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# Compositional changes in free-living bacterial communities along a salinity gradient in two temperate estuaries

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*Abstract*

There is now clear evidence for major differences in heterotrophic bacterial composition between freshwater and marine ecosystems. A fundamental question that remains unresolved is whether the compositional succession occurs by a gradual replacement of the major phylotypes, or whether there are drastic compositional shifts in discrete areas along the gradient from one system to another. The aim of this study is to examine the change in the phylogenetic composition of the free-living bacterioplankton across the salinity gradient in both the Choptank and the Pocomoke Rivers, both subestuaries of the Chesapeake Bay, using fluorescence in situ hybridization (FISH). The proportion of free-living cells that could be detected using FISH varied widely (3 to 80%), and the majority of cells detected belonged to *bacteria,* whereas *archaea* represented less than 3%. The distribution of the different members of *bacteria* exhibited a clear pattern along the salinity gradient, with the dominance of $\alpha$-proteobacteria in the lower, saltwater regions and of $\beta$-proteobacteria in the upper freshwater regions. The *cytophaga-flavobacterium* cluster prevailed in the turbidity maximum located in the middle estuary, and the $\gamma$-proteobacteria showed sporadic peaks along the transect that may have been related to local events. The replacement of $\alpha$- for $\beta$-proteobacteria along the salinity gradient was not gradual but rather occurred rapidly within the turbidity maximum region of the estuaries where the fresh and salt waters mix. The pattern of phylogenetic succession was linked to the development of the turbidity maximum, which is related to rainfall and the ensuing hydrological conditions. There is indication that the phylogenetic succession is accompanied by strong physiological changes in the bacterial assemblage, expressed as a decline in bacterial growth efficiency and community production. The transition appears not to be simply the result of conservative mixing of riverine and estuarine bacterial assemblages, but rather appears to involve cell inactivation and/or death.

Different molecular approaches are converging to show that there are major differences in the phylogenetic composition of bacterial assemblages between terrestrial and aquatic systems, and also among different aquatic ecosystems (Amann et al. 1995; Glöckner et al. 1999; Rappé et al. 2000). For example, studies using fluorescence in situ hybridization (FISH; e.g., Glöckner et al. 1999) have shown that lakes and rivers are generally dominated by the beta-subclass proteobacteria ($\beta$-proteobacteria), whereas the alpha-subclass proteobacteria ($\alpha$-proteobacteria) and the *cytophaga-flavobacterium* cluster (CF) are more prevalent in marine areas. González and Moran (1997) and Crump et al. (1999) have used PCR to show strong compositional changes in the bacterioplankton between freshwater and saltwater portions of the estuaries. Similarly, Murray et al. (1996) suggest a replacement of the phylotypes along the environmental gradient in Tomales Bay and San Francisco Bay. The factors that determine phylogenetic structure are still not well understood, but recent observations suggest key environmental factors that influence the distribution of the different lineage of eubacteria. For instance, the highly contaminated and eutrophic water of Barcelona harbor presents a distinct bacterial assemblage compared to the immediately adjacent coastal waters of the Mediterranean Sea (Schauer et al. 2000). Inorganic nutrients have been hypothesized to drive the seasonal succession of the bacterial assemblages in the Baltic Sea (Pinhassi and Hagström 2000), and shifts in bacterial composition have been linked to changes in the nature of the dissolved organic mater (DOM) pool (Cottrell and Kirchman 2000*a*). Likewise, Rheinheimer (1997) has shown that the composition of the cultivable bacterial communities is strongly correlated with salinity changes in several estuaries.

It is expected that major physical or chemical environmental discontinuities, such as land-water and oxic-anoxic interfaces, and strong temperature, nutrient, and salinity gradients may be areas of particularly dynamic compositional change. Although there are well-defined cross-system patterns in composition, as we mentioned above, there are still very few studies that have investigated in detail the patterns of phylogenetic succession in sharp interfaces, particularly fresh-saltwater discontinuities. Estuaries are ideal environments to examine the compositional succession of bacterial assemblage because of the variety of sharp environmental gradients that typically exist there. The major ecotone in estuaries is often centered in the turbidity maxima created by the interaction between river flow and tidal forcing (Berner and Berner 1996) and is of particular interest because of the dramatic salinity and nutrient gradients that often occur in these regions.

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The main goal of this study was to examine in detail the shifts in phylogenetic composition of the free-living bacterioplankton across the salinity in the Choptank River estuary (Maryland) using FISH. The Choptank estuary is a partially mixed estuarine tributary of the Chesapeake Bay with three main segments (lower, middle, and upper estuary) that can be differentiated largely by salinity and nutrients (Fairbridge 1980). The Chesapeake Bay and Choptank river waters characterize the lower and the upper estuary, respectively. The middle estuary is characterized by sharp physical and chemical gradients (salinity, temperature, nutrients, and total suspended solids), which vary in intensity over the seasons (Ward and Twilley 1986). We sampled another partially mixed estuary tributary of the Chesapeake Bay, the Pocomoke River estuary (Virginia), for comparison to our findings along the salinity transect of the Choptank River estuary.

Our first objective was to determine whether there are consistent differences in the major phylogenetic groups between the freshwater and marine endpoints of the gradient, and whether the differences persist on a seasonal basis. The second objective was to assess whether the phylogenetic transition is gradual along the entire gradient or occurs abruptly at the turbidity maximum, and whether the intensity of the transition (abrupt or gradual) varies according to the physical and chemical characteristic of the gradient itself. Finally, we were interested in exploring whether the phylogenetic transition is accompanied by changes in free-living bacterioplankton metabolism, specifically total carbon consumption, growth efficiency, and the resulting community production.

We performed analyses of the phylogenetic composition of the free-living bacterioplankton assemblage using FISH and oligonucleotide probes targeting domain, division, and subclass levels. Physical characteristics (salinity and temperature) and the distribution and concentration of dissolved inorganic nutrients, dissolved organic carbon (DOC), and total suspended solids (TSS) were used to describe the environmental gradient. In addition, we measured the rates of production and respiration of the free-living assemblage to assess whether the phylogenetic succession was accompanied by measurable changes in community metabolism.

## Materials and methods

*Sampling sites and procedure*—The Choptank River estuary is a partially mixed estuarine tributary of the Chesapeake Bay, located on the coastal plain of the Eastern Shore of Maryland, approximately 185 km upstream from the mouth of the Bay (Fig. 1). The Choptank River has a total length of approximately 115 km from its uppermost freshwater reaches to its confluence with the main channel of the Chesapeake Bay; for this research, we have sampled a transect that covers 100 km and includes most of the freshwater portion and all of the saline portion of the estuary down to the confluence with the Chesapeake Bay. The middle estuary extends approximately from the bridge of Cambridge to the Dover Bridge (km 25 to 60). This ecotone between the upper, freshwater and lower, saltwater ecosystems is characterized by strong environmental discontinuities. The Choptank River estuary has a relatively long water residence time (up to several months; Fisher et al. 1988) compared to its relatively small size, which allowed us to sample rapidly (<8 h) over its entire length and to get a nearly instantaneous picture of the entire system.

The Pocomoke River estuary, the second system we sampled (Fig. 1), is another major tributary of the Chesapeake Bay, which has nevertheless different characteristics. The Pocomoke River estuary has a total length of approximately 60 km (i.e., half of the Choptank River estuary) from its uppermost freshwater reaches to its confluence with the main channel of the Chesapeake Bay. As opposed to the Choptank River, the Pocomoke is characteristically a black water river, draining forests and extensive wetlands, as well as agricultural and poultry operations. For this research we sampled a 30-km transect, which extends from the upper freshwater portion all the way to its confluence with the Chesapeake Bay.

Water samples (20 liters) were collected with a Niskin bottle during a day-long cruise along a transect spanning the salinity gradient in March, May, July, and September 2000 in the Choptank River estuary (stations 1 to 19) and in May 2000 in the Pocomoke River estuary (stations 7 to 23, Fig. 1, inset). Samples were kept in the dark at in situ temperature and processed at Horn Point laboratory within 3 h.

*Hydrological conditions and water residence time calculation of the Choptank River estuary*—Rainfall was recorded at the Horn Point Lab meteorological station (Cambridge, Maryland; www.cbos.org). The major freshwater discharge into the Choptank River estuary comes from the upper watershed (Gauged area) and is recorded by the U.S. Geological Survey at Greensboro, Maryland, Maryland (USGS 01491000) (Fig. 1). The freshwater residence time ($Rt$) was estimated for the whole estuary and for each of its main sections: the upper, the middle and the lower estuary. For a given section $i$, $Rt$ was defined by

$$Rt_i = \left(V_i \frac{14 - S_i}{14}\right) \Bigg/ \left(F_g \frac{A_i}{A_g}\right),$$

where $V_i$ is the water volume of the section ($m^3$), $S_i$ the mean salinity of the section, and $A_i$ is the watershed area considered for the section ($km^2$). $F_g$ is the mean freshwater discharge at Greensboro for three weeks prior to sampling, and $A_g$ is the drainage area of the Gauged basin in $km^2$ (293 $km^2$; Lee et al. 2000). We did not estimate the freshwater residence time of the Pocomoke River estuary due to lack of information on the watershed characteristic and freshwater inputs.

*Chemical analyses*—Inorganic nutrient concentrations measured at each station include dissolved inorganic nitrogen (N) and dissolved inorganic phosphorus (P). Water samples for dissolved nutrient analysis were filtered in the field through a Whatman GF/F glass fiber filter. The filtrate was placed on ice, and then frozen immediately upon return to the laboratory for subsequent determination of dissolved nutrients. Dissolved nutrient concentrations were determined

