Factors influencing the abundance, community composition and activity states of bacterioplankton from the tidal freshwater James River

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Factors influencing the abundance, community composition and activity state of bacterioplankton from the tidal freshwater James River

A thesis submitted in partial fulfillment of the requirements for the degree of M.S. at Virginia Commonwealth University

by

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Aquatic bacteria respond to changing environmental conditions through a variety of mechanisms including changes in abundance, shifts in community composition and variable activity states. In the tidal-freshwater James River, variation in bacterial abundance was linked to nutrient availability and autochthonous production with highest bacterial densities associated with low-nutrient, high-chlorophyll a conditions. Laboratory experiments revealed that bacterial growth rates were nutrient limited at the low-nutrient site, while co-limitation (nutrients, glucose, light) was apparent at the high nutrient site. Despite large differences in abundance, community composition was similar based on TRFLP and 16S rDNA pyrosequencing. Community similarity was lower among rRNA libraries suggesting that variable activity states are prevalent in natural communities. Rare taxa were more likely to be metabolically active and were capable of dramatic growth under microcosm conditions.
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Chapter 1

Introduction

Heterotrophic bacteria account for the bulk of energy and material cycling within ecosystems. They regulate the availability and cycling of inorganic nutrients (Kirchman, 1994) and organic matter (OM; Azam et al., 1983; Sherr and Sherr, 1988; Apple et al., 2004), which in turn determines estuarine delivery to coastal regions where eutrophication can lead to anoxia, destruction of habitat, and losses to commercial fisheries. Aquatic bacterial growth and production are regulated by numerous factors including resource availability, climatic variables and hydrologic conditions. Mechanisms of bacterial community response to these factors are poorly understood, but include shifts in bacterial abundance, community composition, and activity state.

Quantity and quality of OM is often a limiting factor for bacterial growth rates and abundance (i.e., cell density). Allochthonous OM, which has been partially degraded in terrestrial soil systems, is considered a poor substrate for bacteria because of chemical recalcitrance (Hessen and Tranvik, 1998). However, algae release a variety of labile compounds (Hellebust, 1974; Fogg, 1983; Jorgensen, 2009), which are readily utilized by bacterioplankton (Descy et al., 2002; Malinsky-Rushansky and Legrand, 1996). Hence, bacterial production (Lancelot and Billen, 1984; Cole et al., 1988; White et al., 1991; del Giorgio and Pace, 2008) and growth efficiency (del Giorgio and Cole, 1998) frequently correlate with net primary production (NPP). In some systems, however, bacteria are primarily supported by terrigenous materials (Coffin et al., 1989; Hullar et al., 1996; Kelley et al., 1998; Berggren et al., 2010) and coupling between bacterial and primary production
is weak or non-existent (Ducklow and Kirchman, 1983; Painchaud and Therriault, 1989; Schultz et al., 2003; Shiah and Ducklow, 1994).

Nutrients (N and P) can also impact bacterioplankton directly by limiting growth rates or indirectly through their effects on phytoplankton growth and production of autochthonous OM. For example, dissolved organic nitrogen availability has been shown to affect bacterial extracellular enzyme production (Stepanauskas et al., 1999) and sources of nitrogen can affect bacterial community composition (Frette et al., 2009). Alternatively, phytoplankton production is often nutrient-limited (Graneli et al., 1990; Ryther and Dunstan, 1971) which reduces the pool of autochthonous OM available to bacteria.

Finally, in some systems, physical conditions are more important than nutrients and OM resources. Salinity has been found to drive bacterial community composition globally (Lozupone and Knight, 2007) and within estuaries (Troussellier et al., 2002; Crump et al., 2004; Henriques et al., 2004). Other studies have found bacterial communities to be primarily controlled by temperature (Shiah and Ducklow, 1994, 1995; Schultz et al., 2003; McManus et al., 2004; Apple et al., 2004) or other seasonal parameters (Kan et al., 2007). In rivers and estuaries, discharge may be an important seasonal variable affecting residence time, which in turn regulates the development of bacterioplankton communities (Crump et al., 2004).

In addition to shifts in abundance and production, bacterial communities may respond to resource and environmental conditions through changes in community composition. The use of molecular-genetic techniques has enabled recent advances in our understanding of factors influencing bacterial community composition. Longitudinal phylogenetic successions have been described in several rivers and estuaries, where freshwater reaches are generally dominated by Betaproteobacteria, Actinobacteria, and Bacteroidetes (Kirchman et al., 2005; Crump et al., 2009, 2004). Within freshwater reaches, bacterial community composition has been found to correlate with various factors including quantity and quality of OM, inorganic nutrients, water temperature and flow rate (Crump et al., 2009; Crump and Hobbie, 2005; Winter et al., 2007; Adams et al., 2010). Bacterial community doubling time and residence time within an estuary may determine the extent to which community composition is dictated by internal (OM and nutrients) versus external (i.e., climate and temperature) factors (Crump et al., 2009, 2004).
Most prior studies of bacterial community composition have relied on the characterization of the microbial DNA present in environmental samples. However, DNA is present in dormant and even dead cells which typically account for a large proportion of bacterial communities (Ogram et al., 1987; Sherr et al., 1999; del Giorgio and Bouvier, 2002; Choi et al., 1999; Cole, 1999). Staining with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) has shown that the proportion of active cells is typically 1-10% (Choi et al., 1996; del Giorgio and Scarborough, 1995; Karner and Fuhrman, 1997). These findings suggest that essential functions of bacterial communities are carried out by a small and unknown proportion of the community that is metabolically active (Bakken and Olsen, 1987; Epstein, 2009). Shifts in activity state have frequently been invoked as an explanation for functional changes being observed along environmental gradients where community composition changes little (Freitag et al., 2006; Crump et al., 2007; Kan et al., 2007).

