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Comparison of Free-Living and Particle-Associated Bacterial Communities in the Chesapeake Bay by Stable Low-Molecular-Weight RNA Analysis†

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Free-living and particle-associated bacterial communities in the Chesapeake Bay estuary were analyzed and compared by using acridine orange direct counts and low-molecular-weight (LMW) RNA analysis. Samples were taken from top and bottom waters at upper- and mid-bay sites in December 1992. Free-living bacteria dominated the bacterial numbers at all sampling sites, although particle-associated bacteria increased in areas with greater particle loads. LMW RNAs (5S rRNA and tRNA) obtained directly from free-living, particle-associated, and total bacterioplankton communities were analyzed by high-resolution electrophoresis. There were distinct differences in the migration distances between LMW RNAs of free-living and particle-associated communities taken from the same site, indicating that the two communities differ in composition. In addition, LMW RNA profiles differed minimally with depth for all of the communities examined, presumably because of vertical mixing. 5S rRNAs of free-living communities from the upper- and mid-bay regions differed considerably. Particle-associated RNAs, on the other hand, were very similar, suggesting consistent environmental conditions on particles that select for similar community members. Lastly, several isolated bacteria had 5S rRNAs that were not detected in their respective extracted community 5S rRNAs, indicating that these isolated organisms were not representative of dominant members.

Most aquatic environments contain not only free-living bacteria but also bacteria that are attached to suspended particles. Distinct differences in cell abundance, morphology, and metabolic activity between free-living and particle-associated bacteria have been documented. Free-living bacteria predominate in total numbers and total biomass (24, 33, 40). Although their respective significance varies with each specific aquatic system, in areas where particulate material is common (i.e., freshwater and estuarine systems), attached bacteria make up a significant proportion of the total bacterial numbers (24) and account for most of the bacterial production and activity (10). Often, attached bacteria are larger in cell size and are present in higher local concentrations than those found free in the water (3). Cellular uptake of specific nutrients, like sugars and amino acids, can differ significantly between free-living and attached communities (24). Attached bacteria are often more active on a per-cell basis than surrounding free-living bacteria (8, 12, 24, 28, 32, 40), while specific growth rates and carbon production are more equivalent (21, 33, 40). Higher specific exoenzyme concentrations have also been found with macroaggregates (23, 35).

Morphological and metabolic differences between free-living and particle-associated bacteria could be due to different environmental conditions in the water column and within particulates, respectively. In particular, nutrient conditions should differ in these two habitats, resulting in different levels and types of metabolic activity and enrichment of different types of organisms.

Traditional metabolic techniques are limited in the ability to address this phenomenon. Most previous comparisons of particle-associated and free-living bacteria have focused on organisms that have been cultured in the laboratory, which account for only ~1% of the total bacterial numbers in situ (6). The development of various techniques based on community molecular analysis has freed researchers from culturing biases and allowed characterization of community structure by molecular analysis of stable RNA molecules (1, 13, 26, 27, 39). Most community studies have focused on bacterioplankton (<10 μm in size) in various aquatic systems (2, 7, 15, 17, 30). Similar community studies on free-living and particle-associated bacteria are underrepresented. One report, based on RNA gene cloning techniques, has shown that specific bacterial populations, distinct from the predominant free-living bacteria, develop on marine phytodetrital aggregates (4). However, molecular comparisons of free-living and particle-associated communities in freshwater and estuarine systems have not been documented.

The Chesapeake Bay, having a watershed covering approximately 64,000 square miles (~166,500 km^2), has a tremendous influx of freshwater, depositions, and particulate material of various shapes, sizes, and compositions. In addition, dying algal blooms in summer months provide organic-rich particulate detritus. Metabolic studies of free-living and particle-associated bacteria in the Chesapeake Bay have revealed marked differences in cellular uptake, with attached bacteria more active on a per-cell basis (8). In addition, extracellular enzyme activities of attached bacteria showed elevated levels on per-cell and per-liter bases (9). By using a technique involving stable low-molecular-weight (LMW) RNA (13, 17), we compared free-living bacterial communities to those associated with particles (>3.0 μm in size) in the Chesapeake Bay to determine whether these differences in activity are related to differences in community composition. In addition, we com-

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pared the LMW RNA profiles of RNAs extracted from bacterial isolates to those from their source communities. In this way, we investigated to what extent communities are represented by isolated, culturable organisms. Studies were performed at different locations in the water column, as well as over one-half of the length of the bay. This technique not only provides information on total community structure but also enables relatively rapid comparison of communities. Samples can be obtained, processed, and analyzed in a shorter time frame than with sequencing-based methods.