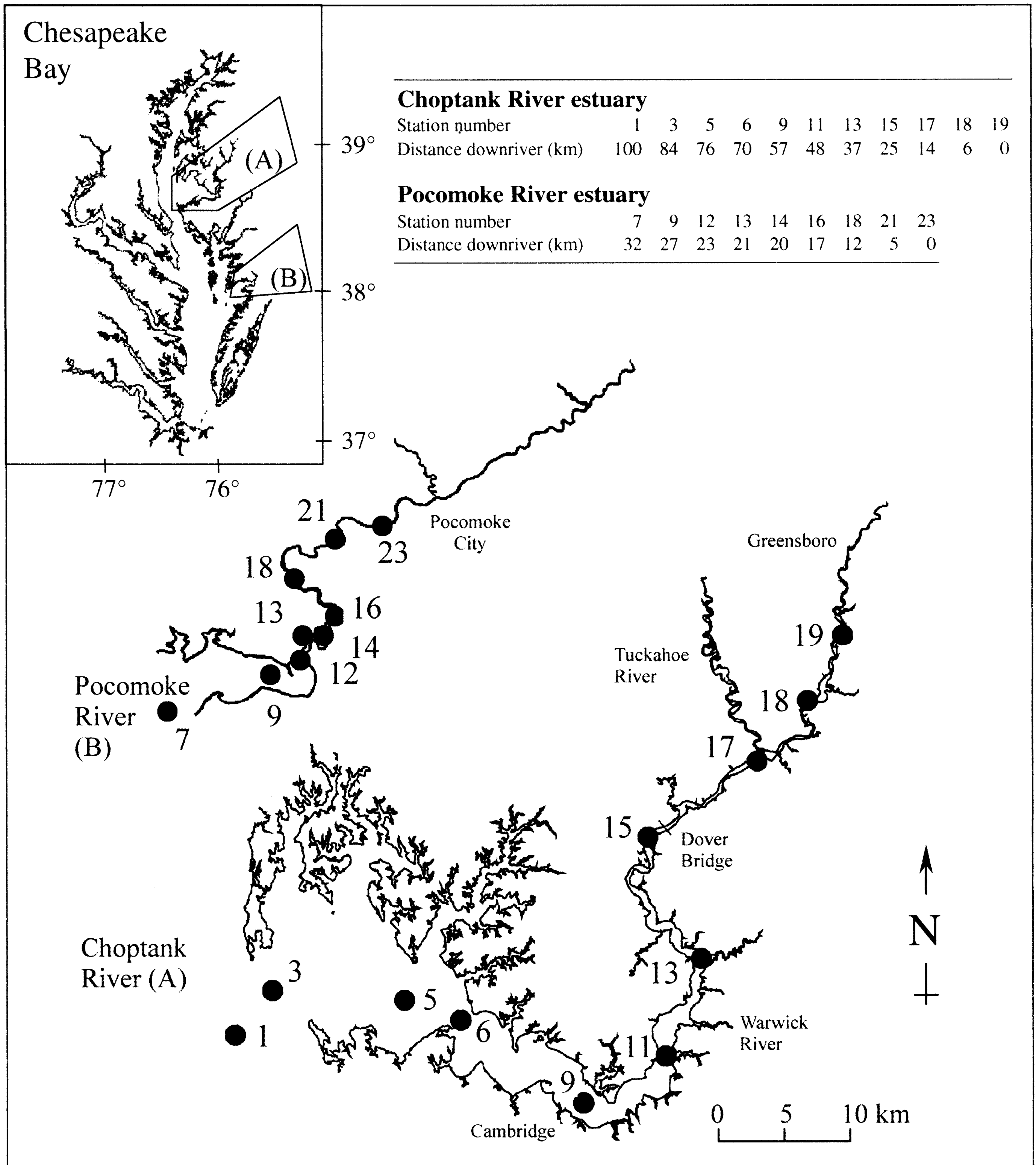


Fig. 1. Geographical location of the stations in the (A) Choptank and (B) Pocomoke River estuaries sampled during day cruises in March, May, July, and September 2000 and in May 2000, respectively. Inset: station numbers with their corresponding distance downriver.

Table 1. Oligonucleotide probes used in this study. rRNA position based on *Escherichia coli* numbering (Brosius et al. 1981). %FA = Percentage of formamide in hybridization buffer. IS: ionic strength (NaCl, M).

| Probe | Target taxa | Probe sequence (5′–3′) | rRNA position | % FA | IS |
|---|---|---|---|---|---|
| EUB338 | bacteria | GCTGCCTCCCGTAGGAGT | 16S (338–355) | 30 | 0.10 |
| NON338 | | ACTCCTACGGGAGGCAGC | 16S (338–355) | 30 | 0.10 |
| ALF1b | alpha-proteobacteria | CGTTCGYTCTGAGCCAG | 16S (19–35) | 40 | 0.44 |
| BET42a | beta-proteobacteria | GCCTTCCCACTTCGTTT | 23S (1027–1043) | 30 | 0.11 |
| GAM42a | gamma-proteobacteria | GCCTTCCCACATCGTTT | 23S (1027–1043) | 30 | 0.10 |
| CF319a | *cytophaga-flavobacterium* cluster | TGGTCCGTGTCTCAGTAC | 16S (319–336) | 30 | 0.08 |
| ARCH915 | *Archaea* | GTGCTCCCCCGCCAATTCCT | 16S (915–935) | 25 | 0.31 |

colorimetrically on a Technicon AutoAnalyzer II system. A raw water sample was placed on ice in the field and processed within 24 h for TSS using vacuum filtration and a modified gravimetric determination (Banse et al. 1963). DOC was analyzed using a high-temperature platinum catalyst in the Shimadzu 5000A TOC Analyzer (Sharp et al. 1993). Chlorophyll *a* concentration was determined spectrophotometrically from ethanol extracts.

*Microbiological analyses*—In this study we focused on the abundance, composition, and community metabolism of free-living bacterioplankton in both the Choptank and the Pocomoke River estuaries. The main reason we focused on the free-living fraction is that we wanted to explore changes in both bacterial respiration (BR) and production (BP) along the gradient and relate these to the phylogenetic succession. In order to measure bacterial respiration it is necessary to physically isolate the bacterial cells from other planktonic components, and we have done this by separating the free-living bacteria from other planktonic components, as we describe below. In addition, there are portions of the river that have high loads of suspended inorganic particles, which make microscopic examination of samples very difficult. However, we measured production on all bulk water samples as well, to have an idea of the behavior of the entire assemblage. The free-living fraction typically accounts for over 60% of the bacterial production, as we explain below.

The water samples were gently filtered upon arrival to Horn Point Lab through 147-mm diameter Millipore AP15 glass fiber filter using a peristaltic pump. In preliminary experiments we tested a wide variety of filters commonly used to separate bacteria, including cellulose and polycarbonate membranes, glass fiber filters, and prefilters with pore sizes ranging from 0.6 to over 5 $\mu$m. We have concluded that the glass fiber Millipore AP 15 filters are the most effective in reducing the number of >2-$\mu$m organisms while maintaining most of the original free-bacterial community structure (del Giorgio and Newell, pers. comm.).

*Fluorescence in situ hybridization*—The filtered water was used to determine the phylogenetic composition of the free-living bacterioplankton using FISH. The FISH protocol is standard except that oligonucleotide probes are labeled with BODIPY green (8-chloromethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene), a fluorochrome synthesized by Molecular Probes. The main reason for using oligonucleotides conjugated with BODIPY, and not the more popular indocarbocyanine dye CY3 for example, is that we are seeking to develop the cytometric detection of hybridized cells, and the excitation and emission spectra of BODIPY fits well the properties of standard, argon-based cytometers. In this paper, however, we report only results based on epifluorescence counts. The oligonucleotide probes were synthesized, conjugated with BODIPY, and purified by PAGE by Synthetic Genetics. The sequences used for the various probes are in Table 1.

The successful use of oligonucleotide probes depends upon key reaction conditions. We tested a variety of conditions on field samples from the Choptank River estuary to optimize the signal strength of each probe by lessening the likelihood of nonhomologous annealing. Our criteria for optimization were brightness of the probe-positive cells and their proportion relative to total bacterial counts. The main variables tested were formamide concentration in the hybridization buffer (from 10 to 60%) and ionic strength (from 0.01M to 5M NaCl). The optimized formamide concentration and ionic strength indicated in Table 1 were employed.

After 1 h of fixation at 4°C with formalin (2%, final concentration), 1 to 5 ml (depending on the sampling period) were filtered onto a 25-mm diameter, 0.2-$\mu$m white, polycarbonate filter (Millipore) under a vacuum of <5 mm Hg. The filters were air dried and stored for less than 2 d in a Petri dish between two aluminum foil pieces at −20°C until hybridization. Each filter was cut into eight equal sections. The filter sections were placed on a 5-$\mu$l droplet of hybridization solution (750 mM NaCl, 100 mM Tris-HCl, pH 7.4, and 5 mM EDTA, 0.01% polyadenylic acid, 0.01% sodium dodecyl sulfate, formamide concentration varying with probe, Table 1) containing 2 ng of probe $\mu l^{-1}$ on a parafilm-covered slide. Slides were incubated at 46°C overnight in an equilibrated chamber. Probes BET42a and GAM42a were used with competitor oligonucleotides as described previously (Manz et al. 1992). The filter sections were transferred to a 1.5-ml microfuge tube containing 0.5 ml of prewarmed (48°C) wash solution (20 mM Tris-HCL pH 7.4, 5 mM EDTA, 0.01% SDS, NaCl concentration varying with probe, Table 1) and incubated at 48°C for 15 min. The filter pieces were dried on Whatman filter paper, placed on slides, covered with 10 $\mu$l of Citifluor solution (Citifluor), and a cover slip. Green BODIPY-labeled cells were counted within 1 d of hybridization by epifluorescence microscopy (Nikon Eclipse E800 equipped with a 100-W high-pressure mercury

bulb) under blue light excitation (488 nm). At least 300 cells were counted on each filter section and the percentage of probe labeled cells was determined from the number of green cells and total DAPI counts.

BODIPY-stained cells fluoresce green, but there is some overlap with the emission spectrum of DAPI. Therefore, samples were not counterstained with DAPI, but rather total counts were obtained from a separate aliquot that was stained with DAPI. Formalin-fixed samples were stained with 4,6-diamidino-2-phenylindol (1 $\mu$g ml$^{-1}$ DAPI final concentration). After 15 min, the samples were filtered onto 0.2-$\mu$m pore-size black polycarbonate membranes under vacuum pressure <10 mm Hg. All filters were air dried and mounted on microscope slides. Slides were stored at −20°C prior to examination. Total bacterial abundance was determined by epifluorescence microscopy (Nikon Eclipse E800 equipped with a 100-W high-pressure mercury bulb) under UV excitation. At least 500 cells were counted per sample on 10 to 20 randomly chosen fields. The proportion of cells scored for the different phylogenetic groups was corrected by subtracting the counts for the control probe NON338.

*Bacterial community metabolism*—Ten liters of filtered water were used to fill two 4-liter acid-washed Erlenmeyer flasks. One flask was placed on a stand and was connected by acid-washed silicone tubing to a lower flask, so that a siphon could be established. The upper flask was open to the atmosphere, whereas the lower flask was sealed and had a sampling port. All the flasks were placed in a large walk-in incubation chamber in the dark at ambient field temperature, and up to 14 samples could be set up and processed simultaneously. This system allowed intensive sampling to establish detailed time courses for oxygen consumption and bacterial production with minimal handling of the sample water. Samples were retrieved from the lower flask by opening the valve that connects the upper and lower flasks and allowing 10 ml of water to overflow through a sampling port before collecting the samples. Each flow-through system was sampled every 3 h, and at each sampling time a maximum of 40 ml of water was retrieved, which was used to determine $O_2$ concentration and leucine uptake as described below. The volume replaced at each sampling thus represented <1% of the total volume of the incubation flask, and because the gas concentrations in the upper container were only marginally different from those in the incubation flask, there was no detectable effect on gas concentrations.