While most DNA-based methods cannot distinguish active from inactive taxa, simultaneous characterization of community DNA and RNA may provide a means for identifying the metabolically active fraction of bacterial communities. Bacterial growth rates correlate with cellular rRNA content (Poulsen et al., 1993; Wagner, 1994; Rosset et al., 1966; Manefield et al., 2002; Felske et al., 1998); therefore, the presence of bacterial 16S rRNA indicates taxa which are metabolically active. This approach has been utilized in community fingerprinting studies which provide information on community similarity but not specific taxonomic identification (Moeseneder et al., 2001, 2005; Troussellier et al., 2002; Winter et al., 2007; Rodriguez-Blanco et al., 2009; Koizumi et al., 2003; Delbes et al., 2001; Juottonen et al., 2008; Pennanen et al., 2004). A recent paper by Jones and Lennon (2010) is the first to combine the DNA-RNA approach with high-throughput sequencing. They assembled RNA and DNA libraries from two samples collected in nearby lakes to compare the taxonomic composition of the communities and their metabolically-active components. Community dominants differed among the RNA- and DNA-based libraries indicating highly variable activity states among taxa. Rare taxa were more likely to be metabolically active based on their relative contribution to the RNA and DNA libraries (RNA%:DNA%). This approach has yet to be tested among multiple samples collected from a single site or in an experimental setting.

In this study, we characterized bacterial responses to varying resource and environmental conditions based on changes in abundance, activity state and community composition. This research focused on the tidal-freshwater James River (Virginia) where seasonal and
spatial variation in phytoplankton production and terrigenous inputs provided a dynamic range of OM and nutrient resource conditions. The upper portion of the tidal-freshwater segment of this estuary is dominated by high nutrients but low algal biomass, whereas the lower segment exhibits high algal biomass and low nutrients (Bukaveckas et al., In review). Our project exploited these longitudinal gradients in nutrient availability and autochthonous OM sources to explore how bacterial communities respond to resource availability. In order to directly test for resource effects, we also conducted a series of microcosm experiments in which nutrients and OM availability were amended to test for effects on bacterial abundance, community composition and activity state.
Chapter 2

Methods

We utilized observational and experimental approaches to investigate microbial responses to resources and environmental conditions. Environmental sampling entailed monthly collections at two sites in the tidal freshwater James River, which differed with respect to nutrient availability and autochthonous production. Microcosm experiments were conducted wherein river water collected from the two sampling locations was subjected to manipulations of nutrients and labile organic matter. Microbial community responses were characterized by measuring changes in abundance, community composition, and activity/dormancy.

2.1 Field methods

Samples from the James River were collected monthly throughout the peak growth season (May–September 2009) from two main channel sites, JMS99 and JMS75, and two adjacent near-shore sites (VCU Rice Center Pier and Osborne Landing). The two sites were selected based on prior work that revealed differences in chlorophyll a (CHLa), net primary production (NPP) and bacterial abundance (BA; Bukaveckas et al., In review). These sites are also part of the monthly monitoring network for the Chesapeake Bay Program. Water was collected near the surface (<1 m) with a Van Dorn water sampler and transported in 2-L opaque Nalgene bottles back to the laboratory at ambient temperatures to avoid disruption of bacterial communities. Vertical profiles of underwater irradiance were
determined by taking measurements at 0.5-m intervals with a LI-COR model LI-1400 data logger. These profiles were used to calculate light attenuation coefficients (Kirk, 1994) and average irradiance within the water column (Gosselain et al., 1994) in order to determine appropriate irradiance for incubating microcosms and primary production samples (Bukaveckas et al., In review). Ancillary water quality data (temperature, pH and dissolved oxygen) were collected using YSI instruments. Water for establishing microcosm experiments was collected from the near shore sites and transported back the laboratory in 20-L carboys.

2.2 Microcosm experiments

Microcosm experiments were performed each month wherein water collected from JMS99 and JMS75 was incubated under five treatment conditions: 1) ambient conditions of light, nutrients and DOC (to test for enclosure effects), 2) enhanced light (to stimulate phytoplankton OM production), 3) elevated glucose (to provide a labile OM source), 4) elevated nutrients (N and P), and 5) a combination of enhanced light, glucose and nutrients (to test for co-limitation). Triplicate microcosms, consisting of 1-L river water in sterile 2-L Erlenmeyer flasks with foam stoppers, were used for each treatment group (total = 15 microcosms per site per month) and incubated at ambient river temperatures for 48 hours. Glucose treatments consisted of an addition of 1.0 mg L$^{-1}$ glucose-C, equivalent to 20% of ambient DOC concentrations in the James River Estuary (c.f. Hoikkala et al., 2009). Nutrient treatments consisted of both phosphorous ($\text{Na}_2\text{HPO}_4$) and nitrogen ($\text{NH}_4\text{Cl}$ and $\text{NaNO}_3$) additions to approximately double ambient nutrient levels (for JMS99 experiments: 33 µg L$^{-1}$ P–PO$_4$, 67 µg L$^{-1}$ N–NH$_4$, and 400 µg L$^{-1}$ N–NO$_3$; for JMS75 experiments: 15 µg L$^{-1}$ P–PO$_4$, 60 µg L$^{-1}$ N–NH$_4$, and 50 µg L$^{-1}$ N–NO$_3$). At the conclusion of the experiment, water was harvested from microcosms for the analyses described below.
### 2.3 Sample analysis

Samples collected from the river and from microcosms were analyzed for Dissolved Organic Carbon (DOC), turbidity, inorganic nutrients, CHLa, bacterial abundance and turbidity. DOC samples were filtered through Whatman GF/A filters and acidified to a pH of 2 with concentrated HCl for later analyses using a Shimadzu TOC analyzer. Turbidity was measured optically with a Hach Turbidimeter. Nutrient concentrations (PO$_4^{3-}$, NO$_3^-$ and NH$_3$) were determined colorimetrically using a Skalar Segmented Flow Analyzer (APHA 1992). Phytoplankton biomass was measured as CHLa by filtration onto Whatman GF/A filters and extraction in 10 ml aqueous (90%) acetone at 4$^\circ$C for 24 hours. Pigment concentrations were measured using a Turner Designs fluorometer, which is calibrated annually with a primary standard and checked at each use using a secondary standard.

Water collected from the river was also used to determine primary production and respiration. Whole water was dispensed into 60 ml biological oxygen demand (BOD) bottles. Three replicates were preserved immediately to establish initial values, 3 replicates were incubated under in-situ light conditions for 8 hours, and 3 replicates were incubated in the dark for 24 hours. Initial and final dissolved oxygen (DO) concentrations were measured using the Winkler titration method (Dawes, 1988). Increased DO in the “light” bottles gave the rate of NPP while decreases in DO in the “dark” bottles gave the rate of community respiration (CR).