MATERIALS AND METHODS

Sample collection. Environmental samples were collected at two locations along the middle axis of the Chesapeake Bay, at latitudes 39°08′N and 37°56′N, on 3 December 1992 aboard the R/V *Cape Henlopen* (Fig. 1). Collecting stations were designated 908 and 756 and represent the upper bay and the mid bay, respectively (41). At each station, water was collected at two different depths by using 10-liter Niskin bottles attached to a General Oceanics rosette (General Oceanics, Miami, Fla.). Water collection depths were 1 m below the surface and 1 m above the bottom. The samples were designated top water and bottom water, respectively. Total depths of bottom water samples were 6 and 14 m for stations 908 and 756, respectively.

To separate the particle-associated and free-living communities, water was filtered under vacuum (200 to 500 mm Hg [26,664 to 66,661 Pa]) onto a 3.0-μm-pore-size polycarbonate filter (47-mm diameter; Millipore, Bedford, Mass.) until saturation. Several filters were collected per station and depth. Microorganisms retained on the 3-μm filters were operationally defined as the particle-associated community. The filtrate (<3.0-μm pore size) was then collected onto 0.22-μm-pore-size Sterivex filters (Millipore) until saturation by using a peristaltic pump (36). These filters were prepared in duplicate and contained the free-living community. Total bacteria at station 756 were harvested by filtering water onto 0.22-μm Sterivex filters until saturation. All filters were frozen on dry ice immediately after processing until returning to the laboratory the next morning. At that point, they were stored at −20 or −80°C.

Determination of cell numbers. Total and free-living bacteria for each sample were counted directly under a microscope by acridine orange direct counting (11). Subsamples of pre- and post-filtered (3.0-μm filters) water from each depth and station were fixed in a final concentration of 1% formalin and refrigerated. Fixed cells were stained with acridine orange (1.0 mg/ml in 5% formalin) at a final concentration of 0.1 mg/ml for 5 min in an Eppendorf tube. Cell preparations were vortexed briefly and filtered onto 0.2-μm-pore-size membrane filters (25-mm diameter; Poretics Corporation, Livermore, Calif.) by using a 100-mm Hg (13,332-Pa) vacuum. The filter was removed and mounted in oil under a coverslip. Cells were counted at a magnification of × 1,250 under an Axioplan epifluorescence microscope (Zeiss, Thornwood, N.Y.). At least 15 fields and 300 cells were counted per slide. Free-living bacterial numbers were represented by the stained 3-μm filtrate, while particle-associated bacteria were calculated by subtracting the free-living counts from the total bacterial counts (obtained without prefiltration).

Isolation of culturable cells. Water samples at each depth and station were plated directly onto 0.1× tryptic soy agar plus salts medium (TSA/10; 3 g of tryptic soy broth, 10 g of NaCl, 15 g of Bacto Agar per liter of deionized water). Plates were incubated at 20°C for 2 weeks. Morphologically different colonies from each depth were isolated onto fresh TSA/10 medium and preserved by freezing at −80°C in a 30% glycerol–0.5% NaCl solution. Numbers of selected isolates ranged between 5 and 12 per depth, depending on the station. A few isolates lost culturability over time and were excluded from the data. For extraction of RNA, cells from frozen cultures were cultured on TSA/10 at 20°C, harvested with a sterile spatula, and suspended in 500 μl of sterile phosphate-buffered saline in sterile 1.5-ml Eppendorf tubes. Cells were then pelleted at 8,000 × *g* for 5 min and stored at −20°C until processed.