Samples of water for oxygen concentration were taken directly from the flasks by inserting the outflow plastic tube into the bottom of a 7-ml glass tube and allowing the water to overflow. Replicate or triplicate tubes were filled this way for every time point taken during the incubation. Each tube was poisoned with 8 $\mu$l saturated HgCl solution and then capped with a ground glass stopper. The tubes were kept immersed in water at 4°C for later gas analysis in the lab. Oxygen concentration in the samples was measured using membrane-inlet mass spectrometry (Kana et al. 1994) within a maximum of 4 d of collection. Briefly, the method is based on the spectrometric determination of the ratio of argon to oxygen in the sample, after the gases in the sample have been allowed to diffuse through a permeable membrane and collected in a stream of helium. The oxygen concentration is then derived from this ratio by determining the solubility of argon corrected for salinity and temperature. The rates of oxygen consumption were calculated from the slope of the $O_2$ versus time relationship fitted to an ordinary least square regression. Rates of oxygen consumption were converted to $CO_2$ production assuming a respiratory quotient (RQ) of 1 (del Giorgio and Newell, pers. comm.).

Rates of bacterial production were estimated from the uptake of $^3$H-leucine following the centrifugation method of Smith and Azam (1992). Three replicate microfuge tubes plus a killed control were incubated for 1 h for each production measurement. There were three individual measurements of leucine uptake in the filtered fraction during the 6-h incubation, and these individual measurements were averaged to obtain a mean rate of bacterial leucine uptake for the incubation period. Rates of leucine uptake were measured in all the unfiltered water samples as well, to have an estimate of the total production, which includes the free-living as well as attached bacteria. Rates of leucine uptake were then converted to rates of C production assuming a conversion factor of 3.1 Kg C Mol leu$^{-1}$ (Kirchman 1993). Total bacterial carbon consumption was calculated as BP + BR, and bacterial growth efficiency (BGE) was calculated as BP/(BP + BR).

*Correlation analyses*—To examine in detail the relationship between the nutrient concentrations and the dominant subclass of the *Proteobacteria,* correlation analyses were performed on the entire database. Because the biological variables did not have a normal distribution (Kolmogorov-Smirnov test), they were log-transformed prior to calculations. In any correlation analysis of estuarine data, variables are bound to be internally correlated because of their strong common link to physical variables such as salinity (Riley 1939). The influence of salinity on the correlations between these variables was assessed using partial correlation analysis according to the Sokal and Rohlf (1995) procedure. The coefficients of partial determination (the square of the coefficients of partial correlation) were used as a measure of the fraction of the total variance of the bacterial variables explained by their correlation with nutrients (Legendre and Legendre 1983).

## Results

*Hydrological, physical and chemical description in the estuaries*—The hydrological conditions varied markedly between sampling periods in the Choptank River estuary. In March and in September, several days of heavy rains (around 6 cm over 1 week at Horn Point Laboratory, Cambridge, Maryland) preceded the cruise (Fig. 2). In May and July, rainfall during the preceding weeks was much lower (around 2 cm). As a result, the freshwater discharge was higher in March and September than in May and July (data not shown), and the *Rt* of the river greatly shortened (Table 2). During period of high rainfall and freshwater discharge, the water was renewed in less than 42 d. During period of low freshwater inputs, *Rt* was much longer, over 100 d. On the other hand, due to the geomorphological characteristic of the

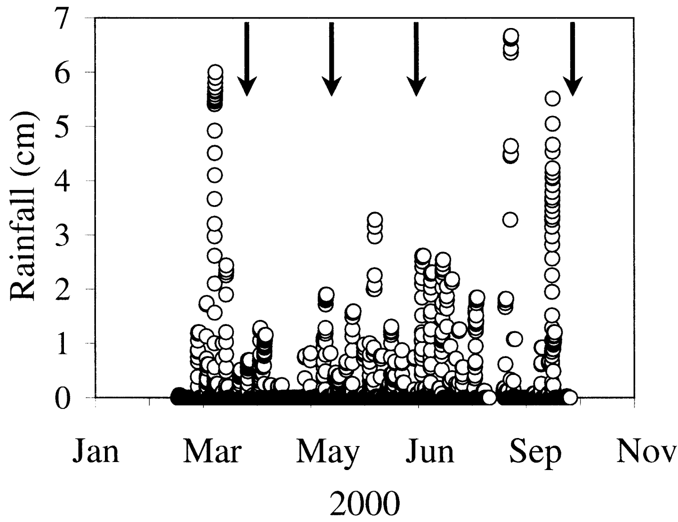


Fig. 2. Rainfall at Horn Point Laboratory (Cambridge, Maryland). Data recorded each 30 min (www.cbos.org). Arrows point to sampling periods.

Table 2. Freshwater residence time (days) of the whole Choptank River estuary (Chop. R.) and in its upper, middle, and lower regions over the months in 2000.

| | Chop. R. | Upper | Middle | Lower |
|---|---|---|---|---|
| March | 20 | 1 | 3 | 16 |
| May | 100 | 3 | 20 | 77 |
| July | 180 | 5 | 28 | 147 |
| September | 42 | 1 | 7 | 34 |

river, *Rt* varies within the gradient (Table 2). In the midestuary, which includes the turbidity maximum region, it ranges from 3 to 7 and 20 to 28 d during high and low water discharge, respectively.

All physical and chemical parameters measured showed strong variations along the Choptank River estuary transect. The salinity within the 100-km transect varied from 0 to 14 (Fig. 3). The most compressed mixing zone occurred during March and September with a mixing zone no longer than 30 km (from km 45 to 75) in the middle section of the estuary. The TSS concentrations varied from 5 to 60 mg $L^{-1}$ and increased toward the freshwater portion. In general, a region of high TSS concentration developed in the midregion of the estuary, whereas the lowest concentrations were found near

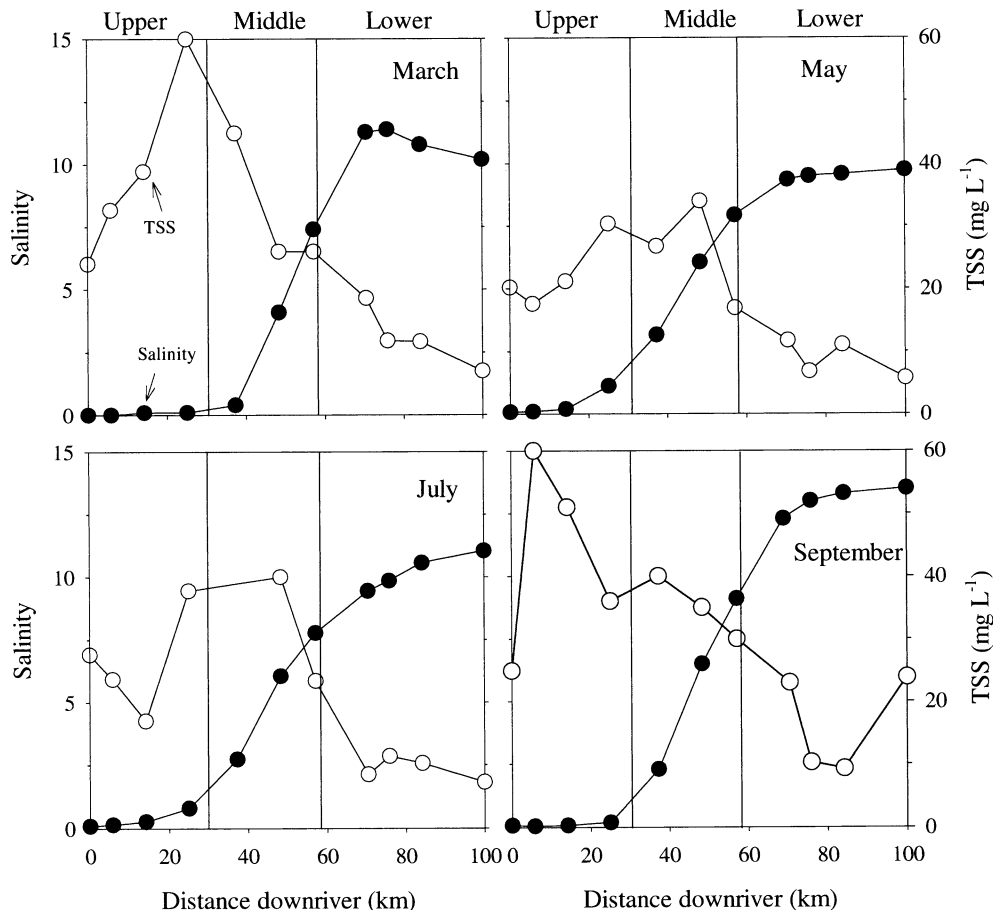


Fig. 3. Distribution of salinity and TSS from upstream (km 0) to the mouth of the Choptank River estuary (km 100) in March, May, July, and September 2000.

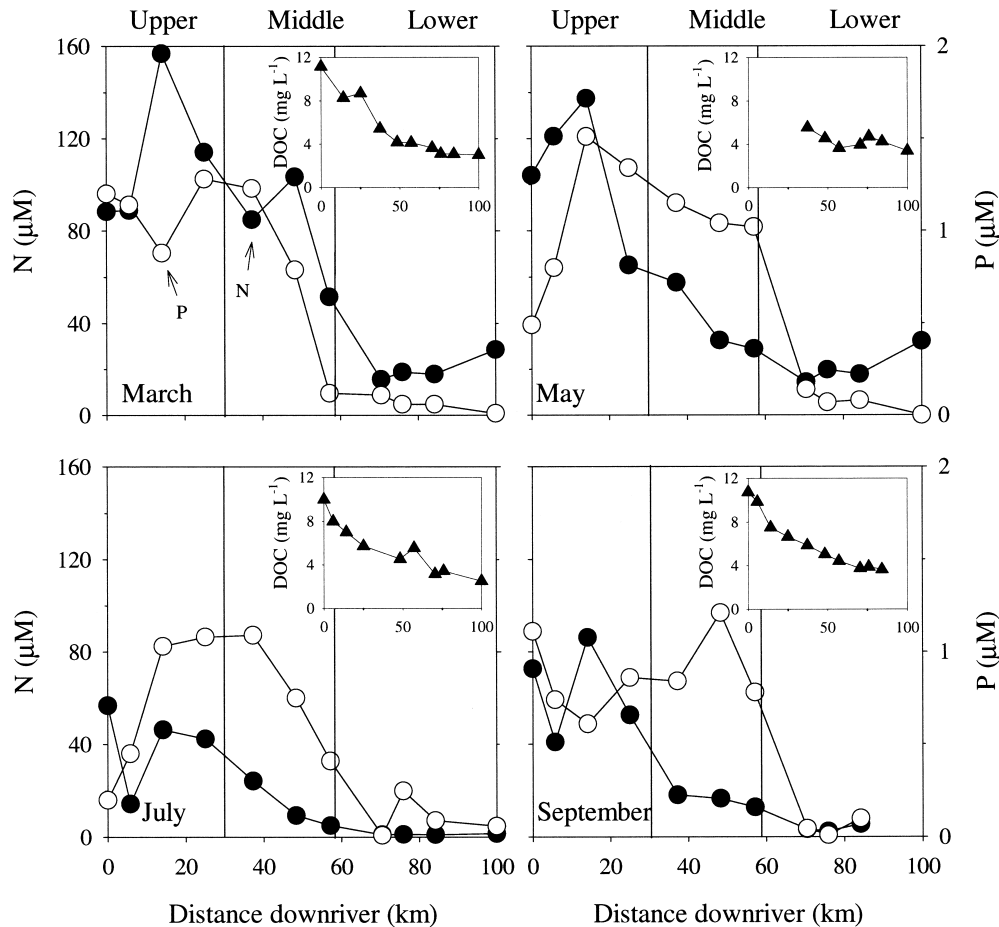


Fig. 4. Concentrations of N and P along the Choptank River estuary in March, May, July, and September 2000. Inset: DOC concentration.

the confluence with the Chesapeake Bay (Fig. 3). The TSS maximum generally coincides with the region of mixing of fresh and salt waters. It spreads from km 25 to 75 in the middle estuary. Temperatures decreased seaward with a gradient located at the beginning of the lower estuary with amplitudes from 2°C in July to 4.5°C in March. Within the freshwater and saline portions of the transect, the temperature varied very little (from 0.1°C in July to 0.8°C in March).