Both total bacteria and metabolically active cells were enumerated. Cells were stained using the LIVE/DEAD® BacLight™Bacterial Viability and Counting Kit (Invitrogen) and counted manually under epifluorescent microscopy, yielding counts of “live” and “dead” bacteria (i.e., intact cells and those with compromised membranes). Triplicate 1-ml samples of river water (or diluted river water if bacterial abundance was high) were incubated for 10 minutes in the dark with 1 $\mu$l red-fluorescent propidium iodide and 2 $\mu$l SYTO® 9 dye. Stained cells were filtered onto 0.2 $\mu$m black polycarbonate membrane filters (Millipore). Filters were affixed to slides with non-fluorescing immersion oil and viewed under 1000x magnification (oil immersion) on a uv-equipped Olympus BX41 microscope. Ten random fields or 250 cells were counted. Metabolically active cells, or those that were actively respiring, were enumerated by staining with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Sigma-Aldrich) and counting as described above. Two ml aliquots were incubated...
with 200 µl CTC solution (15.15 mg ml\(^{-1}\)) in foil-wrapped 15 ml centrifuge tubes for 4 hours on a rotary shaker set to 25 rpm. After incubation, samples were fixed by adding 53 µl formalin (37% solution of formaldehyde). Slides were prepared in a darkened room and fluorescing cells were counted within 24 hours.

2.4 Genetic analysis

Samples of bacterial communities were preserved for DNA- and RNA-based molecular analysis. Triplicate 150 ml samples were filtered through 0.22 µm nitrocellulose filters (Millipore). Filters for RNA analysis were saturated with 1 ml RNAlater (Ambion) to prevent RNA degradation. All filters were stored at −80°C for later use.

Extraction of DNA from bacterial samples was carried out using a WaterMaster DNA Purification Kit (Epicentre Biotechnologies) according to manufacturer’s protocols. DNA purifications were verified by gel electrophoresis and quantified using a Nanodrop Spectrophotometer. 16S rDNA fragments for terminal restriction fragment length polymorphism (TRFLP) were amplified by polymerase chain reaction (PCR) as follows: initial denaturation at 95°C for 3 minutes, 25 cycles of denaturation at 94°C for 45 seconds, initiation at 57°C for 1 minute, and extension at 72°C for 2 minutes, and a final extension at 72°C for 7 minutes. PCR reaction mixtures consisted of 1X PCR Buffer II (Applied Biosystems), 3 mM MgCl\(_2\), 0.2 mM (each) deoxynucleoside triphosphates (GeneAmp), 0.2 µM forward and reverse primers, 0.4 µg bovine serum albumin (Roche), 2.5 units Taq polymerase (Amplitaq, Applied Biosystems), and 0.5 ng template DNA in a final volume of 50 µl. The primers used for TRFLP were 27F-FAM, with a fluorescently-labeled 5’ end, and 1492R (Invitrogen, Polz and Cavanaugh, 1998, TableA.1). PCR products were visualized by gel electrophoresis to check that products were suitable for TRFLP. Products were then purified using a Qiagen MinElute kit. Restriction digest reaction mixtures consisted of 7 µl purified PCR product, 1 µl NEBuffer 4 (Biolabs), and 20 units MspI restriction enzyme (Biolabs) in a final volume of 10 ul. Reaction mixtures were incubated for 6 hours at 37°C followed by a 20 minute incubation at 65°C. Products were again purified with a Qiagen MinElute kit and were run through capillary electrophoresis on a MegaBACE 1000/4000 Series Sequencer (Amersham Biosciences) with an injection period of 100 sec at 3,000 V and a total run of 180 minute at 10,000 V.
DNA was prepared for pyrosequencing by amplifying with PCR using the barcoded primers listed in Table A.1 and the PCR protocol described above except that 1 ng template DNA was included in each reaction and the annealing temperature was 50°C. Primers were designed in PRIMROSE (Altschul et al., 1990) to span the V5-V7 hypervariable regions. RNA was extracted using a RiboPure-Bacteria™ kit (Ambion) according to the manufacturer’s protocol and checked by gel electrophoresis. cDNA (hereafter referred to as rRNA for clarity) was synthesized from RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with one of the four forward primers and was then amplified by PCR as described above. PCR products were sequenced on a Roche 454 GS-FLX pyrosequencer by the VCU Nucleic Acids Research Facility.

2.5 Data analysis

Correlations between environmental variables were identified by simple linear regression with Sidak corrected significance levels in R. Principle component analysis was performed in PAST (Hammer et al., 2001). Significant differences between experimental treatment groups and sampling sites were identified by one-way and two-way ANOVA using JMP 8.

TRFLP-generated electropherogram peaks were analyzed with Fragment Profiler Version 1.2 (Amersham Biosciences) software. Peaks were binned according to read number with a minimum of three peaks required for each bin and minimum bin size of 0.5 bp. Shoulder peaks, peaks outside the 50-1000 bp range, and peaks less than 75 rfu in height were excluded. Peak data was then converted into a binary matrix with 1 denoting presence and 0 denoting absence of a particular terminal restriction fragment. The resulting matrix was analyzed using non-metric multidimensional scaling (NMDS) with Jacquard similarity measures in PC-ORD (Mather, 1976; Kruskal, 1964). Significant differences between groups were identified by ANOSIM in PAST (Hammer et al., 2001). Correlations between peak patterns and environmental variables were identified by Mantel tests with distance between environmental measurements determined using Gower indices.

Tag pyrosequences were trimmed and assigned to a taxonomic hierarchy using the Ribosomal Database Project (RDP) 10 Pyrosequencing Pipeline and Classifier (Wang et al., 2007). Similarity between sequences libraries were described by NMDS with Sorensen
distance measures and tested by multiple response permutation procedure (MRPP). The relationship between rDNA-based rank and the relative recovery of rRNA and rDNA was tested by binomial logistic regression in JMP8.
Chapter 3

Results

3.1 Conditions in the tidal freshwater James River

3.1.1 Physical conditions

The study period was generally characterized by warm water temperatures and declining river discharge (Figure B.1). Water temperatures ranged from 18°C (May) to 30°C (August) and were similar at both sampling locations. Discharge decreased from 541 m³s⁻¹ in May to 34 m³s⁻¹ in September. Turbidity was highest during months with elevated discharge (May and June) and, during periods of low discharge, was higher at river mile 75 relative to 99 due to its proximity to the estuarine turbidity maximum. Differences in water clarity (light attenuation) followed patterns in turbidity (Table A.2). Inorganic nutrient concentrations were similar at the two sites during months with elevated discharge (May-June). During July-August, SRP and DIN concentrations were higher at the landward site (JMS99) relative to JMS75. Differences in DIN were driven by NO₃⁻, which was significantly higher at river mile 99 ($p < 0.05$; one-way ANOVA).

