
PO_4 x FLB	NA	NA	NA
Chl a x FLB	NA	NA	NA
AICc	-50.108	-50.983	-49.396
R^2	0.911	0.913	0.909
p value	<0.001	<0.001	<0.001
weighted probability	0.306	0.475	0.215

Table 2 Potential multivariate models for the prediction of *Acartia*-associated bacteria, assessed by AIC. Models within 3 of the minimum AICc are presented. 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. Asterisks denote significant values: * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

	Model Number					
	78	**80**	**108**	**112**	**114**	**116**
Intercept	11.913***	20.757***	21.577***	14.000*	25.08***	-0.815
Temp	-0.683***	-0.381	-0.425	0.322	3.248	1.077**
Sal	-0.191	-0.521*	-0.591*	-0.222	-0.904**	-----
NH_4	2.564	-5.018***	-2.163	-4.197***	-----	-2.813*
PO_4	19.047	------	-32.051	-----	-58.93***	15.06**
Chl *a*	-----	-2.565**	-2.358**	-2.023	-6.865**	1.433**
FLB	-----	-----	-----	-2.605e-6	-1.862e-5	1.919e-6
temp x sal	0.040***	0.016*	0.020*	-0.014	-0.014	-----
temp x NH_4	-0.010*	-0.034***	-0.040***	-0.045**	-----	-0.041
temp x PO_4	-0.729***	------	0.079	-----	0.017	-1.672**
temp x chl a	-----	-0.043***	0.026*	0.016	-0.032	-0.141**
temp x FLB	-----	-----	-----	3.298e-8	-5.741e-8*	-2.08e-7*
Sal x NH_4	-0.153	0.255***	0.131	0.233***	-----	-----
Sal x PO_4	-0.697	-----	1.377	-----	2.644***	-----
Sal x Chl a	-----	0.099**	0.101**	0.089	0.338**	-----
Sal x FLB	-----	-----	-----	7.578e-8	9.125e-7	-----
NH_4 x PO_4	4.318***	-----	-----	-----	-----	3.669***
NH_4 x Chl a	-----	-0.041	-----	-----	-----	0.344***
NH_4 x FLB	-----	-----	-----	-----	-----	9.846e-7***
PO_4 x Chl a	-----	-----	-----	-----	-----	-----
PO_4 x FLB	-----	-----	-----	-----	-----	-----
Chl a x FLB	-----	-----	-----	-----	-----	-----
AICc	126.078	123.147	125.816	125.816	125.816	125.816
R^2	0.587	0.597	0.610	0.610	0.610	0.610
p value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
weighted probability	0.083	0.359	0.095	0.095	0.095	0.095