Dissolved nutrients showed strong variations that were related to water mixing and freshwater inputs (Fig. 4) in the Choptank River estuary. Concentrations of P and N were always highest in the upward, freshwater portions of the river, with a strong decline in the midsection within the turbidity maximum. However, this decline was much steeper for N than for P. In the lower estuary, both N and P concentrations were generally $<20$ $\mu$M and 0.2 $\mu$M, respectively, throughout the months investigated. The DOC concentration varied from 2.5 to 11.1 mg $L^{-1}$ (mean = 5.8; SD = 2.5; $N$ = 36) with a steep decrease downstream (Fig. 4). Recurrently over the months, the highest chlorophyll *a* concentrations were observed in the lower part of the estuary and declined within the turbidity maxima (del Giorgio and Bouvier, 2002).

The physical and chemical patterns along the river transect were similar between months, but the location and steepness of the main gradients varied with meteorological and tidal conditions. The gradients were more compressed and the differences between the regions were more pronounced during periods of high rainfall (March and September).

In the Pocomoke River estuary, the salinity within the 30-km transect varied from 0 to 14, with the greatest changes occurring between km 20 and 25 (Fig. 5a). The TSS concentrations ranged from 14 to 58 with the highest concentration at km 25 (Fig. 5a). There were major differences with regards to the Choptank River estuary; P decreased more rapidly than N along the transect, especially in the freshwater section. Additionally, the DOC concentrations were 2 times higher in the freshwater section of the Pocomoke than the Choptank River, and decreased seaward with a sharper gradient (Figs. 4 and 5a).

*Domain-specific distribution of free-living bacteria*—Most FISH samples examined along both the Choptank and the Pocomoke River estuaries showed bright hybridization signals and clear distinction among probe-conferred signals and the background. Over the Choptank River estuary, the fraction of autofluorescent cells and cells nonspecifically stained with the control probe (NON) was low in all sam-

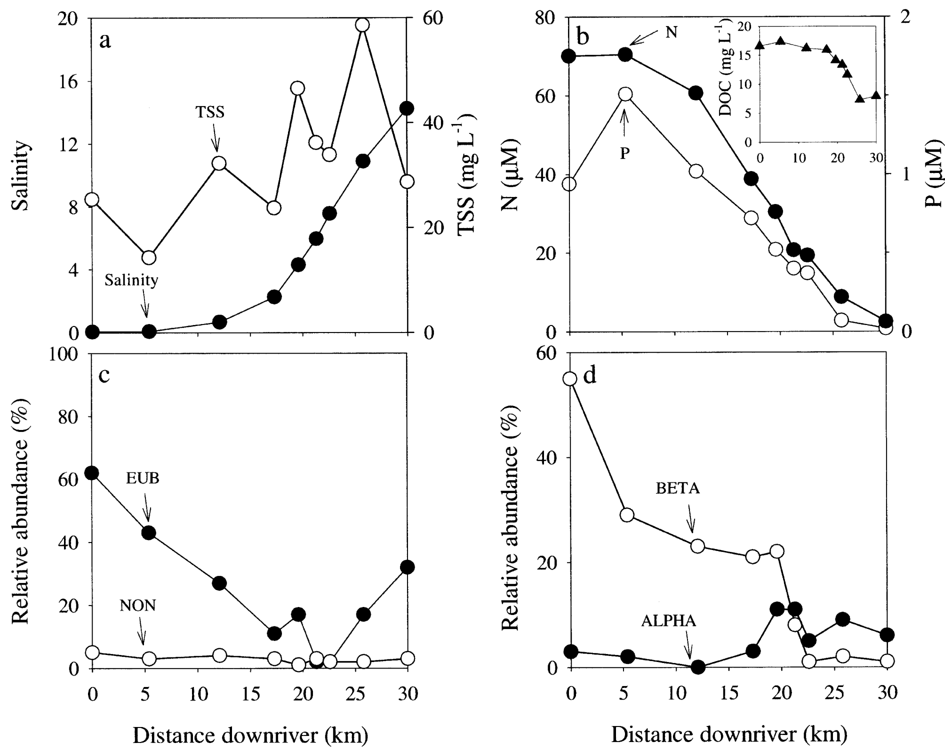


Fig. 5. Distribution of the (a) salinity and TSS concentration, (b) N, P, and DOC concentration, (c) domains *bacteria* (EUB) and the negative control (NON), and (d) $\alpha$- (ALPHA) and $\beta$- (BETA) proteobacteria across the Pocomoke River estuary in May 2000.

ples, averaging less than 2% of DAPI-counts cells (Fig. 6). The probe targeting members of the *archaea* detected on average less than 3% of DAPI counts along the estuary (Fig. 6). The highest relative abundance of *archaea* was observed in the lower estuary near the mouth of the river. The probe complementary to a region of the 16S rRNA conserved in most bacteria (EUB) detected on average 32% of the total number of cells determined by DAPI direct counts in the Choptank River estuary. There was a wide range in the percent of cells hybridized with the eubacterial probe (from 3% to 80%; SD = 21; $N$ = 51), and although most samples were above 40%, there were samples with extremely low percentage hybridization that influence the overall average. The average proportion of hybridization for the entire Choptank River estuary transect varied between months, but the variation in percentage hybridization within a given transect was greater than the differences recorded between months, and there was a recurrent spatial pattern of hybridization (Fig. 6). Both the upper (from km 5 to 25) and lower (from km 60 to 100) estuary regularly had >35% hybridization (mean = 35%, SD = 18, $N$ = 16 and mean = 43%, SD = 25, $N$ = 15, respectively), but in every month the percent of EUB decreased dramatically in the midestuary within the turbidity maximum (around km 50), to values as low as 3% of total DAPI counts (mean = 13%, SD = 10, $N$ = 12). These changes in the proportion of cells that could be hybridized with the eubacterial probe may have been related to changes in community metabolism as we discuss below.

Similar spatial patterns were observed in the Pocomoke River estuary. The control probe (NON) detected less than 2% of DAPI-counts cells over the transect (Fig. 5c). Members of the *bacteria* accounted on average for 20% of DAPI-counts cells. They were more abundant in the upper and lower estuary with percentage hybridization >30%. The percent of EUB decreased sharply in the midestuary within the TTS maxima to values as low as 2% of total cells.

*Spatial bacterial compositional changes*—We used a set of probes specific to four major phylogenetic groups of the *bacteria*: The $\alpha$-, $\beta$- and gamma ($\gamma$) of the *proteobacteria* and the CF, which together allowed a clear detection of differences between the bacterioplankton composition along the estuaries and over the seasons (Figs. 5d, 7, 8). These differences were most pronounced for the $\beta$- and $\alpha$-proteobacteria. The $\beta$-proteobacteria dominated the bacterioplankton composition in the upper freshwater Choptank River estuary (up to 50% of DAPI-stained cells) in every month, but accounted for less than 22% in the other regions. Their average abundance between both upper and lower estuary were significantly different ($t$-test, $p$ = 0.05). Likewise, the $\alpha$-proteobacteria overwhelmingly dominated the free-living community in the lower salt estuary (up to 48% of DAPI-counts), and were almost nonexistent in the upper estuary (less than 9% of the DAPI counts). Again, their average abundance between both upper and lower estuary were significantly different ($t$-test, $p$ = 0.05). Interestingly, both major groups consistently had their lowest relative abundance in the midestuary from km 25 to 50. Members of the $\gamma$-

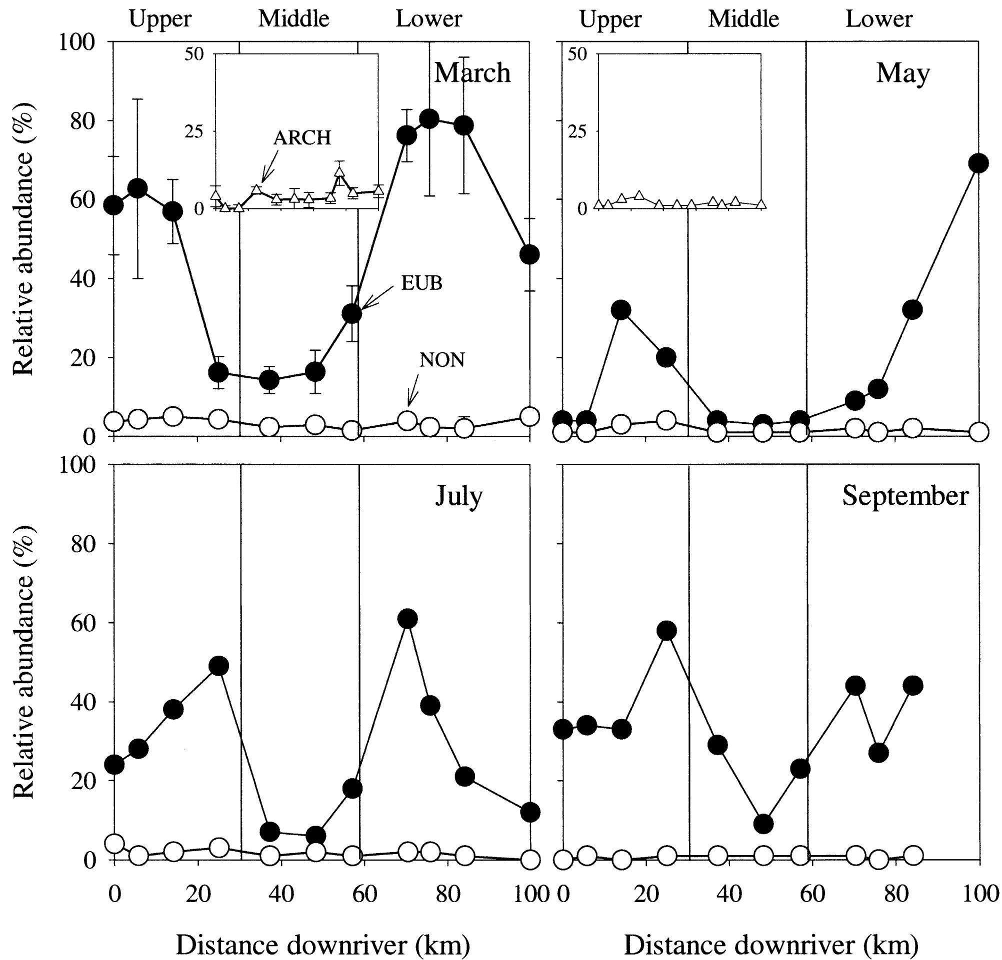


Fig. 6. Distribution of the relative abundance of the domains *bacteria* (EUB), *archaea* (ARCH, inset), and the negative control (NON) along the Choptank River estuary. Error bars: standard deviation based on three different hybridizations on the same sample.

