

Linking the physiologic and phylogenetic successions in free-living bacterial communities along an estuarine salinity gradient

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Abstract

In this paper we assess whether the bacterial compositional succession that occurs along an estuarine salinity gradient is accompanied by changes in both community and single-cell metabolic activity in the free-living bacterioplankton. In addition, we explore whether up- and downstream estuarine communities, which are dominated by different phylogenetic groups, show distinct patterns in single-cell activity and characteristics. We have characterized the physiological succession of the bacterioplankton along the salinity gradient in the Choptank River Estuary (Maryland), using a combination of indices of single-cell activity, which include respiratory activity, membrane polarization and integrity, and DNA and RNA contents, combined with flow cytometry. We have also measured bacterioplankton community production, respiration, and growth efficiency along the estuary. Our data suggest that the sharp phylogenetic succession that occurs within the fresh to saltwater transition region is accompanied by profound metabolic changes both at the single-cell and community levels and that the phylogenetic succession occurs together with measurable physiological stress. The different indices of single-cell characteristics that we have used converge to suggest that within the fresh to saltwater transition zone, there is generalized decline in growth and possibly a loss of activity and even significant cell mortality. At the community level, these changes in single-cell physiology at the site of the phylogenetic succession appear to translate into a generalized decline in bacterial growth efficiency with a decrease in bacterial growth and production but an actual increase in bacterial carbon consumption. Our data also suggest that different phylogenetic groups may have intrinsically different levels of single-cell activity or at least respond differently to the activity assays that we currently use.

There are now a relatively large number of published studies that have described the bacterial composition in a range of aquatic ecosystems by use of a variety of molecular techniques. Overall, these studies have shown, for example, that bacterial communities in fresh- and saltwater systems are often dominated by very different taxonomic groups, even at the broadest phylogenetic levels (Glöckner et al. 1999; Giovanonni and Rappé 2000). The bacterial phylogenetic succession that occurs along environmental gradients, such as salinity gradients, has received considerably less attention than the description of the phylogenetic composition in individual systems. There are at least two fundamental aspects of the phylogenetic succession in bacterioplankton assemblages along environmental gradients that remain to be explored. First, it is still unclear whether the succession proceeds mostly as a physical replacement of major phylogenetic groups or whether the succession proceeds through activation/inactivation of the various groups that are already present in the assemblage. Second, it is still unclear whether

different intrinsic levels of metabolic activity characterize the different major phylogenetic groups or, at least, different responses to the activity assays that we commonly use.

It is difficult to address the first question directly with current molecular approaches. Traditional polymerase chain reaction-based techniques allow the detection of multiple phylotypes but do not generally allow quantification of their relative abundance or their level of cellular activity (Amann et al. 1995). Fluorescence in situ hybridization (FISH), on the other hand, does allow direct quantification of the abundance of phylotypes, but the problem is that not all bacteria present in an assemblage are scored with this method (Glöckner et al. 1999). This leaves the possibility that what appears to be a replacement of groups might actually be the selective activation and inactivation of groups that are already present in the assemblage. Thus, it is possible that the overall composition of the assemblage does not fundamentally change but rather that different major phylogenetic groups are selectively activated and inactivated along the gradient. At the root of this problem is the difficulty in distinguishing between the actual phylogenetic structure of the bacterial assemblage and the structure of the fraction that is metabolically dominant.

The second question—i.e., whether different taxonomic groups are characterized by different intrinsic levels of cellular activity—is also difficult to address directly. The link between hybridization and the level of cellular activity has often been postulated (Kerkof and Ward 1993; Oda et al. 2000) but has seldom been empirically confirmed for natural bacterioplankton. Approaches that combine FISH with autoradiography may allow the exploration of activity (i.e., Ouverney and Fuhrman 1999; Cottrell and Kirchman 2000)

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but do not entirely resolve the issue, because these techniques can only address the question of whether a cell that hybridizes also takes up a given substrate and can be therefore considered active but say nothing about the cells that do not hybridize in the first place. More recent approaches that combine flow cytometry and cell sorting of radiolabeled samples show promise (Lebaron et al. 2001; Servais et al. 2001) but are still limited by the difficulty of determining the identity of the cells that are sorted (Bernard et al. 2000).

Although at present it is difficult to assess the level of activity of the various phylogenetic groups directly, we can at least determine whether the phylogenetic succession is accompanied by detectable changes in metabolic activity, both at the bulk community level and at the level of single cells. The main objective of this research was to assess the physiological and metabolic changes in the free-living bacterioplankton assemblage, both at the single-cell and community level, along an estuarine salinity gradient. In a companion paper (Bouvier and del Giorgio 2002), we show that there is a strong phylogenetic succession along this salinity gradient and that the shift occurs most conspicuously around the transition zone region where fresh- and saltwater mix. In addition, we also noted major changes in community-level metabolism in parallel to the phylogenetic shifts. In this paper, we specifically explore whether the phylogenetic succession that occurs along this salinity gradient is accompanied by measurable changes single-cell characteristics that may explain the changes in community metabolism. In addition, we explore the possibility that communities dominated by different major phylogenetic groups also differ systematically in the levels of single-cell activity. We have characterized the physiological succession of the bacterioplankton that occurs in parallel to the phylogenetic succession along the salinity gradient in the Choptank River Estuary (Maryland), using a combination of indices of single-cell activity, which include respiratory activity, membrane polarization and integrity, and DNA contents, all combined with flow cytometry. We have also measured bacterioplankton community production, respiration, and growth efficiency along the estuary.

Materials and methods

Sampling site and procedure—The Choptank River is a partially mixed estuarine tributary of the Chesapeake Bay, located on the coastal plain of the eastern shore of Maryland, ~185 km from the mouth of the bay (Fig. 1). This estuary extends ~100 km from its uppermost freshwater reaches to its confluence with the main channel of the Chesapeake Bay estuary, with a salinity range of 0 to a maximum of 14 psu. The middle estuary extends approximately from km 25 to 60 and is an ecotone between the upper freshwater and lower saltwater ecosystems. This transition zone is characterized by strong environmental discontinuities (Ward and Twilley 1986). The water residence time of the Choptank River ranges from weeks, at periods of high flow, to several months (Bouvier and del Giorgio 2002).

Water samples were collected with a Niskin bottle during a day-long cruise along a transect spanning the salinity gra-

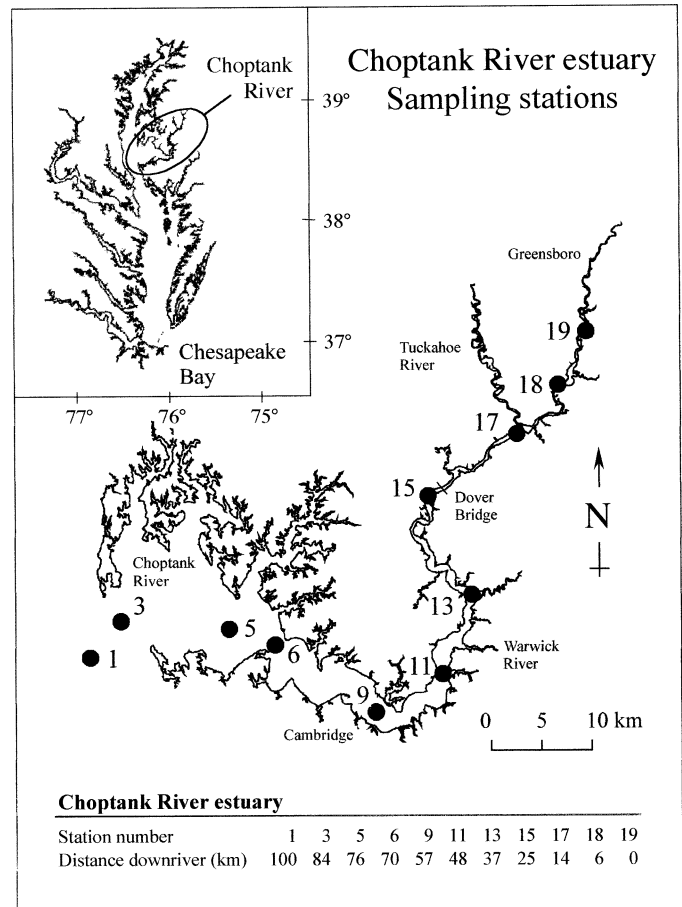


Fig. 1. Map of the study region. The Choptank River estuary is a major tributary of the Chesapeake Bay, which is one of the major estuaries in North America, located in Maryland. The transects spanned almost the entire length of the Choptank River, from the freshwater reaches (sampling locations 19–15), down to its confluence with the Chesapeake Bay (sampling location 1 on the map). The mixing of fresh- and saltwater occurs predominantly around locations 9–13, and we refer to this as the transition zone.

dient of the Choptank River in March, May, July, and September 2000 (Sta. 1–19, Fig. 1). Sta. 3 corresponds to the mouth of the Choptank River and its confluence with the main stem of the Chesapeake Bay estuary. Sta. 19 corresponds to the upper, freshwater portion of the Choptank River. Surface water samples were collected at all stations, kept cool and dark, and brought for processing to the Horn Point laboratory, which is located at the mouth of the river, no more than 3 h after collection.

In this study we focused on the abundance, composition, and community metabolism of free-living bacterioplankton. The main reason for this is that we wanted to explore changes in both bacterial respiration and production 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 that make microscopic examination of samples, particularly for FISH, very difficult. However, we measured bacterial abundance, production, and single-cell activity on all bulk water samples as well, to have an idea of the behavior of the unfiltered assemblage.