Extraction of RNA. Extraction of total RNA from filters and isolates was based on the procedure of Höfle (13). All solutions, glassware, and other working materials were rendered RNase free as described by Sambrook et al. (29).

For the particle-associated population, one 3-μm filter was cut into small pieces by using a clean, sterile scalpel and a glass plate and put into a 15-ml Corex centrifuge tube containing 3.0 ml of extraction buffer (50 mM sodium acetate [NaOAc], 10 mM EDTA, 1% [wt/vol] sodium dodecyl sulfate, pH 5.1). Filter pieces were heated at 100°C for 5 min in extraction buffer and removed with sterile forceps. Cell extracts were immediately extracted with an equal volume of 60°C phenol (containing 0.1% [wt/vol] 8-hydroxyquinoline and equilibrated with 50 mM NaOAc [pH 5.2] until the phenol pH reached <5.2) for 10 min at 60°C. The extract was chilled on ice for at least 2 min, and samples were centrifuged at 12,000 × *g* for 2 min. Aqueous material was extracted in a fresh tube with an equal volume of 60°C phenol-chloroform (4:1) for 5 min and subsequently put on ice for 2 min. Phases were separated by centrifugation (12,000 × *g*, 2 min). Aqueous material was extracted twice more, once with 200 μl of 2.0 M NaOAc and 2 ml of $CHCl_3$ and once with 2 ml of $CHCl_3$. Final extracts were precipitated with 2.8 volumes of precipitation mixture (100% ethanol-NaOAc-$MgCl_2$ in a ratio of 100:10:1) at −20°C. Precipitated RNAs were collected by centrifugation (12,000 × *g*, 20 min) and washed with 70% ethanol. Clean RNAs were dried in a SpeedyVac Concentrator (Savant Instruments, Inc., Farmingdale, N.Y.), resuspended in diethyl pyrocarbonate-treated, distilled, deionized water, and quantified with a Beckman DU-50 spectrophotometer with a Nucleic Acid Soft-Pac Module. Extracted RNAs were stored at −80°C.