proteobacteria and the CF group were generally not abundant over the estuary (Fig. 8). Each of these groups represented on average less than 4% of DAPI counts (SD = 6%; $N$ = 43) in the estuary. In contrast to both $\alpha$- and $\beta$-proteobacteria, the CF group were usually relatively more abundant near the turbidity maximum within the midestuary than on either the freshwater or marine portions, accounting for up to 21% of DAPI-counts from km 25 to 50. $\gamma$-proteobacteria increased locally with spikes at 10, 35, and 70 km downstream from the uppermost freshwater station, reaching 7%, 10%, and 8%, respectively. The probes for $\alpha$-, $\beta$- and $\gamma$-proteobacteria and the CF group together accounted for an average of 80% (SD = 25; $N$ = 43) of the bacteria hybridized with the universal *bacteria* probe (Eub338), so that on average 20% of the domain *bacteria* were unaccounted by the set of probes we used.

In the Pocomoke River estuary, a comparable shift between a dominance of $\beta$-proteobacteria in the freshwater section (up to 60% of the bacterial community) to the dominance of the $\alpha$-proteobacteria in the saline waters (up to 10% of the bacterial community) were observed (Fig. 5d). The $\gamma$-proteobacteria were a minor component of the bacterial assemblage with less than 2% of DAPI counts (data not shown). Similarly, the CF group was usually low (<5% of DAPI counts) over the transect but one high concentration was observed at km 20, i.e., near the TSS max (10% of DAPI counts) (data not shown). Similarly to hybridization in the Choptank River estuary, 16% of the domain *bacteria* were unaccounted for by the set of probes we used.

*Relationship between major groups and environmental factors*—Relationships between the dominant $\alpha$- and $\beta$- subclass of the *proteobacteria* examined by regression analysis are shown in Fig. 9. There was a significant negative relationship between the $\alpha$-proteobacteria and P concentration, in both the Choptank and Pocomoke Rivers ($R^2$ = 0.70, $p$ < 0.001 and $R^2$ = 0.61, $p$ < 0.001, respectively). In both systems, $\alpha$-proteobacteria were only weakly related to either N or DOC. In contrast, in both systems there was a strong positive relationship between the $\beta$-proteobacteria and DOC concentration ($R^2$ = 0.71, $p$ < 0.001 and $R^2$ = 0.69, $p$ < 0.001 in Choptank and Pocomoke River estuaries, respectively). Further examination shows that both $\beta$-proteobacteria-DOC relationships break down toward the lower por-

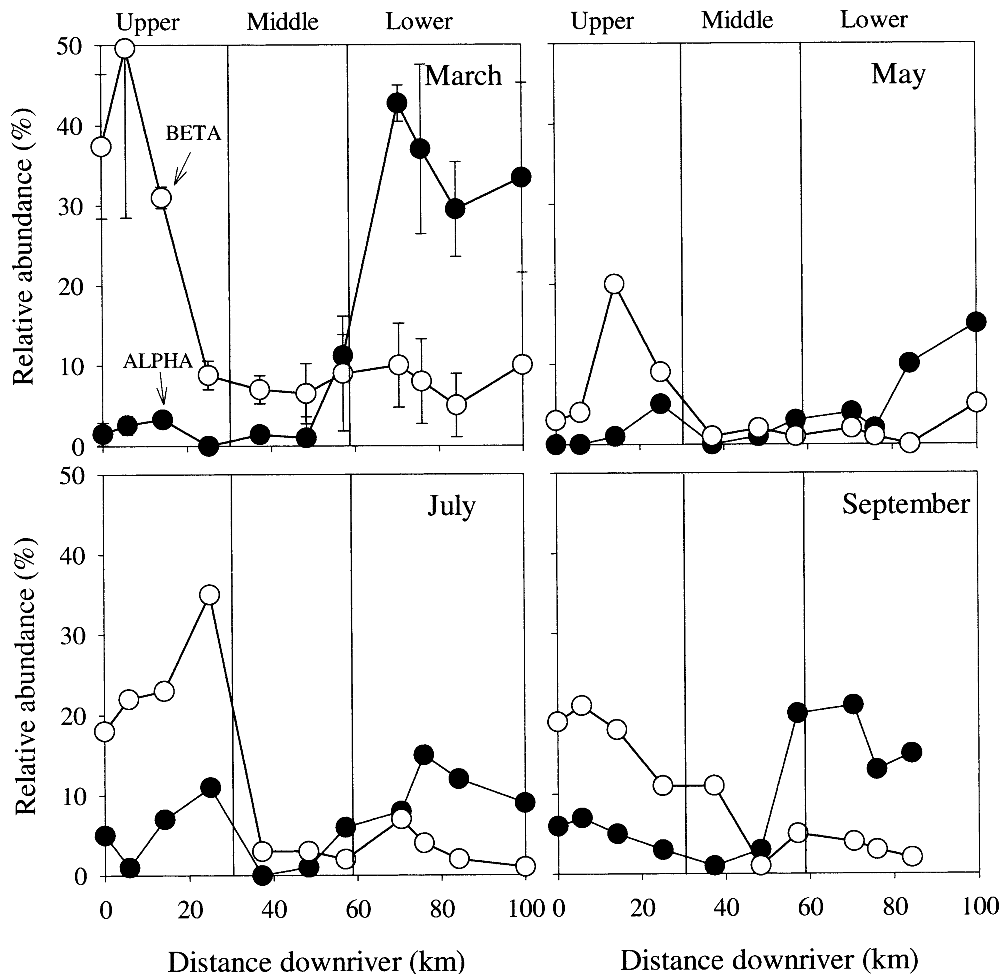


Fig. 7. Distribution of the relative abundance of the $\alpha$- (ALPHA) and $\beta$- (BETA) proteobacteria along the Choptank River estuary in 2000. Error bars: standard deviation based on three different hybridizations on the same sample.

tion of the estuary. At concentration lower than 6 mg DOC $L^{-1}$ and 12 mg DOC $L^{-1}$ in the Choptank and the Pocomoke river estuary, respectively, there were no relationships at all. These DOC concentrations correspond to waters with a salinity of approximately 5 in both estuaries. $\beta$-proteobacteria were only weakly related to dissolved P in either of the two estuaries. These relationships were also significant when data from individual months were considered rather than the mean for the study period.

Most variables considered here covary with salinity to some extent, so that these relationships between composition and P, N, or DOC could be mostly driven by salinity alone. We further explored these relationships using multiple regression analysis, and used the resulting partial regression coefficients to assess the actual portion of the variance explained by P, N, and DOC once the effect of salinity is removed. P still explains a significant portion of the variance in $\alpha$-proteobacteria (12% and 41% in Choptank and Pocomoke, respectively) after having removed the effect of salinity, whereas N and DOC explain virtually none of this variance (Table 3). DOC continues to explain a significant fraction of the variance in $\beta$-proteobacteria once the effect of salinity is removed, but only in the Choptank River estuary (14%). The absence of significant relationship recorded in the Pocomoke River estuary maybe explained by the strong covariation of DOC with salinity ($R^2 = 0.98$, $p < 0.0001$, data not shown), which makes it virtually impossible to separate the effect of the two variables. In the Pocomoke River estuary, 20% of the variance of the $\beta$-proteobacteria was explained by N (Table 3). The evidence suggests a coupling between $\alpha$-proteobacteria and P, and a coupling between DOC and $\beta$-proteobacteria, that is independent of salinity.

*Bacterial compositional transition and hydrological conditions*—The shift in bacterial composition between the upper and lower Choptank River estuary appeared to be strongly associated to the physical and chemical gradients located in the midestuary over the seasons investigated. The intensity of the environmental gradients, which in turn determines the spatial extent of the turbidity maximum within the midestuary zone, changed over the seasons due to differences in rainfall and freshwater inputs. The most abrupt compositional transitions were observed in March and September,

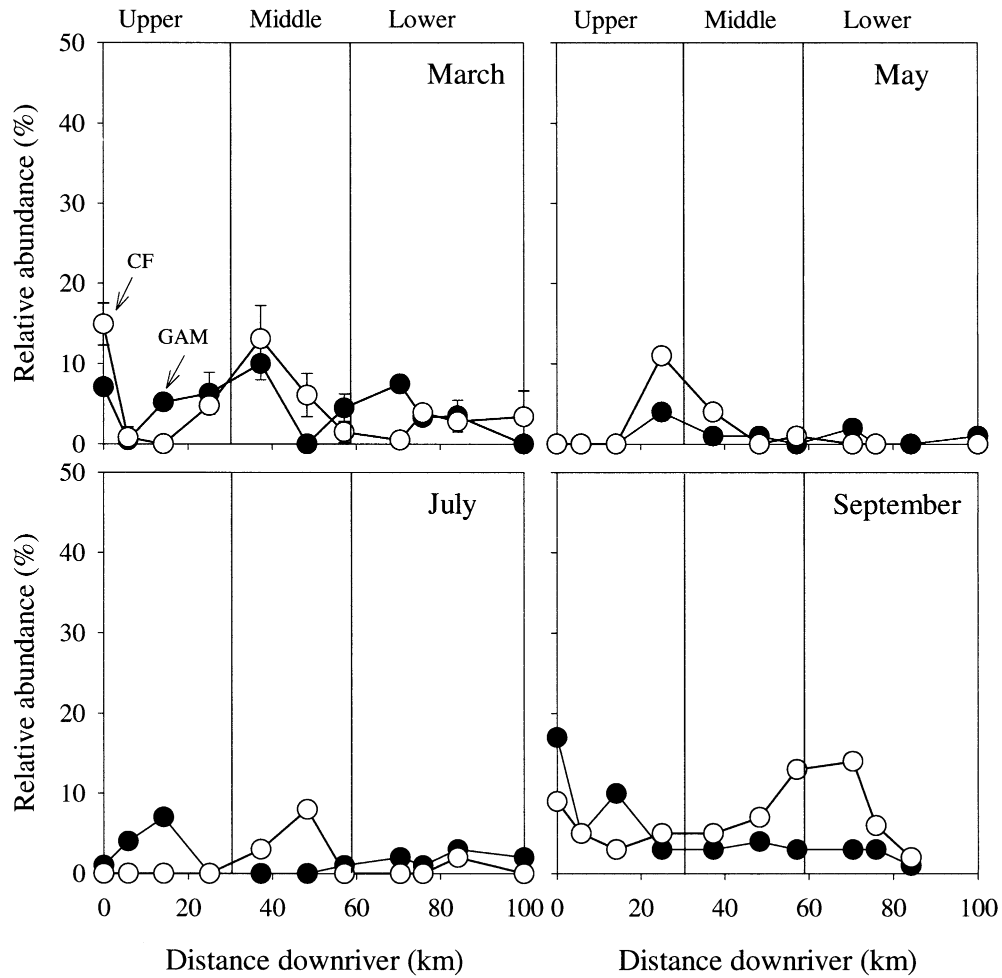


Fig. 8. Distribution of the relative abundance of the members of the $\gamma$-proteobacteria (GAM) and the *cytophaga-flavobacterium* cluster (CF) along the Choptank River estuary. Error bars: standard deviation based on three different hybridizations on the same sample.