Bacterial community metabolism—In the lab, a portion of the bulk water was set aside to perform chlorophyll analysis and to measure bacterial production, abundance, and single-cell activity in the unfiltered water. The remainder was filtered to separate bacterioplankton from other planktonic components to perform measurements of bacterial respiration and production. In previous studies we tested a wide variety of filters commonly used to separate bacteria and concluded that the glass fiber Millipore AP 15 filters are the most effective in reducing the number of picoplankton organisms while maintaining most of the original free-bacterial community structure (del Giorgio and Newell unpubl. data).

Ten liters of water from each site were gently filtered through Millipore AP 15 (15-cm diameter) filters by use of a peristaltic pump and acid-washed tubing, and the filtrate was used to fill two 4-liter, acid-washed Erlenmeyer flasks. One flask is placed on a stand and is connected by acid-washed silicone tubing to a lower flask, so that a siphon can be established. The upper flask is open to the atmosphere, whereas the lower flask is sealed and has a sampling port. All the flasks were placed in a large walk-in incubation chamber, which was kept dark at ambient field temperature; up to 12 samples could be set up and processed simultaneously. This system allows intensive sampling to establish detailed time courses for oxygen consumption and bacterial production with minimal handling of the sample water. Samples are retrieved from the lower flask by opening the valve that connects the upper and lower flasks. Each flow-through system was sampled every 2 h, and at each sampling time a maximum of 40 ml of water are retrieved, which were used to determine O₂ concentration, leucine uptake, and bacterial abundance and single-cell activity, as described below. The volume replaced at each sampling thus represents 1% of the total volume of the incubation flask, and there is no detectable effect on gas concentrations.

Samples of water for oxygen concentration were taken directly from the flasks by inserting the outflow tube into a 5-ml glass tube and allowing the water to overflow. Duplicate or triplicate tubes were filled this way for every time point taken during the incubation. Each tube was poisoned with 8 μ 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 by use of membrane-inlet mass spectrometry (Kana et al. 1994) within a maximum of 3 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. Bacterial respiration (BR) was estimated from the rates of oxygen consumption

calculated from the slope of the O₂ versus time relationship fitted to an ordinary least-square regression. Rates of oxygen consumption were converted to CO₂ production assuming a respiratory quotient of 1, which we have empirically confirmed in parallel studies (del Giorgio and Newell unpubl. data).

Rates of bacterial production (BP) were estimated from the uptake of ³H-leucine according to the centrifugation method of Smith and Azam (1992). There were three measurements of leucine uptake in the filtered fraction during the incubation, at 0, 3, and 6 h, 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 gather an estimate of the total community production, which includes the free-living and attached bacteria. Rates of leucine uptake were then converted to rates of C production under the assumption of 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).

Bacterial enumeration and single-cell characteristics—All enumerations reported here are from fresh, unfixed samples processed immediately upon arrival to the laboratory after collection in the field and during incubations. Total bacterial abundance was determined by flow cytometric analysis (del Giorgio et al. 1996). The flow cytometric analysis of bacterioplankton samples stained with SYTO 13 allows in addition the discrimination of different bacterial populations according to green fluorescence (FL1) and light side scatter (SSC) (Lebaron et al. 1998; Troussellier et al. 1999; Gasol and del Giorgio 2000). There are usually two major identifiable fractions (Li et al. 1995): a first population consists of cells with high FL1 and SSC (high-DNA cells) and a second population of cells with lower mean FL1 and somewhat lower SSC (low-DNA cells). We routinely determined the abundance and characteristics of cells in each of these subpopulations.

The abundance of respiring bacteria that have high rates of metabolism was determined by use of the 5-cyano-2,3-ditoly tetrazolium chloride (CTC) assay, an indicator of the respiratory electron transport system activity (Sherr et al. 1999). Active cells reduce the tetrazolium salt CTC to its fluorescent formazan, and those cells with a high rate of respiration or cellular metabolism (CTC+) produce enough intracellular red fluorescence to allow detection and enumeration by epifluorescence microscopy (Rodriguez et al. 1992) or, as in of the present experiments, flow cytometry (del Giorgio et al. 1997; Sieracki et al. 1999). A stock solution of 50 mM CTC (PolySciences) was prepared daily, filtered through 0.1 μ m and kept dark at 5°C until use. CTC stock solution was added to 0.45 ml of sample to a final CTC concentration of 5 mM and then incubated for 1.5 h at room temperature in the dark. At the end of the incubation, green 1- μ m beads were added, vortexed, and then run in the cytometer. The orange fluorescence of CTC (FL2) and the light SSC emission were used to discriminate the CTC+ cells from CTC- cells and other weakly fluorescent parti-

cles. The percentage of CTC+ cells was calculated relative to the total bacterial counts obtained by SYTO-13 staining.

The proportion of cells lacking membrane polarity was assessed by use of the potential-sensitive dye DiBAC (Molecular Probes), an anionic, lipophilic oxonol that is actively excluded from intact cells but accumulates within cells that have lost membrane potential (Novo et al. 2000). Cells that accumulate DiBAC fluoresce bright green and can easily be detected by use of flow cytometry (Jepras et al. 1995; López-Amorós et al. 1995; Nebe-von-Caron et al. 2000). A 2 μl volume of a stock solution of DiBAC (0.5 mg ml^{-1}) was added to 0.5 ml of fresh water sample, for a final concentration of $1 \mu\text{g ml}^{-1}$. Green 1.0- μm beads were added, and the samples were vortexed and incubated for <10 min before running them in the flow cytometer. Cells stained with DiBAC were enumerated from a cytogram of FL1 and SSC, and the background noise was eliminated by use of a threshold in FL1. To define the cytometric characteristics of depolarized bacterial cells in natural bacterioplankton assemblages, we routinely ran controls that consisted of the same natural water sample that had been incubated with the ionophore Gramicidin S. This treatment resulted in rapid loss of cell polarity, and these samples could then be used to characterize the cytometric signature of depolarized bacterioplankton cells.

We used the CTC and DiBAC assays in the samples taken from the four transects, and in the March transect we also determined the number of cells with damaged or compromised cellular membranes, using the commercial LIVE/DEAD BacLight kit (Molecular Probes). This kit contains a mixture of a cell-impermeant nucleic acid stain (propidium iodide) that only penetrates cells with seriously damaged membranes and a cell-permeant nucleic acid stain (SYTO-9) that acts as a counterstain for all cells; 1 μl of the mixture of the two stains was added to 0.5-ml samples, 1.0 μm beads were added, and the samples were vortexed and incubated at room temperature in the dark for 10 min prior to cytometric analysis. In this assay, cells with compromised membranes are discriminated from intact cells in a cytogram of red versus green fluorescence: damaged bacteria have higher red than green fluorescence because of a greater penetration of the red propidium iodide, whereas intact cells have higher green than red fluorescence (Gasol et al. 1999). To define the cytometric characteristics of depolarized bacterial cells in natural bacterioplankton assemblages, we routinely ran controls that consisted of the same natural water sample that had either been subjected to mild heating or incubated with the ionophore Gramicidin S. Both treatments resulted in rapid loss of cell membrane integrity, and these samples could then be used to characterize the cytometric signature of injured cells.

All the flow cytometric analysis of bacterial populations and single-cell characteristics were performed in a FACSCalibur Flow Cytometer (Becton Dickinson), equipped with an argon laser, at the lowest possible flow rate ($\sim 12 \mu\text{l min}^{-1}$). Samples that had $>1,500 \text{ events s}^{-1}$, which was often the case for total counts, were diluted with 0.1-filtered DI water immediately prior to analysis. We performed total and single-cell activity counts on both the bulk and the filtered water samples.

FISH—The filtered water was used to determine the phylogenetic composition of the free-living bacterioplankton by use of FISH. We used a standard protocol, except that we used oligonucleotide probes labeled with BODIPY green, a fluorochrome synthesized by Molecular Probes. Details of the FISH protocol can be found in a companion paper (Bouvier and del Giorgio 2002). BODIPY-stained cells fluoresce green, but there is some overlap with the emission spectra of 4,6-diamidino-2-phenyl-indole (DAPI). Therefore, samples were not counterstained with DAPI, but rather total counts were obtained from a separate aliquot that was stained with DAPI. At least 500 cells were counted per sample on 10–20 randomly chosen fields. In this article, we focus only on the proportion of cells that could be hybridized and detected with the general eubacterial probe EUB338 (%EUB), but the complete list of probes and sequences that we used are in Bouvier and del Giorgio (2002). All the probe counts have been corrected for nonspecific probe binding by subtraction of the counts for the control probe NON338. We only performed FISH on the filtered water samples.

Chemical analyses—Water samples for dissolved nutrient analysis were filtered in the field through a Whatman GF/F glass-fiber filter (nominal pore size $0.7 \mu\text{M}$). The filtrate was placed on ice and then frozen immediately upon return to the laboratory for subsequent determination of total dissolved nitrogen and phosphorus. A bulk water sample was placed on ice in the field and processed within 24 h for total suspended solids by use of vacuum filtration and a modified gravimetric determination. Dissolved nutrient concentrations were determined colorimetrically on a Technicon Auto-Analyzer II system. Chlorophyll concentration was determined spectrophotometrically from ethanol extracts.

Statistical analyses—The relationships between variables were explored by use of least-squares linear regression analysis. For regression analysis, the variables were log-transformed to attain normality and homoscedasticity. Student's *t* test was performed on the untransformed data to assess differences in mean single cell activity between fresh- and saltwater; a level of 0.05 was considered significant.