3.1.2 Algal and bacterial abundance

Phytoplankton abundance (CHLa) and net primary production (NPP) were higher at river mile 75 compared to river mile 99 (Figure B.2; $p = 0.006$ and 0.008 respectively;
one-way ANOVA of pooled months and sites). Average CHLa was 5-fold higher at JMS75 (mean = 79±17 µg L⁻¹) relative to JMS99 (mean = 15±5.0 µg L⁻¹) with peak concentrations occurring in July (JMS75) and September (JMS99). NPP was 6-fold higher at JMS75 than at JMS99 (mean = 696±154 and 106±8.52 µg O₂ L⁻¹hr⁻¹, respectively). Live BA was higher at river mile 75 with an average value of 80 ×10⁶ cells ml⁻¹ which was almost three times greater than river mile 99 (average = 27 ×10⁶ cells ml⁻¹). There were no significant differences between sites in the proportions of live or active cells (p = 0.23; one-way ANOVA). On average, 84% of cells at both sites were alive (intact membranes) while only 8% of live cells were metabolically active. Bacterial abundance (live and total) increased at both sites throughout the study period, reaching the highest levels in September. At JMS75, live BA was significantly higher in September than in June, July, or August (all p ≤ 0.0002; two-way ANOVA). Linear regression of logarithmically transformed data showed that total, live, and active bacterial abundances were significantly correlated with CHLa, NPP, CR, and DOC (all p ≤ 0.02, all R² ≥ 0.64; Figure B.3).

3.1.3 **Principle component analysis of environmental data**

Principle component analysis (PCA) of main-channel environmental variables (discharge, specific conductivity, secchi depth, turbidity, inorganic nutrients, NPP, CR, CHLa, and DOC) separated samples by both site and month (Figure B.4). PCA identified two composite variables, PC1 and PC2, which explained 71% of the variation in the environmental dataset, with 42% attributed to PC1 and 29% attributed to PC2. PC1 had high factor loadings (≥ ±0.85) for CHLa, NPP, and CR, while PC2 corresponded strongly with specific conductivity and turbidity. The two sampling sites were most similar in May when discharge was high and then diverged as CHLa, NPP and CR increased at JMS75.

3.1.4 **TRFLP-based assessment of community composition in the James River**

Non-metric multidimensional scaling of TRFLP results revealed that bacterial communities from the same month were more similar than bacterial communities from the same site.
Chapter 3. *Results*

(Figure B.5, combined $R^2 = 0.56$, stress = 0.20). Community composition in May differed significantly from July, August, and September (all $p = 0.002$; ANOSIM). September communities differed significantly from May, June, and July communities ($p = 0.0015$, 0.005 and 0.0016 respectively). Overall, our findings show that bacterial abundance varied by both site and month while community composition varied primarily by month. Based on differential staining, the proportion of cells that was metabolically active did not vary among sites or dates.

3.2 Results from microcosm experiments

3.2.1 Factors affecting bacterial abundance in microcosms

In all three JMS75 experiments, CHLa increased under nutrient and combined treatments, reaching values of 150-300 $\mu$g L$^{-1}$ (Figures B.6,B.7,B.8). Phytoplankton responses in JMS99 experiments were more variable. CHLa did not respond in July, but responded to nutrient additions in August and to enhanced light in September. NPP tended to mimic CHLa patterns, with high rates of production (1500–2500 $\mu$g O$_2$ L$^{-1}$hr$^{-1}$) under nutrient and combined treatments in the JMS75 experiments and under nutrient treatments, light treatments, or both in the JMS99 experiments. CR was similar across treatment groups, rarely exceeding 100 $\mu$g O$_2$ L$^{-1}$hr$^{-1}$.

Bacterial abundances responded to nutrient and combined treatments in all three experiments at JMS75, although this response was significant ($p < 0.05$) only in September. In the August JMS75 experiment, BA also responded to the glucose treatment. The proportion of cells that was metabolically active exhibited a significant response to the combined treatment (July experiment; $p = 0.04$; Tukey–Kramer HSD) and to the nutrient and combined treatments (August; $p = 0.01$ and 0.009 respectively; Tukey–Kramer HSD). At JMS99, bacteria abundances responded to all treatments during the August and September experiments and to the combined treatment only in the July experiment. Elevated light, glucose, and nutrients led to 200%, 115%, and 180% increases in BA respectively in August and 162%, 113%, and 146% increases in September. In both experiments, BA was significantly higher in the combined treatment group ($p = 0.037$ in August, $p = 0.006$ in
September; Tukey–Kramer HSD). The proportion of cells that was metabolically active (∼10%) was not responsive to any treatments in the JMS99 experiments.

### 3.2.2 TRFLP-based assessment of community composition in microcosms

TRFLP analysis of experimental samples showed that differences among treatment groups were small in relation to intra-group variability (Figure B.9). At both sites, the light, nutrient, and combined treatment groups separated but not significantly from the control group (all \( p = 0.1 \); ANOSIM). Grouping between the control and initial samples was weak indicating some degree of enclosure effect. When community TRFLP profiles from both experiments were plotted in the same ordinate space, the JMS75 experiment showed a greater degree of divergence, especially between the control and treatment groups, perhaps indicating a greater community response to experimental treatments (data not shown).

Overall, the experimental results show strong and consistent effects of nutrient addition on NPP, CHLa and BA at JMS75. In two of the three JMS75 experiments, increases in bacterial abundance were accompanied by increases in the proportion of active cells. BA, but not CHLa or the proportion of active bacteria, was responsive to combined treatments in all three JMS99 experiments. TRFLP did not indicate a significant shift in community composition during either the JMS75 or JMS99 September experiment.