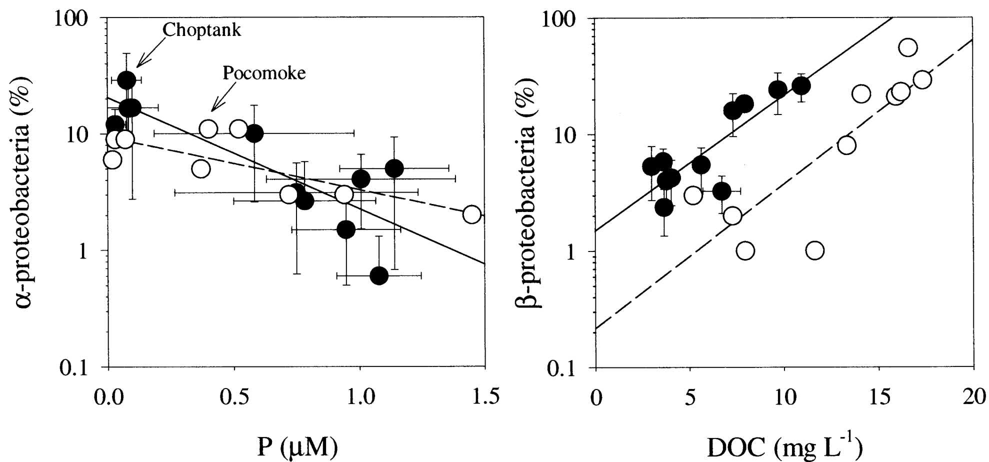


Fig. 9. Relationship between the percentage of $\alpha$-proteobacteria and $\beta$-proteobacteria versus P and DOC concentration, respectively. Solid and dash line are regression lines for the Choptank and the Pocomoke Rivers estuaries data set, respectively. Error bars: Standard deviation of monthly average values.

Table 3. Coefficient of partial determination of P, N, and DOC and log-transformed biological variables from all stations in both estuaries. $n$: number of samples.

| | Choptank | | | Pocomoke | | |
|---|---|---|---|---|---|---|
| | $n$ | Alpha | Beta | $n$ | Alpha | Beta |
| P | 42 | 0.12* | 0.07 | 10 | 0.41* | 0.06† |
| N | 42 | 0.01 | <0.01 | 10 | 0.04 | 0.20* |
| DOC | 42 | 0.02† | 0.14* | 10 | 0.04 | 0.02 |

* $p \leq 0.01$.
† $0.01 \leq p \leq 0.05$.

when the salinity and nutrient gradients were steepest. Conversely, in May and July, when the salinity gradient was less compressed and expanded across the estuary, the bacterial transition stretched out within the same spatial range (Figs. 2, 3, and 4). Interestingly, the transition between the $\alpha$- and $\beta$-proteobacteria occurred in the same salinity range (between 4 and 10), regardless the extent of the region involved.

*Bacterioplankton community metabolism*—Free-living bacterial production exhibited strong seasonal variability. BP was higher on average in May and July, ranging from 0.4 to 1.4 $\mu$g C L$^{-1}$ h$^{-1}$ (mean = 0.6 $\mu$g C L$^{-1}$ h$^{-1}$; SD = 0.3; $N$ = 22), than in March and September, when it ranged from 0.2 to 0.8 $\mu$g C L$^{-1}$ h$^{-1}$ (mean = 0.3 $\mu$g C L$^{-1}$ h$^{-1}$; SD = 0.2; $N$ = 21). There was a clear spatial pattern in unattached BP along the estuary, with a large decline in the midestuary in every month studied (Fig. 10). The decline in BP within the turbidity maximum relative to upstream and downstream stations ranged from 67% to 89%, and was larger during the high rainfall period in March and September (89% and 77%) than during low rainfall time in May and July (67% and 69%). On average, the free-living fraction accounted for 69% of the total BP (Fig. 11). Although the suspended particle load greatly increased in the turbidity maximum region, the proportion of total BP accounted for by the free-living fraction declined only slightly in this region. The bulk production in unfiltered samples followed a similar pattern to that measured in the filtered samples, and in fact declined even more steeply than the filtered fraction, suggesting an overall decline in bacterial production in both the free and attached fractions within this region of the estuary (Fig. 11).

Bacterial respiration varied from 0.8 to 7.0 $\mu$g C L$^{-1}$ h$^{-1}$ in the whole area over the seasons (Fig. 10). The pattern in bacterioplankton respiration was very different to that of production, and BR was generally higher in the midestuary than in either the upper or lower portions of the estuary (Fig. 10). The respiration rate did not vary drastically over sea-

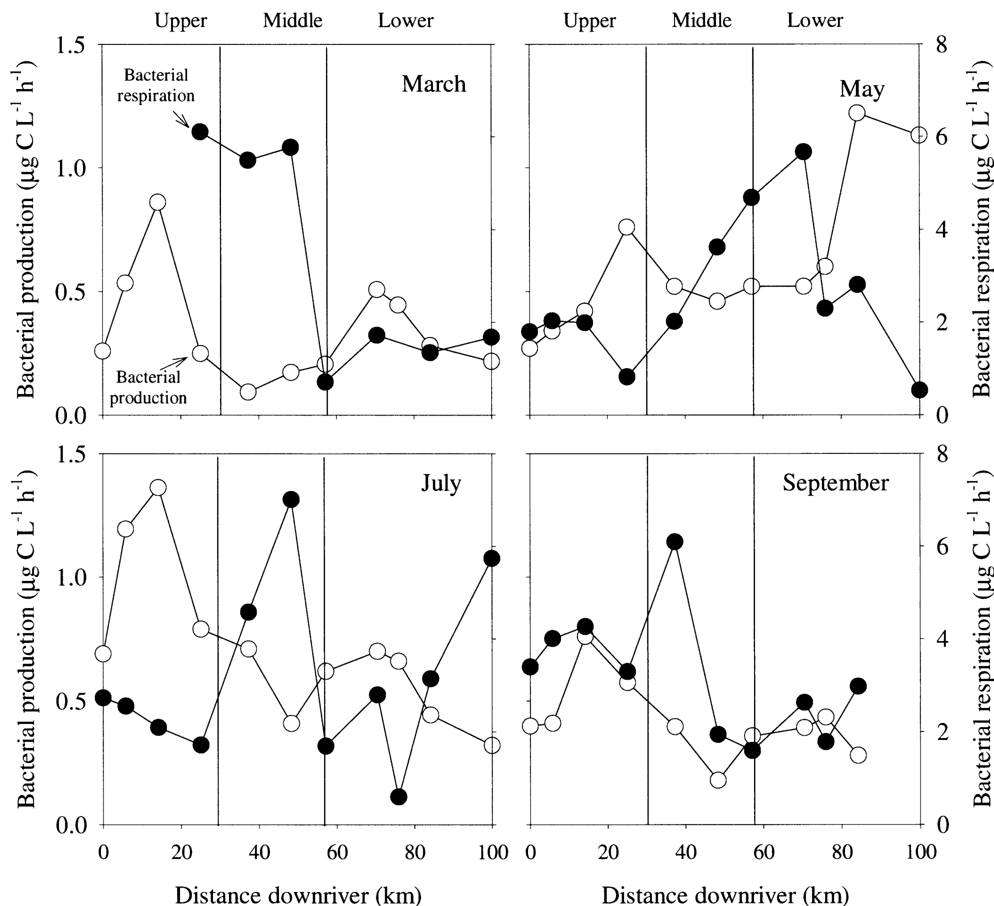


Fig. 10. Changes of the bacterial production and respiration along the Choptank River estuary in 2000.

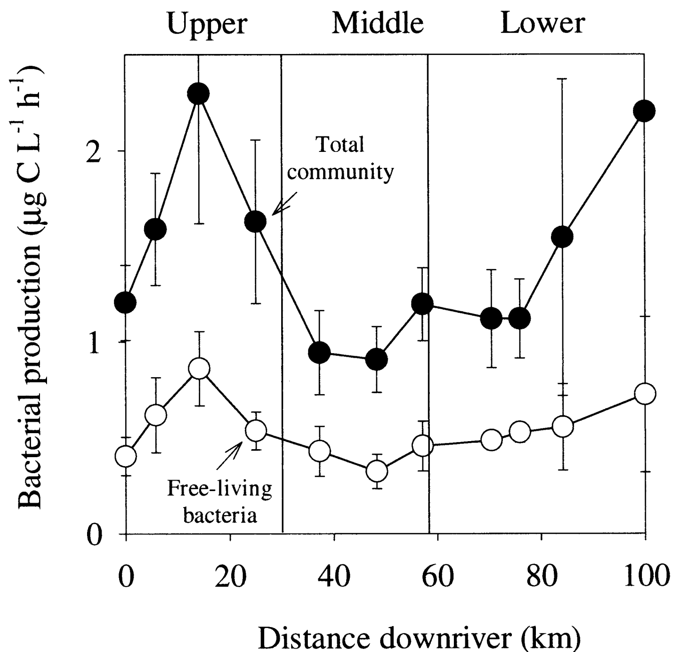


Fig. 11. Average March, May, July, and September 2000 distribution of the bacterial production of the total and the free-living portion of the bacterial community along the Choptank River estuary. Error bars: standard deviation based on seasonal variability.

sons, with a mean rate of 3.0 $\mu$g C $L^{-1}$ $h^{-1}$ (SD = 2.0; $N$ = 43). In most of the stations and over the different sampling periods, the respiration rate greatly exceeded the production rate. Therefore the fluctuation of the total bacterial carbon consumption (BP + BR) across the estuary followed the pattern of respiration, with the highest values occurring in the turbidity maximum within the midestuary region (Fig. 12). However, the difference between average BP + BR in the upper and the lower estuary was not statistically different over seasons ($t$-test, $p$ = 0.05). As a result of the patterns in bacterial production and respiration rates along the estuary, the bacterial growth efficiency (BGE) displayed a strong spatial variation, ranging from 0.02 to 0.67, with the lowest values consistently observed in the center of the turbidity maximum within the midestuary (Fig. 12)

## Discussion

*Phylogenetic composition of free-living bacterioplankton in the Choptank estuary*—The *archaea* were a small fraction of the assemblage, usually less than 3% along the entire gradient, and contributed very little to the overall phylogenetic succession observed in the river. Our results corroborate earlier findings on the low occurrence of *archaea* in estuarine systems (e.g., Crump and Baross 2000). *Archaea* are primarily allochthonous and therefore cannot grow under estuarine environmental conditions or grow too slowly to establish an estuarine community. The slight peaks observed near the mouth of the river might be the result of a water intrusion from the Chesapeake Bay bottom water in the Choptank entrance (Sanford and Boicourt 1990) or could simply be an enhanced local abundance due to the interface between the Bay and the River.