Results

The Choptank River estuary environmental gradient—This study covered 80 km in the lower portion of the Choptank River estuary, starting in the freshwater water upper portion to its confluence with the main stem of the Chesapeake Bay Estuary (Fig. 1). Along this gradient, salinity ranged from 0 to 14 psu, with the sharpest change within the middle portion, where the saline estuarine waters mix with the freshwater riverine waters (Fig. 2A). There were other strong gradients within this transition region, including sharp peaks in turbidity and suspended solids (Bouvier and del Giorgio 2002), total dissolved P, and N (Fig. 2B,C). Dissolved organic carbon (DOC) declined continuously along the salinity gradient (Fig. 3A), and the concentrations at any given station were relatively constant across months. In contrast, the estuary often had high and extremely variable levels of chlorophyll *a* concentration, with peaks of up to 30

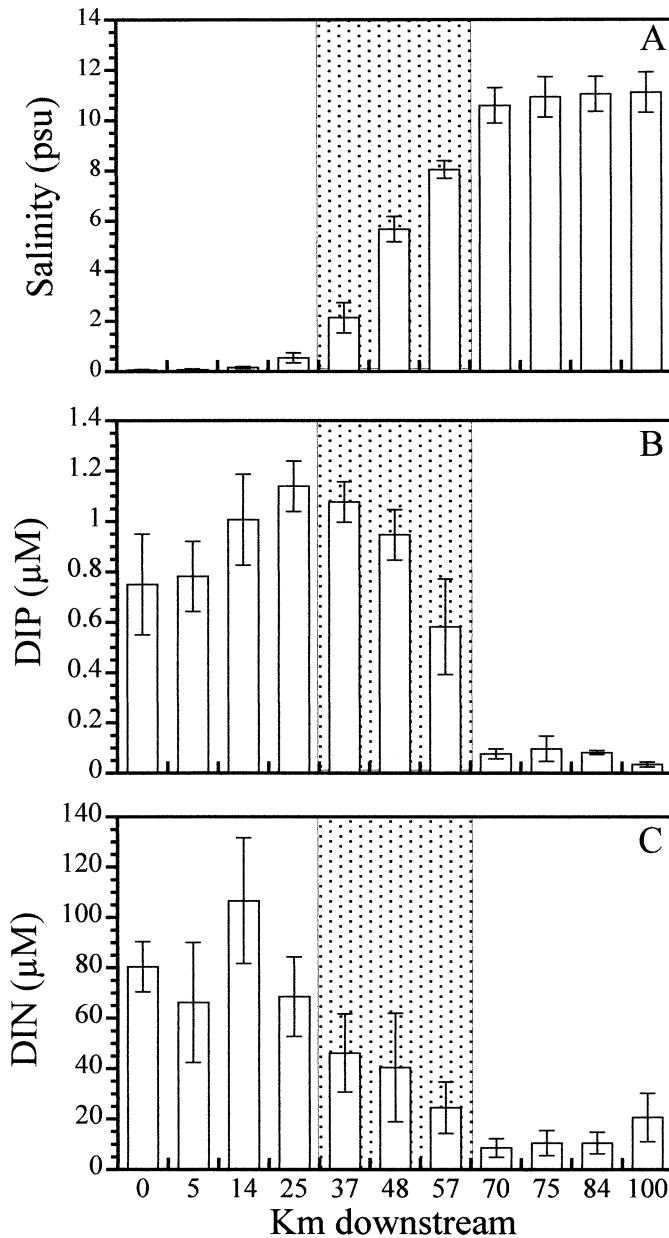


Fig. 2. (A) Salinity, (B) dissolved inorganic phosphorus, and (C) nitrogen along the estuarine gradient. Each bar represents the average (\pm SE) for each sampling station of four samples taken in May, July, August, and September 2000. The average extent of the transition zone where fresh- and saltwater mix is indicated by the dotted area.

$\mu\text{g L}^{-1}$ both in the upper and lower portions (Fig. 3B). There was no relationship between DOC and Chl *a* concentrations, which suggests that the high DOC levels in the freshwater reaches originate from the drainage basin and wetlands and not from in situ algal production.

Changes in free-living bacterial abundance—For brevity and clarity, we have averaged the data of the 4 months for each station. The average pattern in all variables was essentially repeated every month, with minor variations in abso-

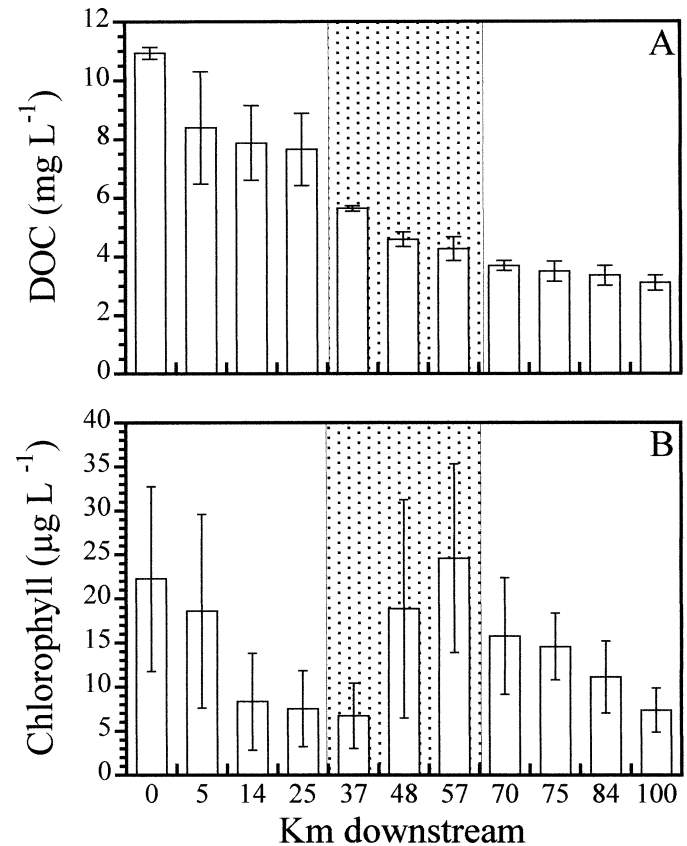


Fig. 3. (A) DOC concentration and (B) Chl *a* concentration along the transect. Each bar represents the average (\pm SE) for each sampling station of four samples taken in May, July, August, and September 2000. The average extent of the transition zone, where fresh- and saltwater mix, is indicated by the dotted area.

lute numbers and in the exact location of peaks. Total free-living bacterial abundance (BA) varied <10 -fold along the transect and over the entire sampling period. Within a given transect, BA varied less than threefold: it was, on average, lower in the upper, freshwater sites than in the lower saltwater sites (Fig. 4A). There was a greater range of variation in the abundance of cells scored positive with the CTC assay cells (Fig. 4B), and, of interest, the abundance of CTC+ cells was on average higher in the freshwater portion. There was an abrupt decline in the abundance of CTC+ cells within the saline reaches of the transition zone (Fig. 4B).

Variations in single-cell activity in the free-living assemblage—The five indices of bacterial single cell activity showed mutually coherent patterns along the transect, and they all suggest strong physiological changes in the free-living bacterioplankton within the mixing or transition region. Figure 5 summarizes the evolution of each of the indices along the salinity gradient. There was a steep decline in the proportion of CTC+ cells from the freshwater to the estuarine portion, ranging from an average of 10% in the upper portion to $<4.5\%$ in the mouth of the estuary, with a minimum within the mixing zone (Fig. 5A). The proportion of high-DNA cells oscillated $\sim 50\%$ and was less variable than the %CTC+ along the gradient, but there was also a

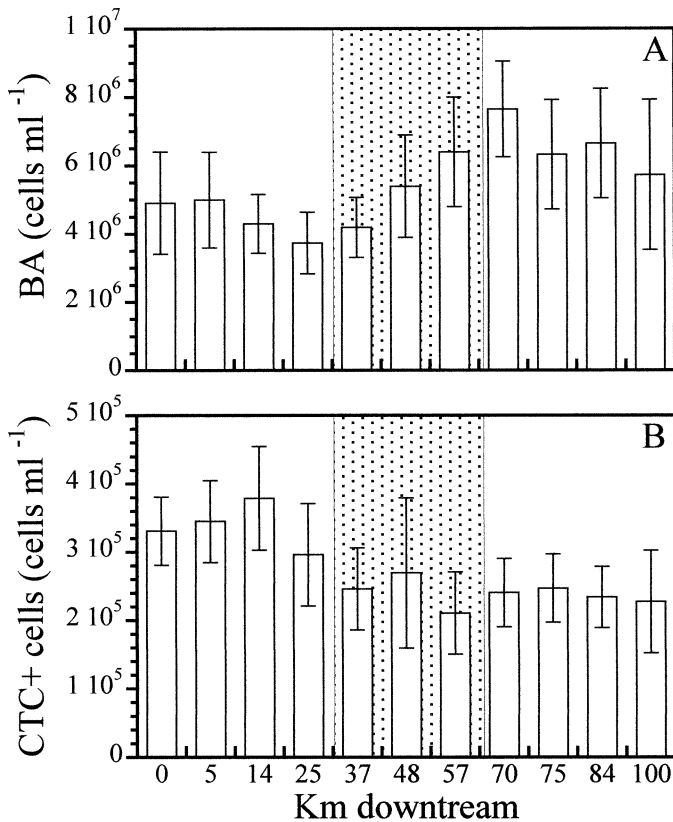


Fig. 4. (A) The total bacterial abundance and (B) the abundance of CTC+ cells along the transect. Each bar represents the average (\pm SE) for each sampling station of four samples taken in May, July, August, and September 2000. The average extent of the transition zone, where fresh- and saltwater mix, is indicated by the dotted area.

systematic decline within the mixing zone (Fig. 5B), which occurred during every month that we sampled. In contrast to both CTC+ and high-DNA cells, the proportion of depolarized cells (DiBAC+) was generally low (<10%) in both fresh- and saltwater and greatly increased within the transition zone (Fig. 5C). The increase in the proportion of DiBAC+ cells within the transition zone occurred during every month investigated, with a peak of >35% in the March transect, which suggests that a large fraction of the assemblage may have lost membrane potential within this region. Likewise, the proportion of cells responsive to propidium iodide when the BacLight assay was used also peaked within the transition zone and was much lower in either the fresh or saltwater portions (Fig. 5D). The BacLight assay was used only in the March transect, and we thus do not know whether this pattern was recurrent, as it was for the other single-cell characteristics discussed above. By far the largest variation in single-cell characteristics was that of the proportion of cells that could be detected with FISH and %EUB. There was a dramatic decline within the transition zone, from an average of 41% in the upper and lower regions to an average of 14% within the mixing region (Fig. 6).