### 3.3 Results from pyrosequencing of 16S rDNA and rRNA

#### 3.3.1 Sequencing statistics and similarity among libraries

After trimming sequences and discarding poor reads, 156,872 sequences were assigned to a hierarchy using RDP’s Bayesian classifier. Of these, 85% were assigned to phyla, 81% were assigned to classes, and 38% were identified to the genus level. Twelve sequences were classified as putative chimeras. Libraries contained on average, \( 4900 \pm 200 \) sequences...
and had a richness of 137±4 taxa. rDNA-based libraries had significantly higher evenness and Shannon-Weiner diversity relative to rRNA libraries \((p = 0.02\) and 0.007 respectively; one-way ANOVA). Biological replicate libraries were prepared for three samples: JMS75 May rDNA, JMS75 August rDNA and JMS75 August rRNA. A frequency distribution of Sorensen distance measures was generated to compare the similarities among replicates to the overall sample pool. Libraries for the May and August DNA replicates were highly similar (Sorensen distances in the bottom 1% and 10% respectively) whereas the RNA replicates exhibited greater dissimilarity (Sorensen distances in the bottom 25%).

rDNA and rRNA libraries were separated in ordination space \((p = 0.002; \text{MRPP})\) with rRNA samples exhibiting greater dispersion than rDNA samples (Figure B.10). Consistent with TRFLP results, pyrosequencing revealed greater variation among months than between sites \((p = 0.04\) and 0.33 respectively; MRPP). Samples from microcosm experiments also exhibited consistent separation between rDNA and rRNA libraries across all treatment groups. Similar shifts were observed in response to experimental treatments for both sites and both libraries with the combined treatments located at the greatest distance from the initial and control samples (Figure B.10).

### 3.3.2 Sequencing-based assessment of community composition

Cyanobacteria and Betaproteobacteria were the dominant classes, accounting for \(\sim 40\%\) of rDNA sequences from environmental samples (Figure B.11). Sphingobacteria were also moderately abundant at both sites. Betaproteobacteria peaked at JMS99 in June with an astonishing relative abundance of 75%, whereas Cyanobacteria appeared to be a major component of increasing bacterial density in late summer at both sites. In environmental samples, 48% of those rDNA sequences identified as Betaproteobacteria were classified as Burkholderiales and another 37% were unclassified. Of those rDNA sequences identified as Cyanobacteria, 96% were unclassified Deferribacterales. Cyanobacteria Family 1.1 always accounted for a disproportionate number of rRNA sequences with 35% of Cyanobacteria sequences attributed to Prochlorococcus.

In both the JMS99 and JMS75 September experiments, community composition in control microcosms differed significantly from that in combined treatment microcosms \((p = 0.006;\)
MRPP). Increasing Betaproteobacteria was an important contributor to overall increasing bacterial abundance (Figure B.11). Betaproteobacteria were only moderately abundant in initial samples (~15%), but became dominant in the combined treatment microcosms (50%). Although Burkholderiales were important in environmental-derived rDNA libraries, Neisseriaceae, primarily Chromobacteria, were more prominent in the experiments, accounting for over 40% of Betaproteobacterial sequences and over 20% of all bacterial sequences in both the JMS75 and JMS99 combined treatment microcosms.

3.3.3 Assessment of activity state

Following Jones and Lennon (2010), we classified taxa as being active or inactive based on their ratio of rRNA:rDNA. This metric uses the ratio of a taxon’s relative contribution to each library (i.e., rRNA%:rDNA%) to avoid potential bias arising from differences in the number of sequences obtained for each sample. Based on this criterion, the proportion of active taxa averaged 52±4% with no significant differences between sites or between environmental and microcosm samples. The number of active taxa was highest in May and August at JMS75 and in July at JMS99 (Figure B.12). In both experiments, the control microcosms had the highest number of active taxa. Binomial logistic regression (all libraries pooled) was used to relate the likelihood of a given taxa being active to its rDNA-based rank. This analysis showed that for each drop in rank, the odds ratio of a taxa being active increased by 1.01±0.001 (p < 0.0001) and indicates that rare taxa were more likely to be active. Because abundant taxa are less likely to be active, the proportion of active cells was always lower than the proportion of active taxa, with the exception of the JMS75 combined treatment microcosm. Active taxa were weighted according to their contribution to the rDNA library yielding an estimate 32±3% of cells that were active (average for all samples). However, this estimate was sensitive to the presumed threshold for distinguishing active cells (rRNA%:rDNA% > 1). For example, use of a higher threshold (rRNA:rDNA > 2) indicated that only 7±1% of cells were active. Using this method of estimation, active cells were most abundant at JMS75 in August (active taxa peaked at same time) and in the combined treatment microcosms in both experiments (Figure B.12).
Chapter 4

Discussion

Our use of combined rDNA and rRNA sequencing and traditional, microscopy-based methods has enabled us to examine the mechanisms of microbial response to resource availability along environmental gradients and in laboratory microcosms. We will first consider microbial responses to environmental gradients in the tidal-freshwater James River and then results from laboratory experiments. Lastly, we will assess the strengths and weaknesses of using combined rDNA and rRNA sequencing to assess bacterial activity states.

4.1 Environmental characterization

Bacterial abundance (both live and active), correlated strongly and positively with CHLa, NPP and DOC (Figure B.3). Coupling between phytoplankton and bacterial production has previously been proposed as an explanation for the distribution of bacterioplankton in the tidal-freshwater James River (Beckwith, 2009) and other systems (Lancelot and Billen, 1984; Cole et al., 1988; White et al., 1991). Analysis of DOC at river miles 75 and 99 has found significantly lower C:N ratios at JMS75 than at JMS99 indicating higher nutritional quality (Beckwith, 2009). Correspondence between CHLa, low C:N ratios, and bacterial abundances suggest that algal exudates may be an important factor determining bacterial abundance in the James River Estuary. Alternatively, both bacteria and algal may be responding to site-specific effects.
Despite large differences in abundance, bacterial community composition was similar at the two sites suggesting that transit times may have been too short for the development of distinct communities. Transit times for this segment of the James River were previously estimated to be 15 days during typical discharge conditions (long-term annual mean = 200 m$^3$s$^{-1}$; Shen et al., 1999; Shen and Lin, 2006). Actual transit times were likely shorter during May and June (discharge = 200-800 m$^3$s$^{-1}$) and longer in July, August, and September (discharge < 100 m$^3$s$^{-1}$). However, even under high discharge conditions, transit times were longer than the 48 hour incubation period used in the microcosm experiments and should have been sufficient for differences in community composition to develop. Kan et al. (2007) attributed seasonal patterns in community composition in the Chesapeake Bay to the presence of similar selective pressures by environmental parameters shared by both sites. Adams et al. (2010) likewise found that temperature rather than source of OM drove shifts in community composition. Although we were unable to identify significant correlations between community structure and environmental variables, we feel that bacterial community structure is most likely regulated by a factor or group of factors that vary seasonally, i.e. water temperature or discharge.