The *bacteria* accounted for most of the cells that were detected using FISH, and in turn the four probes within the domain *bacteria* that we used ($\alpha$-, $\beta$- and $\gamma$-proteobacteria and CF) together accounted for on average 80% of the cells detected with the eubacterial probe (EUB388). Other groups commonly found in estuaries and not addressed in this work could explain the missing 20%. For example, in the turbidity maximum of the Columbia estuary, 20 to 40% of the free-living bacterial populations were gram-positive cells and *Verrucomicrobiales* (Crump et al. 1999). In addition, it is clear that our FISH protocol failed to characterize a fairly large fraction of the assemblage, because the sum of the *archaea* and *bacteria* rarely exceeded 80% of the total counts. We discuss this limitation in sections below.

From the point of view of the phylogenetic composition of the bacterioplankton communities, the most conspicuous feature was the remarkable disparity in the spatial distribution of the different members of *bacteria* all along both the Choptank and Pocomoke River estuaries. $\beta$-proteobacteria overwhelmingly dominated the upper freshwater regions of these rivers, and the $\alpha$-proteobacteria dominated in the lower, saltwater portion, while the members of CF-cluster prevailed in the middle estuary and the $\gamma$-proteobacteria showed sporadic peaks along the transect. These results corroborate the evidence from a number of studies using variety of methods that major differences in microbial assemblages exist between different environments (Murray et al. 1996; Crump et al. 1999; Glöckner et al. 1999).

It is still unclear how and why specific lineages are restricted to precise environments (Methé et al. 1998), but our data allow us to at least generate hypotheses to explain the prevalence of different bacterial groups in various portions of the estuary. The $\alpha$-proteobacteria include genera regarded as marine and oligotrophic (Morgan and Dow 1985) and are, therefore, an important member of bacterioplankton in open ocean (Giovannoni and Rappé 2000). In the lower Choptank and Pocomoke River estuary, at salinities ranging from 8 to 14, this group accounted for as much as 48% of the total bacterial community. This was not unexpected, since the occurrence and even the dominance of this subclass of *proteobacteria* is already reported in similar estuarine regions of the southeastern U.S. coast at salinities comparable to those found our studies. For instance, 13% of the total bacterial DNA at salinity 14 were $\alpha$-proteobacteria, but this group becomes undetectable at salinities below 5 (González and Moran 1997). However, the dominance of the $\alpha$-proteobacteria is still somewhat surprising in the lower Choptank River estuary since the site we sampled and adjacent area (the Chesapeake Bay) are characterized by a markedly different trophic status from that found in open sea systems: the Chesapeake Bay and its tributaries are among the most productive coastal temperate ecosystems in the world (Monaco and Ulanowicz 1997; Fisher et al. 1998). As mentioned above, it is known that the adaptation to substrate and nutrient-deprived conditions characterize the *$\alpha$-proteobacteria* and explain in part their survival and growth at very low inorganic and organic nutrient-source concentrations (Zavarzin et al. 1990). Recently, nutrient concentrations have been pro-

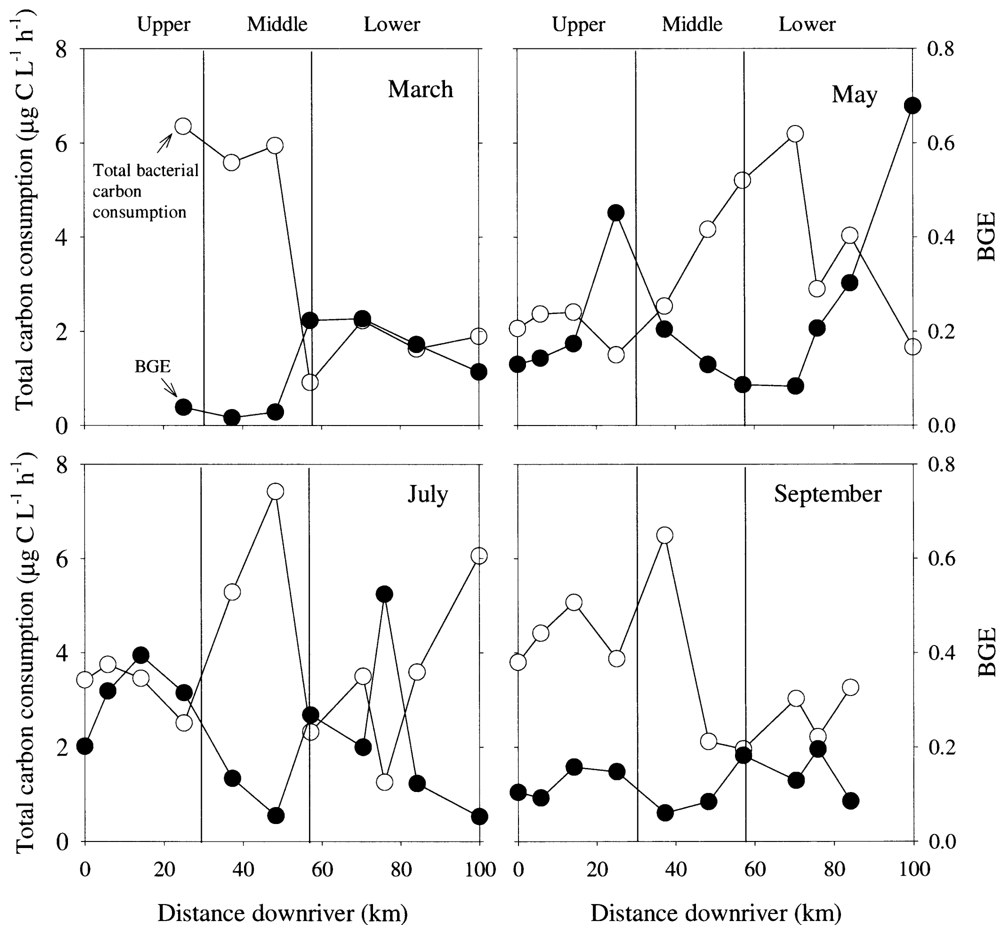


Fig. 12. Total bacterial carbon consumption and BGE across the Choptank River estuary in 2000.

posed as a factor driving the seasonal succession of the bacterioplankton assemblage in the Baltic Sea, and, in particular, low phosphorous concentrations ($<0.05$ $\mu$M) appear to play a major role in determining composition (Pinhassi and Hagström 2000). In both the Choptank and Pocomoke estuaries, the relative abundance of $\alpha$-proteobacteria is inversely correlated to P concentration, and this relationship persists even after removing the confounding effect of salinity. However, removing the effect of salinity in both systems showed there was no relationship with either N or DOC. Interestingly, although the $\alpha$-proteobacteria increased systematically toward the lower estuary where both the N and P concentration were low, this group only peaked at phosphate concentrations below 0.08 $\mu$M (Figs. 4 and 6). The same pattern was found in the Pocomoke River estuary, although these two systems differ in many major properties. Within their range of tolerance to salinity, the $\alpha$-proteobacteria may thus have a competitive advantage at low nutrient concentrations, particularly P, and we hypothesize that the P concentration, rather than the overall trophic status of the system, may influence the distribution of the $\alpha$-proteobacteria.

The $\beta$-proteobacteria typically constitutes a dominant fraction of the bacterial community in freshwater systems (Hiorns et al. 1997) but are often found in coastal bacterioplankton communities as well (e.g., Rappé et al. 2000). The $\beta$-proteobacteria include bacteria that are capable of using reduced gases, such as $H_2$ and methane, and also nitrifying bacteria (Zavarzin et al. 1990). Whereas the first two nutritional processes remain trivial in terms of total fluxes in the water column, nitrification appears as a general and essential process in the freshwater part of estuaries (Bianchi et al. 1999; de Wilde and de Bie 2000). Nitrification has been observed in the upper Choptank (Ward and Twilley 1986) and the Potomac River estuaries (Elkins et al. 1982). In our study, however, the $\beta$-proteobacteria were only clearly related to N distribution in the Pocomoke estuary (Table 3), and it is therefore difficult to link this subclass of *proteobacteria* in the nitrogen cycling of the freshwater section of the estuaries.

More interestingly, we observed in both estuaries a highly significant relationship between the $\beta$-proteobacteria and the DOC concentration (Fig. 9), and partial correlation analysis showed that this relationship is not driven by salinity alone (Table 3). These observations suggest that the distribution of the $\beta$-proteobacteria may be linked to DOC, although it is unclear what aspect of DOC is relevant. Previous studies have shown that the abundance of $\beta$-proteobacteria is related to allochthonous input of organic carbon in lakes (Pernthaler

et al. 1998). There is also increasing evidence that there are systematic differences in the patterns of use of specific compounds among major phylogenetic groups (Cottrell and Kirchman 2000*a*).

Our results suggest that it is not the concentration of DOC per se that affects the prevalence of $\beta$-proteobacteria in these estuaries. Both estuaries showed similar positive relationships between DOC and the proportion of $\beta$-proteobacteria, although the Pocomoke River had on average twice the DOC concentration. It is more likely that bacteria are responding to compositional changes in the DOC pool that are common to both estuaries, and that these changes might be relatively independent of the actual concentration. Major qualitative changes in the DOC pool along salinity gradient in estuaries have been reported, with the upper, freshwater portions dominated by terrigenous materials and the lower, saline portions dominated by algal-derived DOC (Mannino and Harvey 2000).

Although there are significant, positive relationships between DOC and the proportion of $\beta$-proteobacteria in both estuaries when all the data are considered, further inspection shows that both relationships break down toward the lower portion of the estuaries with salinity of approximately 5. This analysis suggests that salinity could be an additional controlling factor limiting the development of the $\beta$-proteobacteria, in addition to quantitative and qualitative changes in the DOC pool. Sodium inhibition has previously been suggested to explain their occurrence in freshwater and low-salinity coastal waters (Hiraishi et al. 1991; Rheinheimer 1997).