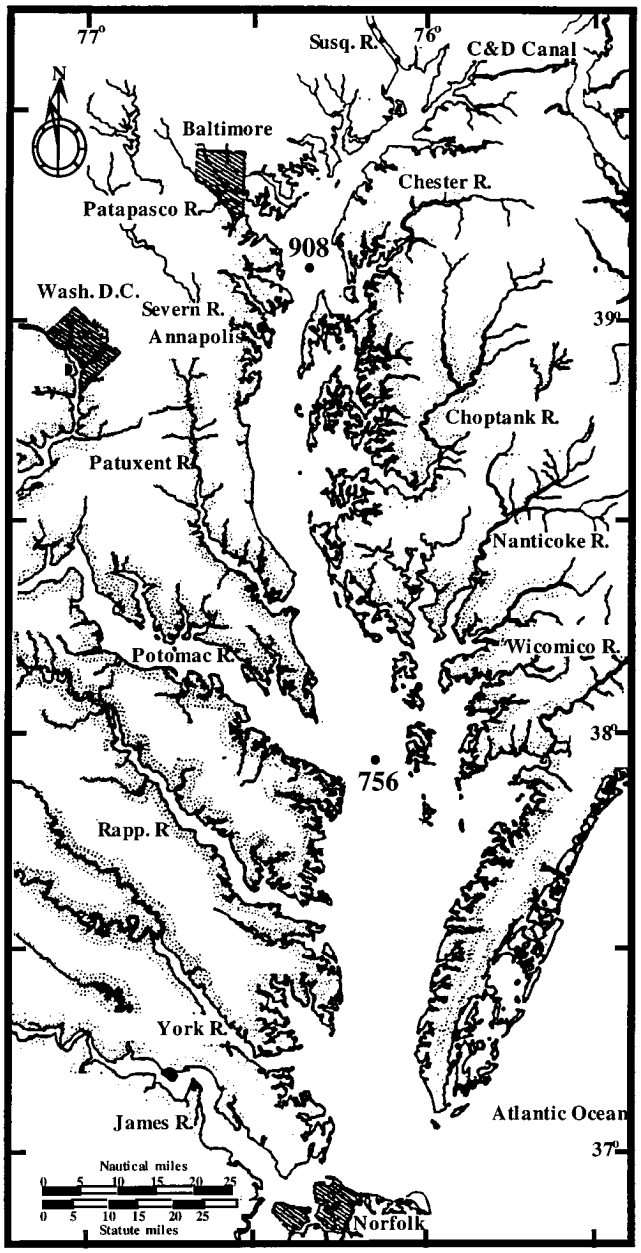

FIG. 1. Map of the Chesapeake Bay illustrating the positions of sampling stations 908 and 756.

Total RNAs from free-living and total communities, harvested onto Sterivex filters, were extracted by using the same procedure as for the particle-associated bacteria, except for the following: with a 3.0-ml syringe (Becton Dickinson, Cockeysville, Md.), 2.0 ml of extraction buffer was added to the Sterivex housing and recapped with a luer-lock mechanism (36). Filters were heated to 100°C for 5 min. Lysate was withdrawn with a clean syringe and put into a 15-ml Corex tube. Filters were rinsed with 1.0 ml of 50 mM NaOAc–10 mM EDTA, pH 5.2. All other subsequent steps were identical to those used for the particle-associated community.

Total RNA from pure culture bacteria was isolated as described by Höfle (13).

Analysis of total RNAs. Total RNAs precipitated from all environmental

TABLE 1. Bacterial abundances at stations 908 and 756 in the Chesapeake Bay[a]

Station	Location	Community[b]	Mean no. of cells ml^{-1} (10^5) ± SD	% of total
908	Top	FL	15.5 ± 0.23	82
		PA	3.4 ± 0.42	18
	Bottom	FL	20.7 ± 0.32	65
		PA	11.3 ± 0.69	35
756	Top	FL	24.6 ± 0.38	>99
		PA	0.13 ± 0.64	<1
	Bottom	FL	14.5 ± 0.18	86
		PA	2.4 ± 0.42	14

[a] Counts were obtained by the acridine orange direct counting procedure (11).
[b] FL, free-living bacteria; PA, particle-associated bacteria.

samples were 3′ end labeled with cytidine 3′,5′-[5′-^{32}P]bisphosphate (Amersham, Arlington Heights, Ill.) by using T4 RNA ligase (United States Biochemical, Cleveland, Ohio) (5, 17). Labeling reactions were carried out at 5°C for 10 h and subsequently stopped with 2 μl of 0.5 M EDTA. The amount of incorporated label (labeling efficiency) was determined by standard procedures (29). Five-microliter aliquots of each sample were pipetted onto two Whatman GF/C filters. One of the filters was rinsed (three times) under vacuum with 5% trichloroacetic acid–20 mM sodium PP_i and represented the incorporated label. The other filter, representing the total label, was not rinsed. Filters were counted in a Beckman LS 1801 scintillation counter to determine labeling efficiency and specific activities of labeled total RNA. Radioactively labeled RNA was separated from the unincorporated label with Sephadex G-50 spin columns (29).

Equal counts of labeled RNA were subjected to denaturing high-power polyacrylamide gel electrophoresis. Polyacrylamide gels (14% polyacrylamide; size, 550 by 170 by 0.4 mm; acrylamide–*N*′,*N*-methylene bisacrylamide ratio, 29:1 [wt/wt]; 7 M urea in TBE buffer [100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.5]) were run by using a Sequi-sen sequencing gel apparatus (Bio-Rad, Hercules, Calif.). After a prerun of 1 to 2 h at 40 W of constant power, gels were run at 40 W of constant power for 1 h to separate high-molecular-weight RNA from LMW RNA. Gels were then run at 80 W of constant power and at 60°C until xylene cyanol reached the bottom (~2 to 3 h). Separated RNA was fixed by using 0.1% acetic acid for 30 min, rinsed with deionized water, and dried onto chromatography paper at 80°C under a vacuum for ~45 min. Dried gels were exposed to Xar-5 film (Kodak, Rochester, N.Y.) for 1 to 5 days at −80°C and developed with an M35A X-Omat automated developer (Kodak). Duplicates of autoradiographs were made with X-Omat C Duplicating Film (Kodak). Total RNA from isolates was visualized by using both 3′ end labeling and a modified ammoniacal silver staining procedure (5, 25).