Prominent taxa in our samples were typical of the freshwater environment, with a few exceptions. Although, Bacteroidetes are common in other studies, the prevalence of Sphingobacteria in our environmental samples was unusually high (Selje et al., 2005) and may be due to bacterial associations with algae (Ueda et al., 2009). Our samples revealed an high prevalence of Cyanobacteria, supporting previous work showing that density of Cyanobacteria is higher in the tidal-freshwater James River than in other Chesapeake Bay tributaries and that Cyanobacterial biomass in the James River has risen significantly over the past 20 years (Marshall et al., 2009). Overall, our findings suggest that bacterial abundance was most closely linked to autochthonous production which varied between sites and likely influenced the quantity and quality of organic matter. Bacterial response to the resource gradient between river miles 99 and 75 did not appear to have involved a shift in community composition or changes in active bacterial abundance.
4.2 Microcosm experiments

Bacterial abundance was responsive to nutrient amendments at JMS75 (low nutrient site) but not at JMS99 (high nutrient site). It is unclear whether these results support coupling between autochthonous production and heterotrophic respiration or simply indicate that both bacteria and phytoplankton were limited by inorganic nutrients. In the JMS75 CHLa and NPP were also responsive to nutrient additions, potentially providing microcosm bacteria with additional labile OM. Bacterial abundance and algal biomass did not increase in the JMS99 experiments under enhanced nutrients due to the high ambient nutrient levels. Instead, JMS99 bacteria were responsive to combined treatments, suggesting co-limitation of bacterial growth at this site. Light limitation due to the deep, narrow channel morphometry at JMS99 (Bukaveckas et al., In review) seemed to be an important component of this co-limitation, while the relative importance of organic matter and inorganic nutrients remains unclear.

We were surprised that glucose additions did not affect BA in either the JMS75 or the JMS99 experiments, suggesting that James River bacteria were not limited by the availability of organic matter. There are several alternative explanations for the lack of response to glucose additions. First, our glucose amendments (1.0 mg L$^{-1}$) were low relative to those in other studies (0.5 mg - 9 g L$^{-1}$; Kirchman et al., 1990; Carlson and Ducklow, 1996; Kogure et al., 1987; Kirchman et al., 1990; Coffin et al., 1993; Shiah and Ducklow, 1994; Carlson and Ducklow, 1996; Cherrier et al., 1996). Choi et al. (1999) reported that yeast extract additions of 20 mg ml$^{-1}$ were necessary to detect differences in active BA. Although our level of treatment was low, it was chosen to be within the range of natural variation in DOC. Second, carbon enrichment has been shown to lead to high cell-specific respiration rates but lower bacterial growth efficiency, a response undetectable by measurement of BA alone (Smith and del Giorgio, 2003). We cannot discount this possibility, but note that we did not see increased CR in response to glucose amendments. In fact, the lack of response by CR even while CHLa and BA increased implies bacterial growth efficiency was higher in the microcosms. Finally, Hasegawa et al. (2005) found bacterial grazing rates were commensurate with bacterial population and controlled bacterial abundance after glutamate enrichment. Prior studies in the James River have reported low zooplankton filtration rates (<10% CHLa per day; Bukaveckas et al., In review) though rates for bacterial ingestion may be higher than those measured for phytoplankton.
Shifts in community composition between control and treatment microcosms were detected by tag pyrosequencing but not by TRFLP analyses. Changes in abundance of rare taxa or shifts between taxa with conserved restriction enzyme loci (i.e. within a single terminal restriction fragment) may not be detected by TRFLP (Avis et al., 2006; Drenovsky et al., 2008). Although several other studies have incubated microcosms for longer periods, ranging from 4 to 11 days (Frette et al., 2009; Pinhassi et al., 1999; Eiler et al., 2003), we found that a 48 hour incubation period was a sufficient length of time to detect changes in community composition in response to treatments while avoiding enclosure effects. Under nutrient and combined treatments, the number of active taxa decreased and the relative abundance of a few taxa increased dramatically, suggesting that with increased incubation periods we might see decreased microbial diversity. Rare taxa were capable of exploiting changes in resource conditions. For example, Chromobacteria had <1% relative abundance in initial samples and >20% relative abundance in the combined treatment. Overall, results from the microcosm experiments suggested that bacterial communities responded to changes in substrate availability via changes in both abundance and community composition.

4.3 Metabolic activity

Having considered how bacterial abundance and community composition respond to resource conditions, we will now consider changes in activity states as revealed by CTC staining and rDNA/rRNA sequencing. In both environmental and microcosm samples, we observed large changes in bacterial abundance despite the low proportion of the community that was detectable by CTC staining. CTC-based estimates for the abundance of active cells have been shown to correlate with bacterial production (del Giorgio et al., 1997; Sherr et al., 1999) and respiration (Smith, 1998), but have come under criticism (e.g. Ullrich et al., 1996; Karner and Fuhrman, 1997; Servais et al., 2001). There is currently no standardized protocol for CTC use and CTC concentrations and incubation times vary between studies (Creach et al., 2003). In order to determine optimal incubation times, we fixed samples at different time points and found that the number CTC+ cells increased until 4 hours but decreased by 6 hours (data not shown). However, other studies have found that cells require longer to reduce CTC (Caro et al., 1999; Choi et al., 1999; Berman et al., 2001; del Giorgio and Scarborough, 1995). Additionally, our CTC concentration
during staining (4.4 mM) is lower than the optimal range, 5-10mM, recommended by Choi et al. (1999) and may have led to low estimates of active cells. Furthermore, the percent of cells which are CTC+ may decrease as a population reaches lag phase (Lopez-Amoros et al., 1995; Creach et al., 2003; Choi et al., 1999). Finally, the proportion of cells that are metabolically active may remain constant even while productivity per cell increases (Almeida et al., 2001). Overall, although the percentages of cells identified as active (mean = 13±1%) were quite low, our estimates were similar to those found in previous CTC-based studies (del Giorgio and Scarborough, 1995; Choi et al., 1996; Karner and Fuhrman, 1997; Choi et al., 1999). If only CTC+ cells were actively dividing, maximum doubling times in our microcosms ranged from 8 to 21 hours, consistent with previous findings (Crump et al., 2007).