Changes in free-living community metabolism along the Choptank River estuary—The seasonal variations in bulk

metabolism are presented in the companion paper (Bouvier and del Giorgio 2002), and herein we present only the average patterns that were found along the salinity gradient in the Choptank River. The average total carbon consumption in the free-living bacterioplankton fraction, calculated as BP + BR, tended to peak in the middle portion of the estuary within the mixing zone and was, on average, remarkably similar between the fresh- and saltwater portions (Fig. 7A). The average BP in the freshwater portions was higher than in the estuarine portions, but BP was always lowest within the mixing zone (Fig. 7B). The decline in BP corresponded to an increase in BR (not shown), and the two combined resulted in the peak in total carbon consumption that consistently occurred in the mixing zone. The steep decline in BP and increase in total carbon consumption resulted from a sharp decline in BGE within the mixing zone (Fig. 7C), which was recurrent during the 4 months studied.

Single-cell and community metabolism in the bulk assemblage—As we have discussed above, in this article we have focused on the patterns in the free-living bacterioplankton fraction, but we also measured BA, single-cell activity, and BP in the bulk, unfiltered water samples, to assess whether the patterns that we describe for the unattached fraction might also apply to the overall community.

Both the total bacterial abundance and the abundance of CTC+ cells in the bulk, unfiltered samples followed essentially the same pattern that we described for the filtered fraction (data not shown), although both were 20%–40% higher than in the filtered sample. The average percentage of CTC+ cells was also higher in the unfiltered sample relative to the filtered sample, which is not surprising given that filtration selectively removes larger cells that tend to be more responsive to the CTC assay (Gasol et al. 1995). However, the average pattern in the percentage of CTC+ cells along the salinity gradient in the unfiltered water samples was essentially the same as described above for the filtered samples: %CTC+ was, on average, higher in the freshwater portions and declined drastically within the transition zone (Fig. 8A). The other indices of single-cell activity also showed patterns in the bulk samples that were similar to those described for the free-living fraction (data not shown). BP in the filtered fraction was, on average, 69% of the production measured in the bulk, unfiltered samples, and, not surprisingly, the percentage of production due to the unattached fraction was slightly lower within the transition region where the suspended solid load peaks. BP in the unfiltered sample followed essentially the same pattern as in the filtered sample: there was a decline in BP in the bulk unfiltered samples within the transition zone, and this decline was of the same magnitude as was noted in the filtered samples (Fig. 8B).

Relationship between single-cell activity and community metabolism—BP in the free-living fraction was only weakly correlated to changes in total bacterial abundance (Fig. 9A) and was more strongly related to the abundance of CTC+ cells (Fig. 9B). The former explained 16% of the total variance in BP, whereas the latter explained 39%. Although the abundance of CTC+ cells was the single best predictor of BP in this system, >60% of the variance remains unex-

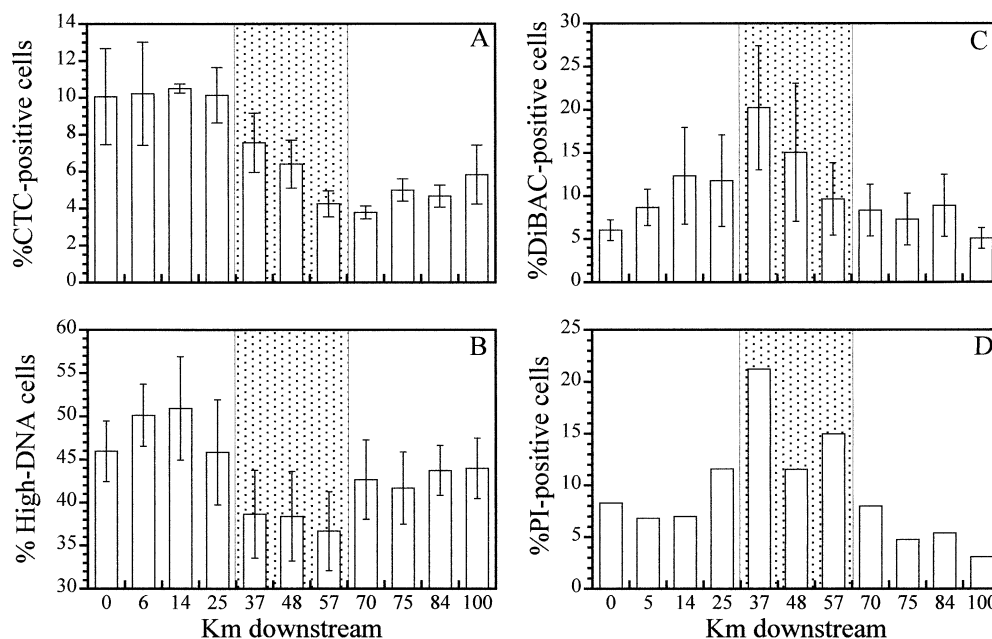


Fig. 5. (A) The proportion of CTC+ cells, (B) high-DNA cells, (C) DiBAC+ cells, and (D) PI+ cells along the estuarine transect. Each bar represents the average (\pm SE) for each sampling station of four samples taken in May, July, August, and September 2000. The average extent of the transition zone, where fresh- and saltwater mix, is indicated by the dotted area.

plained. Further exploration reveals that imbedded in the overall relationship between BP and CTC+ cells are distinctly different relationships. This relationship seems to vary seasonally, but, in addition, the response of bacteria to the CTC assay seems to differ between freshwater and estuarine communities. During 2 of the 4 months examined, the relationship between BP and CTC+ abundance for the freshwater samples was strikingly different from that found for the saltwater samples, within a given transect (Fig. 10A,B). In March, the relationship between CTC+ abun-

dance and BP for fresh- and saltwater both had $r^2 > 0.9$ and had similar slopes, but the freshwater relationship had an intercept that was almost 10-fold lower (Fig. 10A). In July, the relationship for freshwater was equally strong, whereas the slope for the saltwater relationship was not significantly different from 0 (Fig. 10B). In May and September, we did not note such disparity between the fresh- and saltwater, and all points fell approximately within the same diffuse cloud (data not shown).

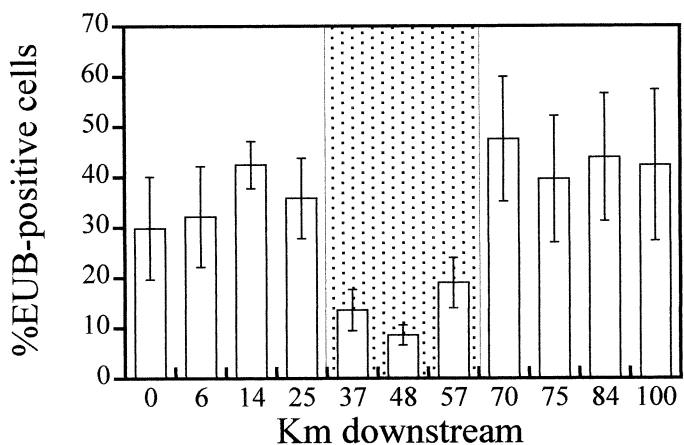


Fig. 6. The proportion of cells that could be detected by use of the eubacterial rRNA-targeted oligonucleotide probe (Eub338) and FISH. Each bar represents the average (\pm SE) for each sampling station of four samples taken in May, July, August, and September 2000. The average extent of the transition zone, where fresh- and saltwater mix, is indicated by the dotted area.

Differences in single-cell characteristics between fresh- and saltwater—We have averaged the data from the four freshwater stations and the four saltwater stations over the study period, to investigate systematic differences between the two end members of the gradient. For this analysis, we have not included the data from the middle stations within the transition zone of the estuary, where the major physiological shifts occurred. The proportion of CTC+ cells was, on average, threefold higher in the freshwater portion than in the saltwater and was significantly different between the two sets of sites (Fig. 11A). In addition, these CTC+ cells had, on average, almost double the orange fluorescence per cell (Fig. 11B), which suggests that these cells either had a higher specific rate of metabolism or simply a greater capacity to reduce CTC to its fluorescent formazan. Specific production, calculated as production divided by the total cell abundance in the filtered fraction, was almost twice as high in freshwater and was also significantly different between the two portions of the river (Fig. 11C). The proportion of high-DNA cells was, on average, 15% higher in freshwater (Fig. 11D).