Commercially available 5S rRNA, $tRNA^{Tyr}$, and $tRNA^{Phe}$ from *Escherichia coli* MRE600 (Boehringer Mannheim, Indianapolis, Ind.) were used as molecular weight markers. They represent 120, 89, and 76 nucleotides (nt), respectively, and correspond to the three different size classes of stable LMW RNA molecules seen, namely, 5S rRNA, tRNA class 2, and tRNA class 1. The silver staining procedure was used with 5S rRNA hydrosylate, $tRNA^{Tyr}$, and $tRNA^{Phe}$ mixed together in the following concentrations: 1, 0.2, and 0.2 μg per lane. The 3′ end labeling procedure used molecular weight markers in the same proportions but with a total of 1.0 ng per lane.

Densitometry. Silver-stained gels and autoradiographs were scanned with a 620 Video Densitometer (Bio-Rad) by using reflectance and transmittance densitometry, respectively. Scans were imported and processed with the 1-D Analyst II Data Analysis Software (Bio-Rad) for Microsoft Windows. All scans were normalized by using their migration relative to that of the 120-nt 5S rRNA marker.

RESULTS

Bacterial numbers. Bacterial numbers at both stations 908 and 756 were dominated by free-living bacteria (Table 1), particularly in surface water (82% at station 908; >99% at station 756). Presence of particle-associated bacteria was minimal at station 756. Less than 1% of the total bacteria were associated with particles in the top water, compared with 14% in the bottom fraction.

RNA analysis and labeling. RNA extracted from natural samples and isolates was of high quality, as determined by UV spectrophotometric measurements. Extracted RNA from natural samples was probably of bacterial origin because we were unable to extract RNA from pure cultures of algal and yeast cells by using this extraction technique (results not shown). Yields of total RNA varied depending on the nature of the sample and the number of cells present (Table 2). Numbers of total, free-living, and particle-associated bacteria per filter were 2.6×10^9 to 9.7×10^9, 1.5×10^9 to 6.1×10^9, and 1.6×10^8 to 5.6×10^8, respectively. RNA yields from free-living and total bacterial communities were considerably higher than those obtained from particle-associated communities. Recoveries were 0.2 to 11 μg per filter for free-living bacteria and 0.6 to 10 μg per filter for total bacteria. This corresponds to ranges of ~0.03 to 7 and 0.1 to 3.3 fg per cell for free-living and total communities, respectively. RNA yields from particle-associated bacteria were very low and could not be determined accurately by UV spectrophotometry. This is due primarily to the low number of bacteria per filter.

TABLE 2. Bacterial numbers obtained per filter and analysis of RNA extracted from environmental samples[a]

Community type	No. of cells $filter^{-1}$ (10^9)	μg of RNA $filter^{-1}$	Labeling efficiency (% of label incorporated)	Sp act (cpm μg of RNA^{-1}, 10^5)
Total	2.6–9.7	0.6–10	36–55	2–6
Free living	1.5–6.1	0.2–11	47–66	2–6
Particle associated	0.16–0.56	ND[b]	6–37	ND

[a] Quantitation of RNA yields was obtained by UV spectrophotometry (A_{260}). RNA labeling data were obtained by selective precipitation with trichloroacetic acid and liquid scintillation counting (counts per minute).
[b] ND, not determined.