The determination of activity state for individual taxa based on the relative abundance of rDNA and rRNA remains problematic. Jones and Lennon (2010) identified taxa with a higher relative abundance of rRNA than rDNA as active. However, this definition is arguably subjective and variables such as rRNA operon copy number further hinder assessment of activity state. Using this metric, our results showed, as in the prior study, that rare taxa are more likely to be active. Disproportionate activity by rare taxa dispels the notion that rare taxa are present accidentally and supports the theory that they represent an important reservoir that is capable of exploiting resources and favorable environmental conditions. Despite problems in identifying active taxa, the disparity found between rRNA libraries, even when their corresponding rDNA libraries were quite similar, emphasizes the importance of shifting activity state as a mechanism of bacterial response to resource gradients. Our rDNA libraries indicate that bacterial communities at JMS75 and JMS99 were largely unchanged throughout the second half of our sampling period. However, rRNA libraries varied greatly from site to site and from month to month. For example, September JMS75 and JMS99 rDNA libraries were quite similar (Sorensen distance in bottom 10%) while rRNA libraries were quite different (Sorensen distance in top 15%). Even replicate rRNA libraries showed greater dissimilarity than replicate rDNA libraries. This finding should be interpreted cautiously as it is based on a single replicated rRNA sample and two replicated rDNA samples. But, as this is the first study to perform such replication, it suggests that further efforts are needed to resolve whether divergence among rRNA samples is due to variable activity states or methodological issues related to rRNA recovery and cDNA production. Overall, our assessment of changes in rDNA and
rRNA relative abundance among sites, dates, and treatment groups shows that changes in abundance and activity state can be important even while community composition remains relatively stable.

4.4 Conclusions

Our results indicate that bacterial abundance in the tidal-freshwater James River was strongly linked to organic substrates (correlations with DOC, CHLa, NPP) which varied over time and between our sampling sites. Community composition varied over the course of the sampling period but was similar among sites that differed in CHLa and bacterial abundance. Microcosm experiments indicated that bacterioplankton were limited by inorganic nutrients at the low nutrient, high CHLa site (JMS75) and were co-limited by some combination of light, labile OM, and inorganic nutrients at the high nutrient, low CHLa site. The proportion of cells that were metabolically active based on differential staining was low and similar among sites, dates and treatments. In contrast, variability among rRNA libraries showed that shifting metabolic activity state is an important adaptive mechanism for bacterial communities. We have identified those taxa that were disproportionately active in the environment and within our experiments. However, this approach is based on an assumed and fixed ratio of %rRNA:%rDNA that is largely untested. A satisfactory metric for identifying metabolic activity state has not yet been developed. Finally, we have demonstrated the importance of rare taxa when bacterial communities respond to favorable conditions in the environment.
## Appendix A

### Tables

**Table A.1:** Primer, adaptor, and barcode sequences used in T-RFLP and tag pyrosequencing.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F</td>
<td>AGAGTTTGATC(C/A)TGGCTCAG</td>
</tr>
<tr>
<td>1492R</td>
<td>TACGC(C/T)TACCTGTGTTACGACTT</td>
</tr>
<tr>
<td>XLR Adaptor and Key</td>
<td>CCATCTCATCCTGCGTGTCTCCGACTCAG</td>
</tr>
<tr>
<td>Barcode 1</td>
<td>CAGATCG</td>
</tr>
<tr>
<td>Barcode 2</td>
<td>AGCTAGCT</td>
</tr>
<tr>
<td>Barcode 3</td>
<td>GTCAGTACA</td>
</tr>
<tr>
<td>Barcode 4</td>
<td>TCGACAGATC</td>
</tr>
<tr>
<td>16S-V5-7-F</td>
<td>CGCAAG(A/G)HTRAAACTCAAAGG</td>
</tr>
<tr>
<td>16S-V5-7-R</td>
<td>ACTAGCGAHTCC(G/A)RCTTC</td>
</tr>
</tbody>
</table>
Table A.2: Comparison of James River mile 75 (JMS75) and 99 (JMS99) main-channel (MC) and near-shore (NS) sites, May-September 2009. Values are means ± standard error. Means were compared by one-way ANOVA where * means $p < 0.01$ and ** means $p < 0.001$. Different groups are denoted by letter. Active bacterial abundance had unequal variance and * denotes $p < 0.01$ by Welch's test. No significant differences were found between main-channel and corresponding near-shore sites.