The two other indices of single-cell activity that we used,

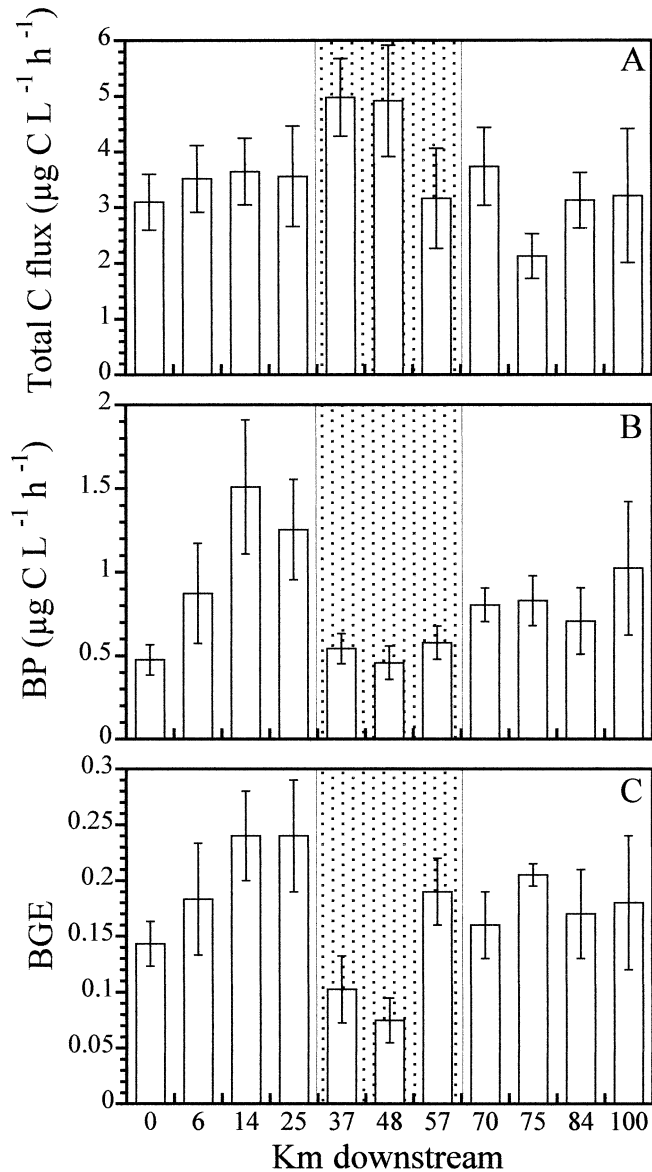


Fig. 7. (A) The total organic carbon consumption by bacterioplankton, calculated as the sum of BP and BR, (B) BP, and (C) BGE along the estuarine gradient. Each bar represents the average (\pm SE) for each sampling station of four samples taken in May, July, August, and September 2000. The average extent of the transition zone, where fresh- and saltwater mix, is indicated with by dotted area. The data are summarized from Bouvier and del Giorgio (2002).

DiBAC and integrity (PI), were generally low (<10% of the total cell count) in both upper and lower portions of the river and only attained significant peaks in the mid-portion within the transition zone region, as we have shown above. But there were nevertheless some apparent differences between fresh- and saltwater portions, with an average higher proportion of both depolarized and injured cells in the fresh water areas (Fig. 11E,F, respectively).

Linking composition and activity in the free-living bacterioplankton fraction—The average phylogenetic composition between fresh- and saltwater was also very different, as we

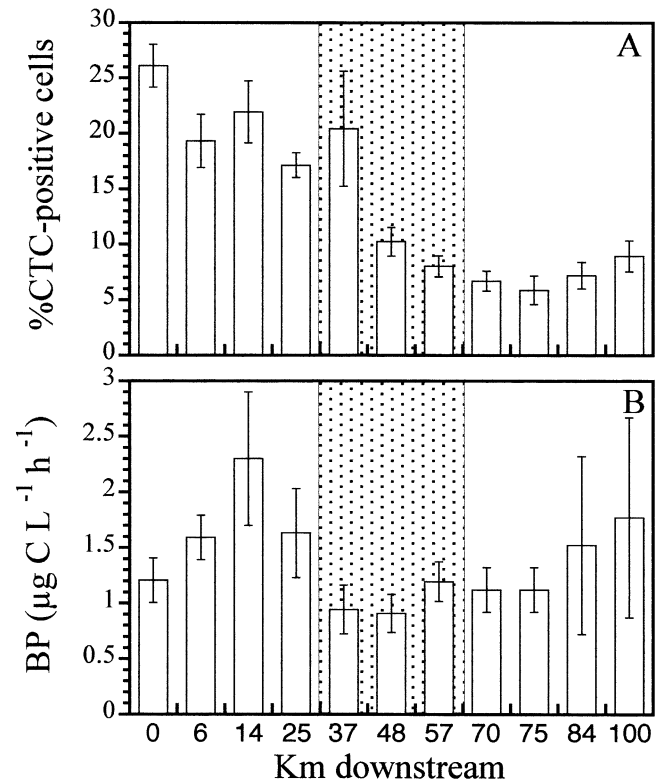


Fig. 8. (A) The proportion of CTC+ cells and (B) BP in the bulk, unfiltered samples, along the estuarine gradient. Each bar represents the average (\pm SE) for each sampling station of four samples taken in May, July, August, and September 2000. The average extent of the transition zone, where fresh- and saltwater mix, is indicated by the dotted area.

have described in detail in the companion paper (Bouvier and del Giorgio 2002). In the context of this article, it is of interest to note that the beta-subclass proteobacteria (β -proteobacteria) were the dominant group in the freshwater portions, whereas the alpha-subclass proteobacteria (α -proteobacteria) dominated the saltwater (Fig. 12). Other phylogenetic groups contributed much less to the overall community composition (Bouvier and del Giorgio 2002). Not surprisingly, some of the differences in single-cell characteristics that we discussed above appear to be linked to the patterns in community composition, and there seems to be a general increase in the level of single-cell activity related to increased dominance of β -proteobacteria (Fig. 13). The proportion of β -proteobacteria was strongly positively related to both the proportion of CTC+ cells (Fig. 13A) and the specific bacterial production (Fig. 13B). These relationships were evident in the pooled data (as shown) and also within individual transects. There was also a marginally significant positive relationship between the proportion of β -proteobacteria and bacterial growth efficiency when all the data are considered (not shown), which on further examination reveals a set of relationships for different months. In the July transect, for example, there was a very strong positive relationship between β -proteobacteria and BGE (Fig. 13C), but the relationship was weaker for the other months, for reasons that are unclear. It should be noted that there was

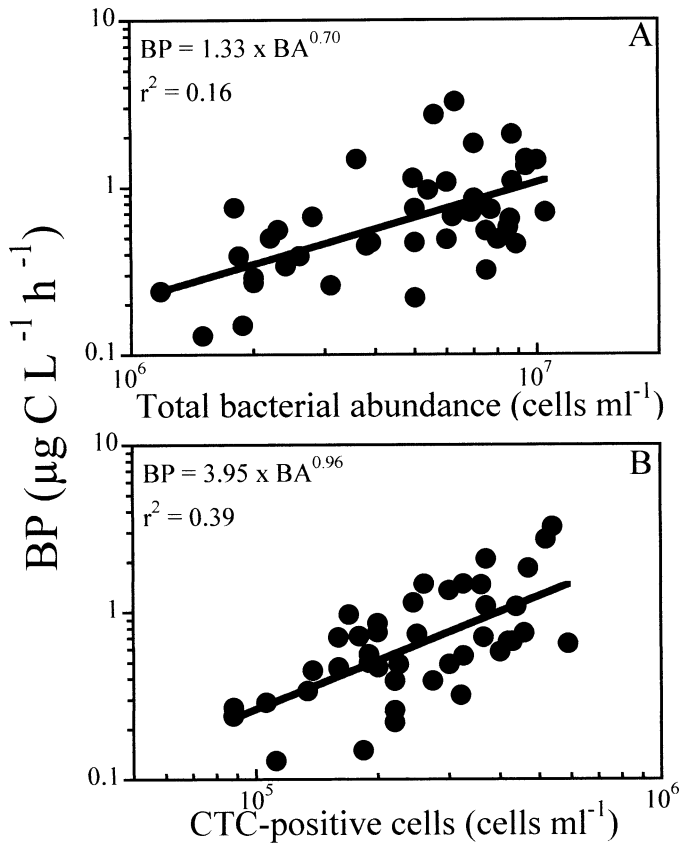


Fig. 9. (A) BP as a function of total bacterial abundance and (B) the abundance of CTC+ cells. Each data point is an individual site along the transect, and the data for the four sampling months are included.

no relationship at all between any of these variables and the relative proportion of any other phylogenetic group investigated. Also, it should be noted that salinity alone explained little of the variance in %CTC, specific production, and BGE in this data set.

There was no significant relationship between the proportion of high-DNA cells and the relative abundance of any phylogenetic group, which suggests that this bacterial fraction does not necessarily have a taxonomic basis. Likewise, there was no relationship between the proportion of low-DNA cells and any of the phylogenetic groups, but, of interest, there was a strong relationship between the proportion of α -proteobacteria and the average green fluorescence of low-DNA cell fraction (Fig. 14).

Discussion

In this article we have focused on two complementary but clearly distinct questions that relate to bacterioplankton function across environmental gradients. The first question is whether the compositional succession along environmental gradients occurs with measurable changes in both community and single-cell metabolic activity. The second major question is whether communities dominated by different phylogenetic groups show distinct patterns in single-cell ac-

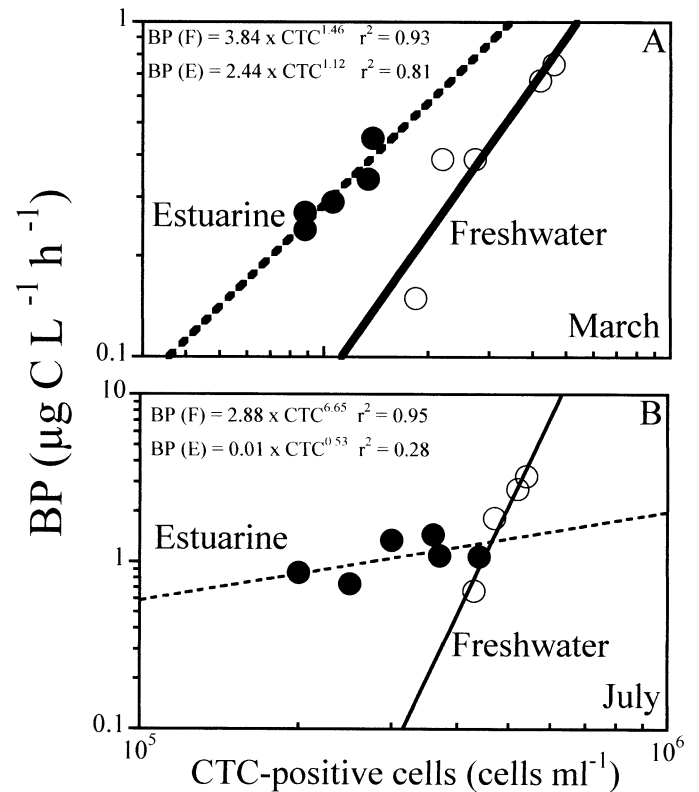


Fig. 10. BP as a function of the abundance of CTC+ cells, but the relationships for (A) May and (B) July are shown separately. The data have been further split into freshwater (open circles and full line) and saltwater (filled circles and dotted line). The lines represent the least-square regression to the data.

tivity and characteristics. We address in detail both questions in sections below, but first we discuss the effectiveness and problems associated with the indices of single-cell activity that we have used in this research to characterize the physiological succession along the Choptank River.