Up to 2.0 μg of total RNA was labeled with cytidine-3′, 5′-[5′-^{32}P]bisphosphate. Labeling efficiencies were reasonably high for all environmental and isolate samples (Table 2). Between 11 and 50% of the label was incorporated into pure culture total RNA, while 47 to 66 and 36 to 55% of the label was incorporated into free-living and total bacterial communities, respectively. Specific activities for these samples were 2×10^5 to 6×10^5 cpm/μg of RNA. Labeling efficiencies for particle-associated bacteria were slightly lower at 6 to 37%. Lower values can probably be attributed both to lower RNA yields and to possible contaminating humic material in RNA preparations. Specific activities were not determined for particle-associated material. Activity was detected after passing labeling products through Sephadex G-50 spin columns. RNA was loaded onto gels with equal counts per minute per lane. It was established that 600 cpm per lane gave good band resolution. In cases in which labeled total RNAs yielded less than 600 cpm, the entire sample was loaded onto the gel. The weight of RNA corresponding to 600 cpm was between 10 and 30 ng, as determined by labeling experiments with pure culture, free-living, and total bacterial RNAs.

General analysis of LMW RNA profiles. Electrophoretic analysis of environmental and pure culture LMW RNAs revealed a clear separation of the three major groups: 5S rRNA and both class 2 and class 1 tRNAs (Fig. 2 and 3). Molecular size ranges of bacterial 5S rRNAs are 107 to 131 nt, as indicated by sequence data (37). Class 2 and 1 tRNAs range from 83 to 96 and 72 to 79 nt, respectively (38). Separately extracted replicate samples were run side by side to estimate the reproducibility of the types and sizes of LMW RNA molecules that were present. In every case, very similar, if not identical, pro-

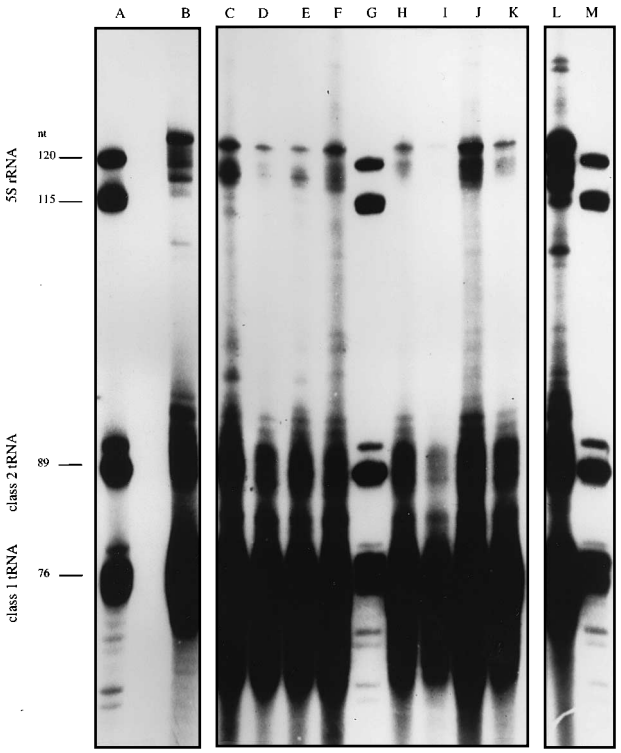

FIG. 2. LMW RNA profiles of free-living (lanes B and L) and particle-associated (lanes C to F and H to K) bacteria at station 908. Lanes B to F represent top water communities, while lanes H to L represent bottom water communities. Lanes A, G, and M contained molecular weight markers (5S rRNA, $tRNA^{Tyr}$, and $tRNA^{Phe}$ from *E. coli* at 120, 89, and 76 nt, respectively). For replicates, each lane (C to F and H to K) represents the same sample type that was extracted and labeled separately. In these samples, the entire yield of total RNA from one filter was labeled and loaded onto the gel.