<table>
<thead>
<tr>
<th></th>
<th>JMS99-MC</th>
<th>JMS99-NS</th>
<th>JMS75-MC</th>
<th>JMS75-NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turbidity (NTU)</td>
<td>10.5 ± 3.0</td>
<td>—</td>
<td>13.5 ± 1.5</td>
<td>—</td>
</tr>
<tr>
<td>Light attenuation (m$^{-1}$)</td>
<td>1.6 ± 0.2$^a$</td>
<td>—</td>
<td>2.7 ± 0.2$^b$</td>
<td>—</td>
</tr>
<tr>
<td>CHLa ($\mu$g L$^{-1}$)**</td>
<td>14.8 ± 5.0$^a$</td>
<td>19.3 ± 8.2$^a$</td>
<td>79.3 ± 16.5$^b$</td>
<td>92.0 ± 10.1$^b$</td>
</tr>
<tr>
<td>NPP* ($\mu$g O$_2$ L$^{-1}$hr$^{-1}$)</td>
<td>106 ± 57$^a$</td>
<td>182 ± 91$^a$</td>
<td>696 ± 154$^b$</td>
<td>688 ± 77$^b$</td>
</tr>
<tr>
<td>CR* ($\mu$g O$_2$ L$^{-1}$hr$^{-1}$)</td>
<td>27 ± 3$^a$</td>
<td>28 ± 8$^a$</td>
<td>48 ± 9$^{ab}$</td>
<td>63 ± 7$^b$</td>
</tr>
<tr>
<td>SRP (mg L$^{-1}$)</td>
<td>0.03 ± 0.006</td>
<td>0.03 ± 0.006</td>
<td>0.02 ± 0.004</td>
<td>0.02 ± 0.005</td>
</tr>
<tr>
<td>DIN (mg L$^{-1}$)*</td>
<td>0.6 ± 0.1$^a$</td>
<td>0.6 ± 0.1$^a$</td>
<td>0.3 ± 0.06$^{ab}$</td>
<td>0.1 ± 0.03$^b$</td>
</tr>
<tr>
<td>DOC (mg L$^{-1}$)</td>
<td>3.3 ± 0.15</td>
<td>3.5 ± 0.21</td>
<td>4.2 ± 0.43</td>
<td>5.1 ± 0.82</td>
</tr>
<tr>
<td>Live BA** ($10^6$ cells ml$^{-1}$)</td>
<td>27.5 ± 7.7$^a$</td>
<td>32.1 ± 1.7$^a$</td>
<td>80.5 ± 38.9$^b$</td>
<td>99.9 ± 17.9$^b$</td>
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<tr>
<td>Active BA* ($10^6$ cells ml$^{-1}$)</td>
<td>3.0 ± 1.1$^a$</td>
<td>9.2 ± 2.9$^a$</td>
<td>5.3 ± 1.9$^{ab}$</td>
<td>17.5 ± 1.4$^b$</td>
</tr>
</tbody>
</table>
Appendix B

Figures
Figure B.1: Water temperature, turbidity, discharge, specific conductivity, soluble reactive phosphorous (SRP) and dissolved inorganic nitrogen (DIN) for main-channel sampling locations in the James River (JMS99 and JMS75.) Discharge values are averages of three days prior to each sampling date from USGS reported values measured at Cartersville, VA monitoring site 02035000.
Figure B.2: Phytoplankton biomass (CHLa), dissolved organic carbon (DOC), net primary production (NPP), community respiration (CR), abundance of live bacterial cells, and the proportion of intact cells that are active for main-channel sampling locations in the James River (JMS99 and JMS75). Points for NPP, CR, live bacterial abundance and proportion intact represent mean of 3 replicates ±1 SE.
Figure B.3: Simple linear regressions of log-transformed data showed significant positive correlation between live bacterial abundance (live BA) and net primary production (NPP), community respiration (CR), chlorophyll a (CHLa), and dissolved organic carbon (DOC) at main-channel sampling sites in the James River (JMS99 and JMS75), June-September 2009.
Figure B.4: Principle component analysis of environmental variables at main-channel sampling locations in the James River (JMS99 and JMS75). Axis 1 explains 42% of variation with high factor loadings for phytoplankton biomass (CHLa), net primary production (NPP), and community respiration (CR). Axis 2 explains 29% of variation with high factor loadings for specific conductivity and turbidity.
Figure B.5: Non-metric multidimensional scaling of microbial communities based on TRFLP peak profiles. Each point represents the mean ±1 SE of all replicates from both JMS99 and JMS75 near-shore sampling sites in the James River for a given month. Cumulative $R^2 = 0.56$. Axes 1, 2 and 3 explain 14%, 20% and 21% of variation respectively.
Figure B.6: Net primary production (NPP), community respiration (CR), phytoplankton biomass (CHLa), live bacterial abundance (BA), and proportion of live cells that are metabolically active for JMS75 and JMS99 July microcosm experiments. Points represent mean values ±1 SE for three replicates in each treatment group.
Figure B.7: Net primary production (NPP), community respiration (CR), phytoplankton biomass (CHLa), live bacterial abundance (BA), and proportion of live cells that are metabolically active for JMS75 and JMS99 August microcosm experiments. Points represent mean values ±1 SE for three replicates in each treatment group.
Figure B.8: Net primary production (NPP), community respiration (CR), phytoplankton biomass (CHLa), live bacterial abundance (BA), and proportion of live cells that are metabolically active for JMS75 and JMS99 September microcosm experiments. Points represent mean values ±1 SE for three replicates in each treatment group.
Figure B.9: Non-metric multidimensional scaling of microbial communities based on TRFLP peak profiles. Each point represents the mean ±1 SE of all replicates in each treatment group. For the JMS99 experiment, cumulative $R^2 = 0.57$ and stress = 0.08. Axes 1, 2 and 3 explain 24%, 20% and 23% of variation respectively. For the JMS75 experiment, cumulative $R^2 = 0.62$ and stress = 0.16. Axes 1, 2 and 3 explain 14%, 22% and 27% of variation respectively.
Figure B.10: Non-metric multidimensional scaling based on 16S rDNA and rRNA tag pyrosequencing-generated community composition and active OTUs for (A) James River near-shore sampling locations (cumulative $R^2 = 0.82$, stress = 0.11, Axis 1 and 2 explain 52% and 30% of variation respectively) and for (B) September microcosm experiments for JMS99 and JMS75 (cumulative $R^2 = 0.80$, stress = 0.14, Axis 1 and 2 explain 59% and 21% of variation respectively).
Figure B.11: Relative abundance of ten most prevalent classes of bacteria at near-shore sampling locations in the James River (JMS99 and JMS75) and September microcosm experiments.
Figure B.12: Active OTUs and active cells (abundance extrapolated from relative abundance of rDNA for taxa where %rRNA:%rDNA > 1) for sampling locations in the James River (JMS99 and JMS75) and September microcosm experiments.
Appendix C

Vita

Catherine Mackay Luria was born on April 30, 1982, in Albany, New York, and is an American citizen. She received her Bachelor of Science in Animal Ecology and Classical Studies from Iowa State University in 2005 and subsequently worked as a lab technician at the USDA National Animal Disease Center in Ames, IA and at Philip Morris USA in Richmond, VA.


Beckwith, M. J., 2009. Coupling of autotrophic and heterotrophic plankton food web components in the tidal-freshwater James River, USA.