The ecological significance of single-cell measurements—The five indices that we have used to characterize the physiological succession target distinct aspects of cellular function and should be viewed as complementary but by no means equivalent. Each of them has its particular limitations and technical problems as well, which we briefly discuss below.

Cytometric analyses of bacterioplankton labeled with nucleic acid stains commonly reveal the existence of at least two distinct fractions, characterized by high and low apparent DNA contents (Li et al. 1995; Troussellier et al. 1999). High-DNA cells are usually larger and appear to be the most dynamic component of the assemblage (Li et al. 1995; Gasol et al. 1999; Gasol and del Giorgio 2000). Recent studies that have used flow sorting of bacterioplankton incubated with radiolabeled substrates have confirmed that high-DNA cells are responsible for the bulk of bacterial community production (Lebaron et al. 2001). The proportion of high-DNA cells thus appears to be an ecologically relevant index of single-cell activity that can be used to describe physiological

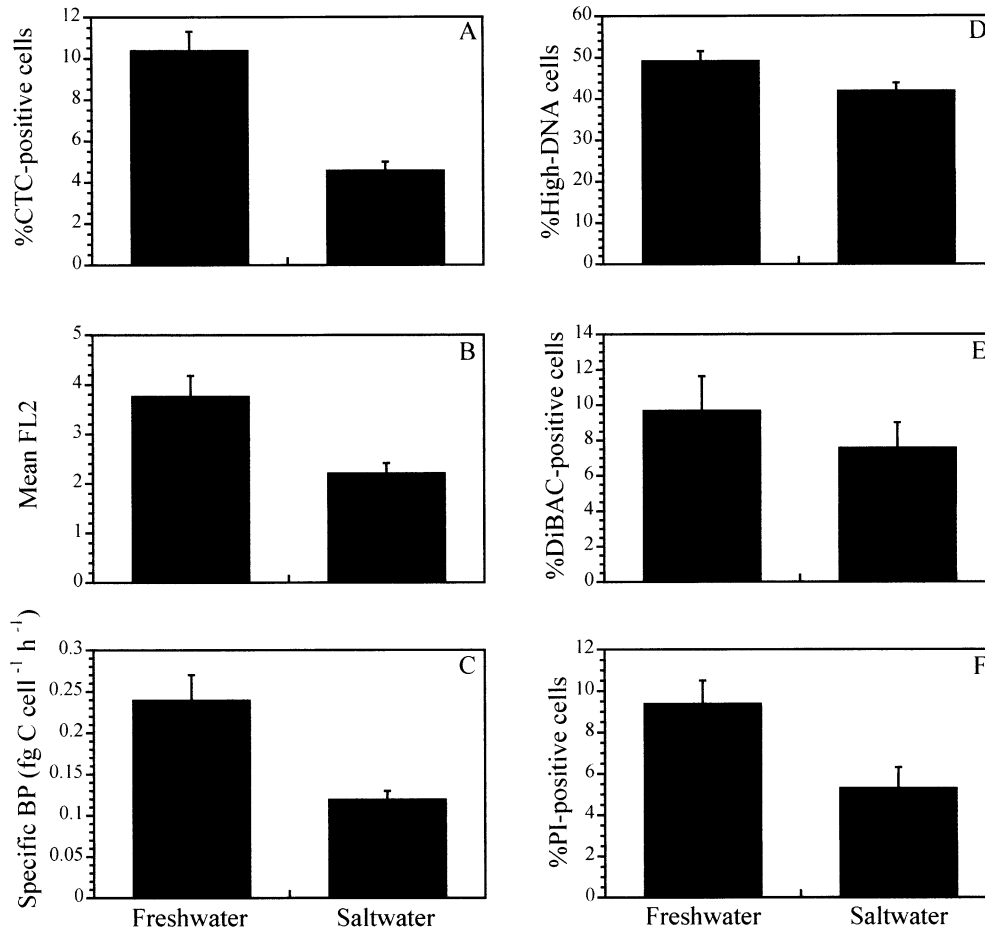


Fig. 11. The average single-cell characteristics in fresh- (upstream) versus saltwater (downstream) sites in the Choptank River estuary. (A) The proportion of CTC+ cells, (B) the average FL2 per CTC+ cell, (C) specific production, calculated as BP/total abundance, (D) the proportion of high-DNA cells, (E) the proportion of DiBAC+ cells, and (F) the proportion of PI+ cells. Each bar represents the average (\pm SE) measurements for all the fresh- or saltwater sites for the four sampling periods. The data from the three sites within the transition zone have been excluded.

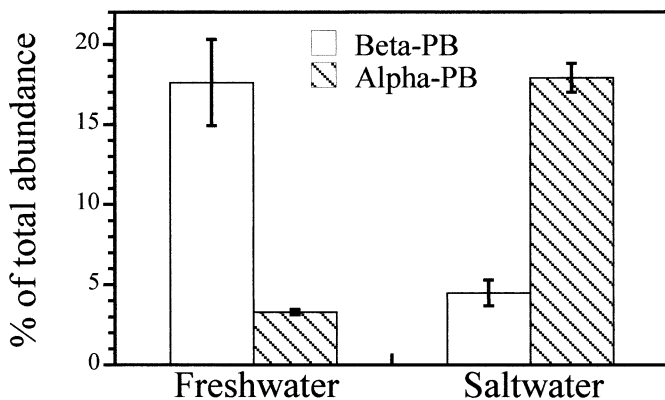


Fig. 12. The proportion of α - and β -proteobacteria in the fresh- and saltwater portions of the estuary. Each bar represents the average (\pm SE) measurements for all the fresh- or saltwater sites for the four sampling periods. The data from the three sites within the transition zone have been excluded. The data are summarized from Bouvier and del Giorgio (2002).

changes in bacterioplankton assemblages. But the separation of high- and low-DNA fractions is often quite arbitrary, because the two fractions are not always distinctly apart and often overlap completely (Gasol and del Giorgio 2000). The proportion of high-DNA cells can be made to vary substantially in experimental manipulations (Gasol et al. 1999), but it is usually much less variable in natural water samples, a factor that might limit its usefulness as a physiological index. For example, in our study, the proportion of high-DNA cells was constrained between 30% and 60% over the seasons and along the estuarine transect, in spite of much larger variations in temperature, chlorophyll, bacterial community metabolism, and even in other single-cell parameters such as the %CTC+ cells. In addition, this fraction is by no means homogenous, and there appears to be a wide range of cell-specific activity associated with high-DNA cells (Lebaron et al. 2001).

The proportion of cells capable of reducing CTC to its fluorescent formazan was much lower than the proportion of high-DNA cells, but was also much more variable. The proportion of CTC+ cells varied from <3% to >50%, and, as

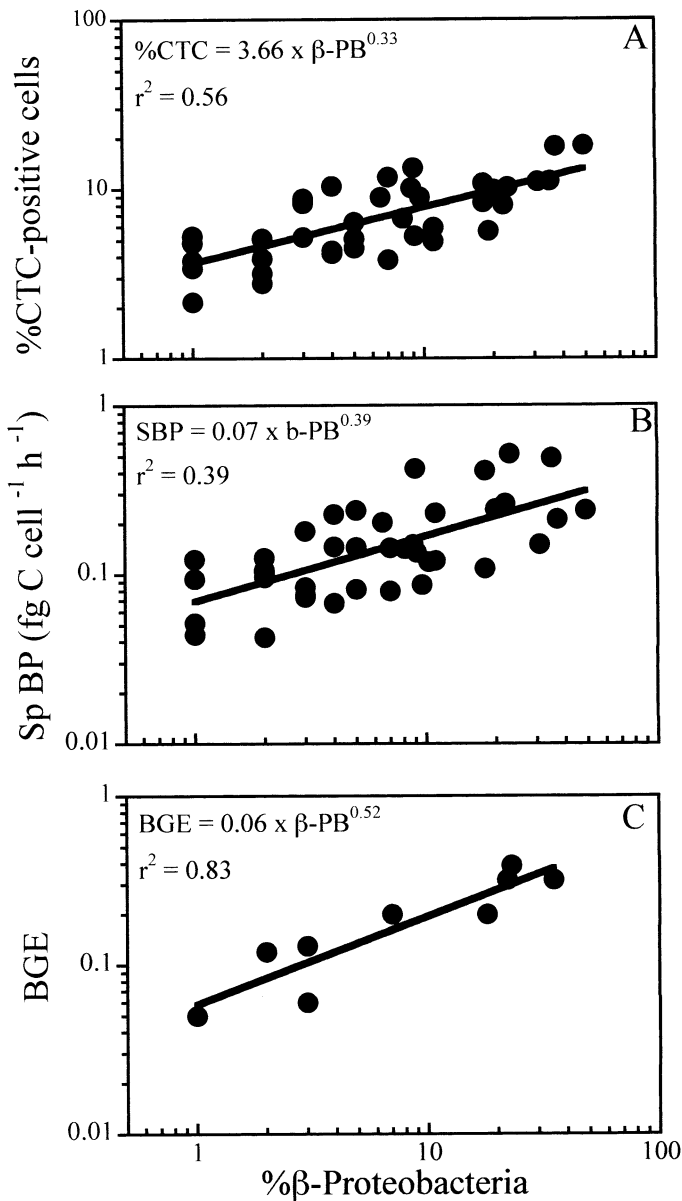


Fig. 13. (A) The proportion of CTC+ cells, (B) specific production, and (C) BGE as functions of the proportion of β -proteobacteria. Each data point is an individual site along the transect, and the data for the four sampling months are included, except in (C), where only the data from the July transect is shown.