The members of the CF group are usually found in all marine and freshwater systems and may sometimes be among the most abundant bacterial groups in marine waters (DeLong et al. 1993). They are often observed attached to organic particles (Rath et al. 1998; personal observation) and are therefore found in abundance in the turbidity maxima of estuaries (Crump et al. 1999). In the present study, the free-living CF group also peaked in the mixing area where TSS concentrations are generally high and became the dominant subclass with up to 21% of DAPI counts. We hypothesize that there was indeed an increase in attached CF within the turbidity maximum and that some of these cells may have detached from particles and were thus included in the filtered sample. Alternatively, the environmental conditions may favor growth of free-living CF independently of the particle distribution.

The $\gamma$-proteobacteria were a minor component of the bacterioplankton across the estuary, and their isolated spikes appeared to be related to nutrient point sources to the river. The preference of many $\gamma$-proteobacteria for high nutrient concentrations (Zavarzin et al. 1990) may explain their local peaks. They were more prevalent at approximately km 15, 40, and 70, coinciding with the locations of three well-known point-source inputs of nutrients (Berndt 1999); the freshwater end-member near the confluence with the Tuckahoe River (~ km 15), the confluence with the Warwick River (~ km 40) into which flows the outfall from the large Twin Cities Wastewater Treatment facility, and the Cambridge Wastewater Treatment Outfall (km 70). The $\gamma$-proteobacteria may therefore be viewed as an opportunist group that takes advantage of transient nutrient enriched conditions. Alternatively, the transient spikes in $\gamma$-proteobacteria may not be the result of in situ growth but rather of loadings of allochthonous communities.

*Phylogenetic succession along the freshwater to saltwater gradient*—Our results show that the phylogenetic succession of the free-living bacteria along the salinity gradient was not gradual, but rather occurred with a sharp replacement of communities in and around the mixing zone where the nutrient concentration and the salinity changed drastically. This pattern occurred recurrently in the Choptank River estuary over the months investigated, and we have observed the same basic pattern in the Pocomoke River estuary, which suggests that the rapid phylogenetic succession may be a general feature of partially mixed estuaries. As we mentioned above, it is unclear what combination of environmental and biological factors determines the distribution of major lineages, or drives the actual phylogenetic succession. We discuss the transition between the dominant $\alpha$-proteobacteria in the lower region and the dominant $\beta$-proteobacteria in the upper region in more detail in the section below.

*Factors driving the phylogenetic succession in the Choptank River estuary*—It has been suggested that the composition of the bacterial communities may be related to changes in salinity (Barcina et al. 1997). In the present study, along the 0–14 salinity transect, $\alpha$-proteobacteria were observed only at salinity higher than 5 and the $\beta$-proteobacteria were observed at salinity lower than 10. These limits varied over seasons but in each case corresponded well with the salinity range of the transition (4 to 10), suggesting that salinity may play a significant role in the transition between $\alpha$ and $\beta$-proteobacteria. However, previous studies have also shown that osmotic changes of 10 typically result in only modest declines in bacterial growth rate (Painchaud et al. 1995). It is thus not clear whether the range in salinity alone observed along the Choptank and Pocomoke River estuaries was enough to drive the bacterial community succession.

Within an entire transect we found as much as 4.5°C of difference in temperature, but a given bacterial assemblage will never experience this range. Within the transition zone where most of the phylogenetic and physiological changes occurred there was a much smaller temperature differential, from 0.1°C in July to 0.8°C in March, and it is very unlikely that this range would drive a shift in the bacterial assemblage.

Inorganic and organic nutrient availability may profoundly influence not only bacterial metabolism but community composition (Morita 1997; Giovannoni and Rappé 2000). Although it is likely that P may play a role in determining the broad distribution of some major groups, as we hypothesize above, it is not clear at all how it might influence the actual succession that occurs within the transition zone. P often peaked in the mixing area whereas N declined considerably and downstream of the transition zone.

The DOC concentration was higher in the freshwater portions and declined continuously downstream, but the total bacterial carbon consumption did not decline correspondingly. Average carbon consumption was statistically similar be-

tween the upper and lower estuary and increased sharply within the transition zone, which suggests that bacteria were not limited by the availability of organic matter. Interestingly, the partitioning of carbon consumed showed systematic shift with a steep increase in respiration relative to production within the transition zone. As a consequence, the lowest values of BGE were generally recorded in the center of the transition zone, where the highest rates of total C consumption were observed. This decline in BGE and BP and increase in total carbon consumption is difficult to explain simply on the basis of changes in DOC and nutrients. Although both DOC and nitrogen concentrations decline within the transition area relative to the upstream freshwaters, as we mentioned above, they continue to decline toward the mouth of the estuary, and yet both bacterial production and BGE strongly rebound downstream of the transition zone. The concentration of P in fact often peaked within the transition zone. Interestingly, the chlorophyll *a* concentration was highest in the lower portion of the estuary and also declined sharply within the transition area, suggesting that there were strong shifts in the phytoplankton community as well. Overall, there was no apparent relationship between bacterial metabolism or composition and the distribution of chlorophyll *a* along the transect (del Giorgio and Bouvier 2002), and there is no evidence that bacterial succession may have been driven by changes in primary production.

The sharp decline in BP in the transition zone is clearly linked to changes in BGE rather than to a decline in overall carbon consumption. We hypothesize that changes in community metabolism, particularly the decline in BGE, reflect changes in the physiological condition of the bacterial assemblage that may not be directly related to DOC or nutrient limitation per se. Rather, it is possible that low community BGE partly reflects higher maintenance energy requirements to regulate the internal pH and osmotic pressure, as well as membrane energization of the cells within the transition zone (del Giorgio and Cole 1998). We propose that the unstable environment that characterizes the transition zone may induce severe physiological stress, and we show elsewhere that this hypothesis is supported by measurements of bacterial single-cell activity (del Giorgio and Bouvier 2002). The freshwater masses do not mix rapidly with the bay waters and oscillate under tidal forcing as they progress downstream. This oscillation creates a continually shifting environment, and although the water residence in this area was sometimes more than sufficient to allow adaptation and development of bacterial assemblage (*see below*), it may be precisely the instability of this region that prevents this development from occurring. The changes in salinity, nutrients or organic matter, or other environmental factors within the transition zone may be less important in triggering the bacterial phylogenetic succession than the intrinsic instability that characterizes the transition zone.

Our data show that the actual patterns of composition and metabolism transition are strongly linked to hydrological conditions. Moderate salinity gradients observed in May and July are clearly distinguished from steep gradients in March and September and are largely driven by river flow. In their study in the turbidity maxima region of the Columbia estuary, Crump et al. (1999) suggest that the free-living bacteria may not develop into a uniquely adapted estuarine community due to a short residence time of water (1 to 2 d). The lack of the development of a bacterial community in the middle Choptank during short residence times (from 3 to 7 d) corroborates their idea. However, during low rainfall periods the retention of water in the middle estuary was theoretically sufficient for development of a bacterial community (20–28 d, Table 2), but still no specific free-living assemblage appeared (Figs. 7 and 8).

That the phylogenetic succession may be accompanied by extreme physiological stress is also evident in the patterns of hybridization along the estuarine transect. The capacity to detect bacteria using FISH has been correlated to RNA content and thus to the state of single-cell activity (Kerkhof and Ward 1993; Karner and Fuhrman 1997; Tolker-Nielsen et al. 1997). In the Choptank River estuary, the average proportion of cells that could be detected with the eubacterial probe (32%; SD = 20; $N$ = 55) was low compared to previous observations in aquatic systems using comparable direct microscopic observations: 49% (Hicks et al. 1992, ponds), 55% (Pernthaler et al. 1998, oligotrophic lake), 56% (Glöckner et al. 1999, lakes, seas, and ocean), 77% (Glöckner et al. 2000, lakes) and 80% (calculated from Cottrell and Kirchman 2000*b,* coastal water). This low average proportion of cells hybridized by eubacterial and more specific probes is in part due to the inclusion of extremely low percentages recorded in the middle estuary (as low as 3%). The average proportion of cells that were detected using FISH in the upper and lower estuary was 39% (SD = 22; $N$ = 31), still in the lower range of the previous data. The overall low hybridization values relative to other studies are most likely due to differences in the fluorescence signal of the probes conjugated to BODIPY relative to other conjugates such as CY3 (Amann et al 1995; Glöckner et al. 1999). But the proportion of cells detected with the same eubacterial probe within the transition zone was much lower still, on average 13% (SD = 10; $N$ = 12), and the dramatic drop in the proportion of cells hybridized within the transition zone was not an artifact of the method: The drop occurred in all months around the same location, and in samples that were processed in the same batches where much higher hybridization was achieved. The underlying mechanisms for the drop in hybridization within the transition zone are still unclear, but we hypothesize that this drop was at least in part due to environmental stress and a general decline in cellular activity (Tolker-Nielsen et al. 1997).

Surprisingly, the proportion of BP due to the free-living fraction did not decline substantially in the transition zone, and both total and unattached production declined in the midestuary and then increased once again in the lower estuary. In fact, the decline in bulk, unfiltered BP within the transition zone was much more dramatic than in the free-living fraction, which suggests that the factors affecting the free-living fraction were strongly affecting the attached bacterial fraction as well. This is an important observation, because it would seem that no phylogenetic group was able to overcome the apparent environmental stress in this region. This also includes the CF group, because their transient increase that we noted in the free-living fraction, and that most likely occurred in the attached fraction as well within the turbidity

maximum, did not compensate for the loss of activity induced by environmental factors within the transition zone.

*Implications on the bacterial community representation*—The FISH method typically detects only a portion of the bacterial community, and there is increasing evidence that it preferentially detects cells that may have a higher level of metabolic activity (Oda et al. 2000). We have found a positive relationship between the response to FISH and the single-cell activity in different aquatic ecosystems (Bouvier et al., pers. comm.). The relationship between the response to FISH and the physiological status of the free-living bacterial population suggests that our description of the bacterial assemblage is biased toward cells that display a certain level of activity. Therefore, it is still unclear whether the phylogenetic succession proceeds as the result of simple mixing of different communities, differential growth, or differential inactivation and activation of existing communities. From our results it is indeed impossible to deduce whether there was an actual physical replacement of the major phylogenetic groups along the salinity gradient or whether the observed succession was simply the result of selective activation/inactivation of groups that were already present. We have shown above that the strongest phylogenetic shifts occur together with shifts in community production, respiration, and growth efficiency, and we have suggested that these shifts in metabolism are evidence that the replacement of phylogenetic groups may be mediated by physiological stress. We have also discussed the relationship between the compositional succession and changes in single-cell metabolic activity in the Choptank River, and further suggest that profound phylogenetic shifts are linked to cell stress, loss of activity, and even death (del Giorgio and Bouvier, 2002). It is most likely that both types of succession, i.e., activation/inactivation and replacement, occur simultaneously, but we are far from understanding the relative importance of these processes in determining bacterial succession and community composition. The other major question that we pose here, and that needs to be further addressed, is whether the environmental conditions that trigger the bacterial succession seen within the mixing area might not be the conditions that determine the actual distribution of the dominant phylogenetic groups.

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