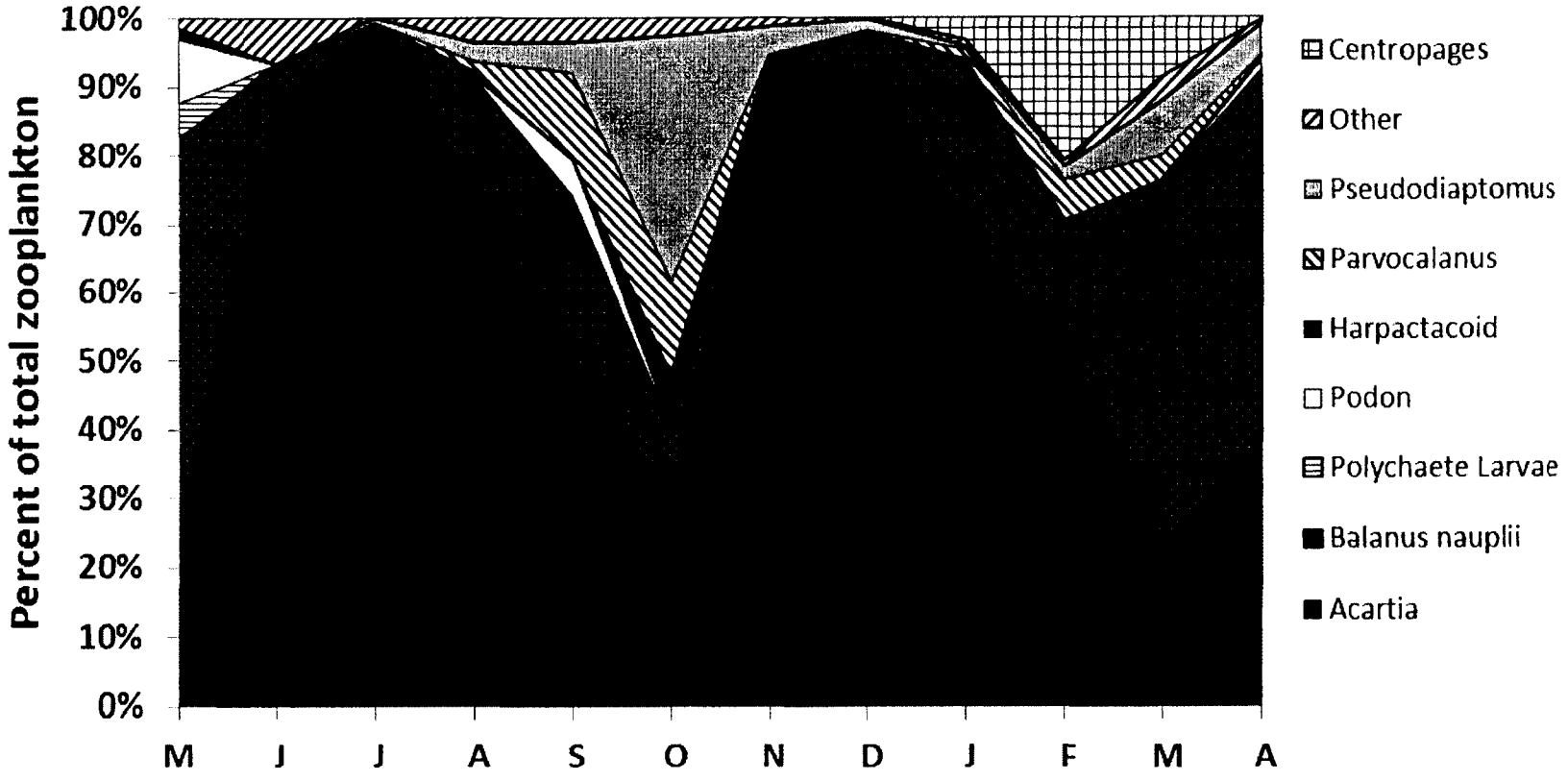
Table 3 Potential multivariate models for the prediction of *Balanus*-associated bacteria, assessed by AIC. Models within 2 of the minimum AICc are presented. Values are the coefficients for each of the predictor variables in the model. Temp = temperature, Sal = salinity, NH_4 = Ammonium, PO_4 = Phosphate, FLB = free-living bacteria, NA = not applicable. Asterisks denote significant values: * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

	Model Number		
	26	**58**	**70**
Intercept	9.045***	8.469***	9.212***
Temp	-----	-----	-----
Sal	-----	0.023	-----
NH_4	0.173***	1.288	0.078
PO_4	2.544***	9.907	2.406***
Chl *a*	-----	-----	-0.053
FLB	-----	-----	-----
temp x sal	-----	-----	-----
temp x NH_4	-----	-----	-----
temp x PO_4	-----	-----	-----
temp x chl a	-----	-----	-----
temp x FLB	-----	-----	-----
Sal x NH_4	-----	-0.059	-----
Sal x PO_4	-----	-0.299	-----
Sal x Chl a	-----	-----	-----
Sal x FLB	-----	-----	-----
NH_4 x PO_4	-0.245	-0.118	-0.265
NH_4 x Chl a	-----	-----	0.031
NH_4 x FLB	-----	-----	-----
PO_4 x Chl a	-----	-----	0.234
PO_4 x FLB	-----	-----	-----
Chl a x FLB	-----	-----	-----
AICc	73.454	75.264	74.743
R^2	0.7067	0.7216	0.7231
p value	<0.0001	<0.001	<0.001
weighted probability	0.2478	0.100	0.120

APPENDIX III

Zooplankton Community Composition

Fig. 1. Relative composition of the zooplankton community within the York River from May 2010 – April 2011.


Percent of total zooplankton
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
M
J
J
A
S
O
N
D
J
F
M
A
Centropages
Other
Pseudodiaptomus
Parvocalanus
Harpactacoid
Podon
Polychaete Larvae
Balanus nauplii
Acartia

VITA

SAMANTHA LYNN BICKEL

Born January 24, 1983 in Watertown, WI. Graduated from Watertown High School, Watertown, WI in 2001. Earned Bachelor of Arts degrees in Biology and Mathematics from Carthage College in 2005. Received Master of Science in Marine Science from College of William & Mary, School of Marine Science (VIMS) in 2009. Entered the Ph.D. program at the College of William & Mary, School of Marine Science (VIMS) in 2009.