we discuss below, this variation was seasonal as well as spatial. It has been suggested in the past that the CTC technique only detects the cells with the highest rates of metabolism within the assemblage and most likely represent a sub-component of the high-DNA fraction (Sherr et al. 1999; Sieraki et al. 1999). Recent studies that have used flow sorting of radiolabeled cells have shown that CTC+ cells do not always have higher specific uptake rates than the bulk assemblage (Servais et al. 2001). This and other studies (i.e., Ullrich et al. 1999) have questioned the utility of the CTC assay as an indicator of single-cell characteristics. In spite of these shortcomings, in our study the abundance of CTC+

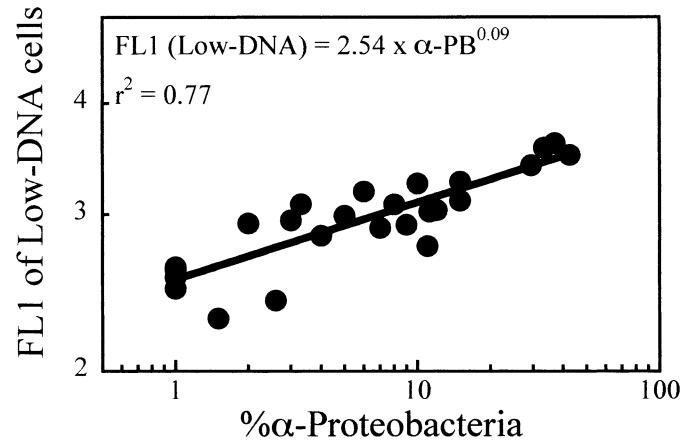


Fig. 14. The average FL1 per cell for the low-DNA fraction as a function of the proportion of α -proteobacteria. Each data point is an individual site along the transect, and the data for the four sampling months are included.

cells explained a much greater portion of the variance in bacterial metabolism than any other single variable that we measured, including the relative and absolute abundance of high-DNA cells. This has been noted for other systems in the past (del Giorgio and Scarborough 1995; Lovejoy et al. 1996; del Giorgio et al. 1997; Yamaguchi and Nasu 1997; Smith 1998; Sherr et al. 1999).

In metabolically active bacteria with intact cytoplasmic membranes, there is typically a difference of electrical potential across the membrane, with the interior negative with respect to the exterior. If the cell dies or if the membrane ruptures or develops large holes, as is often the case under heat, salinity, and other types of stress, the membrane potential is reduced and the cells become depolarized (Novo et al. 2000). Oxonol dyes that have a negative charge, such as DiBAC, are normally excluded from healthy cells by the membrane potential gradient but are accumulated in depolarized cells, which then become fluorescent (López-Amorós et al. 1995; Nebe-von-Caron et al. 2000). Although in many of the samples that we processed the background fluorescence due to DiBAC was extremely high, the population of cells that accumulated the oxonol was usually above the background and could be quantified. The proportion of cells that accumulated DiBAC was usually low (<8%) in freshwater and estuarine samples but was high within the transition zone. We regularly ran controls of the same fresh samples to which we added the ionophore Gramicidin S, and, in these controls, typically >80% of the cells could be detected with DiBAC, which suggests that, in most estuarine and riverine samples, the majority of cells retain membrane potential even if they are not scored as active by other methods.

It is usually assumed that bacterial permeability to exclusion stains such as propidium iodide is associated with the presence of large and often irreparable breaches in the membrane (Novo et al. 2000), although the apparent loss of membrane integrity does not necessarily imply cell death, because there is evidence that cells can recuperate and resume growth under the appropriate conditions (Barer and Harwood 1999). It is likely that membrane damage is also associated to the

loss of the capsular envelope, which in turn has been linked to loss of intracellular integrity in bacterioplankton cells (Heissenberger et al. 1996). The BacLight kit is increasingly being used to assess cell injury and mortality in culture and in environmental samples (Boulos et al. 1999; Gasol et al. 2000). For example, Decamp and Rajendran (1997) found relatively high proportion of “dead” (PI+) cells in eutrophic coastal waters (>20%), and this proportion increased with increasing temperature and decreasing O₂ concentration in the water. Propidium iodide has been used in combination with RNA probes to effectively distinguish dead from active bacteria as well (Williams et al. 1998).

Membrane potential and integrity are two aspects of cellular function that should be a priori strongly linked, but studies have shown that this expectation is not always borne out, because the loss of membrane integrity is not always accompanied by loss of electric potential and vice versa (Novo et al. 2000). In our study, the peaks in both depolarized (DiBAC+) and injured (PI+) cells occurred within the transition zone but not always coincident in the same station, which suggests, as we discuss below, that loss of membrane potential may be spatially separated from cell injury and death.

The capacity to detect bacterial cells by use of oligonucleotide probes and (FISH) is in large part related to the cell-specific rRNA contents (Kerkhof and Ward 1993; Ruimy et al. 1994), which itself has been suggested as an index of cell activity (Karner and Fuhrman 1997; Williams et al. 1998). The brightness of the fluorescent signal obtained with FISH is indeed correlated to RNA content (Oda et al. 2000) but depends on many other factors as well, including the fixation and permeabilization of cells, the hybridization conditions, and the nature of the fluorescent conjugate. For example, in this study we have used oligonucleotide probes conjugated with the green fluorochrome BODIPY, which seems to yield a lower fluorescence signal of hybridized cells than the more commonly used CY3 (Amann et al. 1995; Glöckner et al. 1999). This explains the generally low %EUB that we have obtained, relative to other similar studies (Karner and Fuhrman 1997; Glöckner et al. 1999), but does in no way explain the dramatic and recurrent decline in %EUB within the mixing region. It would seem that the proportion of cells hybridized with the eubacterial probe may not be in itself an absolute index of cellular activity, but the spatial variation in %EUB measured along the salinity gradient when a consistent method is used most likely does reflect changes in cellular physiology in the bacterioplankton.

There is currently an intense debate over the application and meaning of the various indices of single-cell activity that are used in aquatic microbiology (Karner and Fuhrman 1997; Smith 1998; Sherr et al. 1999; Ullrich et al. 1999; Servais et al. 2001), and there is little question that there are major methodological uncertainties and technical difficulties in the determination of bacterioplankton single-cell activity (Nebbe-von-Caron et al. 2000). Much of this debate has often been centered over the apparent lack of agreement between the different methods. Our study provides a good example, because although the various indices broadly agreed in terms of the overall physiological succession, when analyzed pairwise, they were only weakly correlated to each

other, if at all. As we pointed out above, each method targets a unique cellular function, and the links between these are often complex (Joux et al. 1997; Sherr et al. 1999; Joux and Lebaron 2000; Nebe-von-Caron et al. 2000). Another factor that may in some way uncouple the various indices of single-cell activity are intrinsic differences in the response to these assays by different phylogenetic groups. This second factor becomes particularly important when comparing patterns of single-cell activity across communities that differ greatly in composition, which is certainly the case in the Choptank River, as we discuss in the following sections.

The physiological succession along the salinity gradient—

There is a clear pattern of phylogenetic succession along the salinity gradient, with dominance of β -proteobacteria in the freshwater portions and of α -proteobacteria in the freshwater portions (Bouvier and del Giorgio 2002). This succession is abrupt, is restricted to the maximum turbidity region where fresh- and saltwater mix, and is accompanied by large shifts in community metabolism. The sharp decline in BP in the transition zone was linked to changes in BGE rather than to declines in overall carbon consumption, which in fact increased in this zone. The decline in BGE and BP and the increase in BR and carbon consumption within the transition zone are difficult to explain simply on the basis of changes in DOC and nutrients. Although both DOC and nitrogen concentrations declined within the transition area relative to the upstream freshwater, they continued 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 total dissolved P in fact often peaked within the transition zone. Of interest, the Chl *a* concentration was often highest in the lower portion of the estuary and also declined sharply within the transition zone, which suggests that there were strong shifts in the phytoplankton community as well in this area. Overall, there was no apparent relationship between bacterial metabolism or composition and the distribution of Chl *a* along the transect, and there is no evidence that bacterial phylogenetic or physiologic succession may have been driven by changes in primary production.

The changes in community metabolism, and particularly the decline in BGE, may be related to changes in the physiological condition of the bacterial assemblage that may not be directly related to DOC or nutrient limitation per se. We have previously hypothesized (Bouvier and del Giorgio 2002) that the 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). This hypothesis is supported by our results with single-cell activity indices. Membrane depolarization and permeabilization, coupled with an apparent decline in overall metabolic activity as evidenced from declines in CTC+, high-DNA, and EUB+ cells, can be unambiguously interpreted as signs of physiological stress to the bacterial assemblage as a whole. The various indices of single-cell activity suggest that the transition from freshwater to estuarine (saline) conditions is mediated by loss of activity, injury, and even cell death. It appears that at the community level, these

changes in single-cell physiology at the site of the phylogenetic succession translate into a generalized decline in bacterial growth efficiency with a decrease in bacterial growth and production but an increase in bacterial carbon consumption.

Our data also suggest that there may be an actual succession of physiological states along the salinity gradient, where cells undergo different levels of stress along the passage from fresh- to saltwater (Painchaud et al. 1995). The greatest shifts in all indices occurred within the transition zone, but not all the indices showed equally large shifts, and, in addition, the peaks or troughs were often spatially displaced. There seemed to be a progression, which, viewed from downstream to upstream stations, was evidenced by an initial decline in %CTC even before the transition zone, followed by a steep decline in %EUB and %high-DNA and then peaks of depolarized and permeabilized cells (from <8% to >35%) toward the freshwater reaches of the transition zone. It is conceivable that in early stages of stress there is a decline in growth rate and in overall metabolism, evidenced by a decline in CTC+ and EUB+ cells, followed by a general loss of membrane polarity, evidenced by an increase in DiBAC+ cells, and finally a loss of membrane integrity and cell death, evidenced by an increase in PI+ cells.

In some ways, the pattern of single-cell characteristics that we describe along the salinity gradient resembles the succession of physiological states in pure bacterial cultures subjected to starvation or other forms of stress (Joux et al. 1997; Joux and Lebaron 2000; Nebe-von-Caron et al. 2000). Culture studies have shown that the proportion of CTC+ and of growing cells decline steeply on the onset of stress, whereas genomic integrity (DNA contents), ribosomal content, and membrane integrity decline much more slowly (Joux et al. 1997; Caro et al. 1999).

The capacity to detect bacteria by use of oligonucleotide probes based on the rRNA cellular contents has been shown to be extremely sensitive to osmotic stress and carbon starvation but not to cold and other types of physical and chemical stress (Tolker-Nielsen et al. 1997; Oda et al. 2000). As we have pointed out above, there is no evidence for carbon starvation within the transition zone, so the dramatic decline in %EUB is more likely related to osmotic stress of cells within this region of mixing, coupled perhaps with a generalized decline in growth rate. This explanation is consistent with the large increases in depolarized and injured cells that were also observed in this region. The low average %CTC that we found in saltwater may also reflect the effect of osmotic stress on the capacity of bacteria to reduce CTC. But as opposed to the other indices, which changed dramatically within the mixing region but were roughly similar between up and downstream sites, the proportion of CTC+ cells decreased within the mixing region but remained low in all stations downstream. We argue in the section below that there may also be phylogenetic component to the pattern of relative abundance of CTC+ cells.

Linking phylogenetic composition to single-cell and community activity—The second major question that we address in this article is whether communities dominated by different phylogenetic groups show distinct patterns in single-cell ac-

tivity and characteristics. Our results show that some of the single-cell indices, particularly CTC, are significantly different between fresh- and saltwater regions, independently of what their behavior is within the transition zone regions. This is not a methodological artifact because all samples from a given transect were processed in the same way. Previous studies have shown that in lakes the proportion of CTC+ cells oscillates ~10%–30%, even in the least productive waters (del Giorgio and Scarborough 1995; del Giorgio et al. 1997), whereas reports from estuarine and especially marine sites usually show much lower proportion of CTC+ cells (Gasol et al. 1995; Lovejoy et al. 1996; Karner and Fuhrman 1997; Sherr et al. 1999). The low proportion of CTC+ cells in marine waters has actually been used as evidence that the method is ineffective (Ullrich et al. 1999). Herein we suggest that there may be taxonomic basis for the differences in the response to the CTC assay, which may explain the patterns observed across systems. In particular, the %CTC+ cells appears to be related to the proportion of β -proteobacteria in the assemblage. This relationship does not imply that β -proteobacteria are unique in taking up and reducing CTC, but rather that they may do so at a higher rate per cell than other groups. One of the underlying mechanisms to explain the higher proportion of CTC+ cells in freshwater is that cells appear to accumulate more formazan on average, such that more of these cells cross the threshold of detection with the flow cytometer. We found a significantly higher mean FL2 per cell in freshwater, and this higher FL2 does not correspond to a greater average cell size in freshwater, as evidenced by the mean SSC and FL1. It is still unclear whether the CTC+ fraction is phylogenetically distinct, although recent work that has used flow sorting has shown little difference between the CTC+ fraction and the bulk assemblage in a small set of samples (Bernard et al. 2000).

Specific bacterial production was also significantly higher in fresh- compared with saltwater, but, again, this pattern must be interpreted with caution. This result is driven by the fact that bacterial abundance was, on average, lower in the freshwater portion, in spite of comparable rates of bacterial organic carbon consumption and production. Scaling BP to the entire assemblage is only appropriate when the majority of cells are contributing more or less equally to the overall activity, and it is precisely one of the premises of this study that this scaling is completely inappropriate in natural bacterioplankton assemblages, including those in the Choptank River.

Salinity is an overriding structuring factor in the gradient that we have studied, and it is conceivable that the relationships between physiological parameters and the relative abundance of β -proteobacteria are driven simply by the covariation of all these variables with salinity. If this were the case, the relationships with β -proteobacteria would not be of any ecological significance. But salinity alone was only marginally related to either the proportion of CTC+ cells or bacterial specific activity, and there was no correlation whatsoever with growth efficiency, production, or any of the other indices of single-cell activity. Of interest, although α - and β -proteobacteria show roughly inverse patterns of distribution along the estuarine gradient, the relative abundance of

α -proteobacteria was not significantly correlated with any of the physiological variables. In fact, the relative abundance of β -proteobacteria was the best predictor of both the proportion of CTC+ cells and of specific bacterial production. Our results agree with recent reports of phylogenetic differences in the patterns of use of organic substrates (Ouverney and Fuhrman 1999; Cottrell and Kirchman 2000).

It is still unclear whether high- and low-DNA cells represent distinct phylogenetic groups with intrinsic differences in morphology and function or simply represent cells with different rates of metabolic activity or growth with no distinct phylogenetic affiliation. The fact that high- and low-DNA fractions have been described for a wide variety of aquatic ecosystems characterized by very different phylogenetic composition argues for the lack of a taxonomic basis for this attribute of planktonic bacterial assemblages. Our own data show only a slightly higher average proportion of high-DNA cells in fresh- versus saltwater (49% vs. 41%, respectively), which we know are dominated by different major phylogenetic groups, and we did not find any relationship between the proportion of high- and low-DNA cells and the relative abundance of phylogenetic groups. We did find, however, a strong positive relationship between the proportion of α -proteobacteria and the average FL1 of low-DNA cells. It is interesting to discover relations between cytometric parameters of cells and FISH-derived bacterial composition, because there is no methodological connection between them, but it is also difficult to interpret these relationships. Although FL1 is primarily related to DNA contents, it has also been associated to mean cell size (Gasol and del Giorgio 2000), and there is generally a positive relationship between mean cell size and cell activity (Gasol et al. 1995). If the low-DNA fraction were preferentially composed of a particular phylogenetic group, such as slow-growing α -proteobacteria, then a relationship between the mean FL1 of this fraction and the proportion of cells that can be detected with the group-specific probe and FISH would indeed be expected simply on the basis of the level of activity of the cells. The pattern could be interpreted as selective activation along the salinity gradient of this particular bacterial group that was already present within the low-DNA fraction, but this hypothesis remains to be tested through molecular characterization of the low-DNA fraction isolated by use of flow sorting.

Implications to understanding bacterioplankton function—The past decade has seen great advances in our understanding of microbial diversity in aquatic systems and has yielded fairly robust large-scale patterns in microbial biodiversity and in the distribution of major phylogenetic groups (Giovanonni and Rappé 2000). In spite of these advances, we still know little of the phylogenetic succession of bacterioplankton assemblages in aquatic ecosystems, particularly how the transition between phylogenetically distinct assemblages occurs, both temporally within a given system (i.e., Pinhassi and Hagström 2000) and across systems. We contend that the phylogenetic succession along an estuarine gradient, which we have described in detail in a companion paper (Bouvier and del Giorgio 2002), is accompanied by a distinct physiological succession that is also reflected at the

level of community metabolism. We do not know what combination of environmental factors drives the phylogenetic succession in the Choptank River and other similar systems, but it is clear from our data that the succession is at least accompanied by severe physiological stress to the bacteria. It is interesting to note that there does not appear to be a group of free-living bacteria that is capable of exploiting these transient conditions that occur within the region of mixing of fresh- and saltwater.

Our data suggest that members of the β -proteobacteria may be characterized by higher intrinsic rates of metabolism, growth, and perhaps even growth efficiency. These data must be interpreted with caution, because although the patterns of phylogenetic dominance are very clear in the Choptank River, the dominant groups seldom represent >40% of the total bacterial count, and the bulk of the bacterial community often remains uncharacterized phylogenetically. Likewise, the individual single-cell assays seldom account for >50% of the total assemblage, so a relatively large fraction of the assemblage remains phylogenetically and physiologically uncharacterized. Alternatively, our data may indicate that there may be a phylogenetic component in the response of bacterial cells to routine cytometric and metabolic assays. Either way, the potential modulation of single-cell activity and community metabolism by taxonomic composition should be further investigated, because it has profound implications on our understanding of the response of bacteria to the environmental forcing.

All the current approaches used to determine single-cell bacterioplankton activity or physiological status have inherent methodological and conceptual problems. But it is also clear that no single method can capture the breadth of physiological responses that are involved, for example, in the replacement of bacterial phylogenetic groups within sharp aquatic ecotones. Our data further emphasizes that bulk measurements such as total bacterial abundance and production often mask complex and diverse responses of bacterioplankton to drastic changes in salinity or temperature, nutrient and organic matter resources.

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