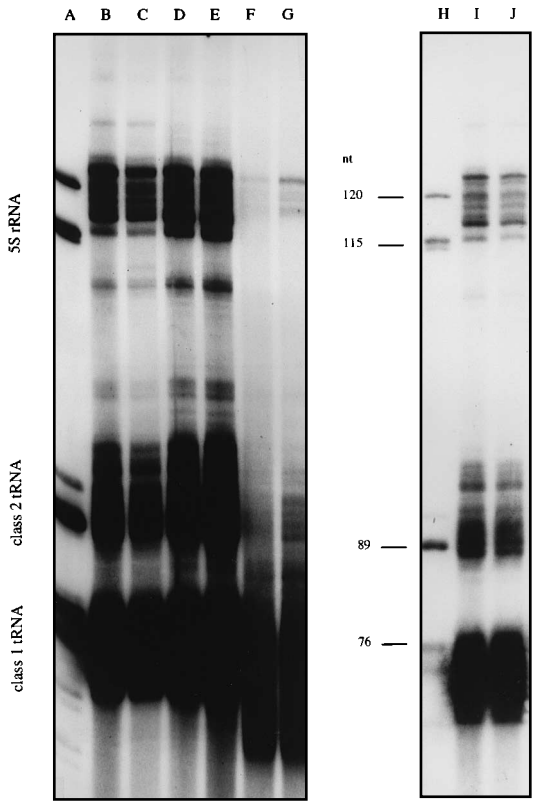

FIG. 3. LMW RNA profiles of total (lanes B to C and I to J), free-living (lanes D to E), and particle-associated (lanes F to G) bacteria at station 756. Lanes B to G represent top water communities, while lanes I to J represent bottom water communities. Only total bacterial community samples were analyzed for bottom water. Molecular weight markers are shown in lanes A and H (refer to Fig. 2 legend). Replicate lanes represent identical sample types that were extracted and labeled separately.

files were obtained for replicate samples (Fig. 2, station 908 particle-associated bacteria [top and bottom; four replicates each]; Fig. 3, station 756 free-living, particle-associated, and total bacteria [two replicates each]). The only differences seen were in band intensity. This probably indicates a difference in the amounts of RNA between replicate samples and could be attributed to small variations in the number of cells per filter during sampling. Densitometry scans of two replicates from the station 756 top water free-living community illustrate this point well (Fig. 4). All peaks are nearly identical in the two scans.

LMW RNA comparisons between free-living and particle-associated communities. Direct comparisons can be made of free-living and particle-associated community LMW RNA profiles for stations 908 (Fig. 2) and 756 (Fig. 3). Results from both top and bottom water samples are given. At station 908, particle-associated and free-living communities have similarly sized bands within the 5S rRNA range which are often present in higher intensities. However, the free-living community possesses unique 5S rRNA bands that signal some major differences in community makeup. More marked differences arise in class 2 and 1 tRNAs, in which particle-associated communities have LMW molecules that are lacking in the free-living community. Exact sizes of the molecules could not be determined, but all three fall into different size ranges (>89, 76 to 89, and ≪76 nt). Differences not discernible by visual inspection or photography of gels can be seen in densitometry scans (Fig. 5), notably in the 5S rRNA range for top water. The most intense 5S rRNA peak for the particle-associated community, representing the most significant 5S rRNA molecule(s), correlates with a trough in the free-living community (indicated by the shaded area in Fig. 5). Additional peaks unique to particle-associated community RNA are easily distinguishable in scans of tRNA regions (arrows in Fig. 5).

Station 756, a mid-bay station, allowed us to compare not only free-living and particle-associated communities but also their relationships to the total community. This was done only for top water. LMW RNA profiles are illustrated in Fig. 3, while their respective scans are presented in Fig. 6. First, it is apparent that the free-living community dominates the total community. Total and free-living community profiles are nearly identical, with some differences in band intensity. This is consistent with the direct counting data obtained at this station (Table 1). Second, free-living and particle-associated RNA profiles differ considerably in some ways but still share common-sized molecules. Many bands are seen in a wide free-living 5S rRNA range, indicating that this may be a complex community. A much narrower range results for the particle-associated community, indicating less complexity. Some bands are shared between the two communities (at 120 nt), but some 5S rRNA bands that are significant in the free-living fraction are not found in the particle-associated fraction (Fig. 6). This is particularly true for 5S rRNA molecules of >120 and ≪120 nt (arrows). As with station 908, very small class 1 tRNA molecules (≪76 nt; arrows) indicate key differences between

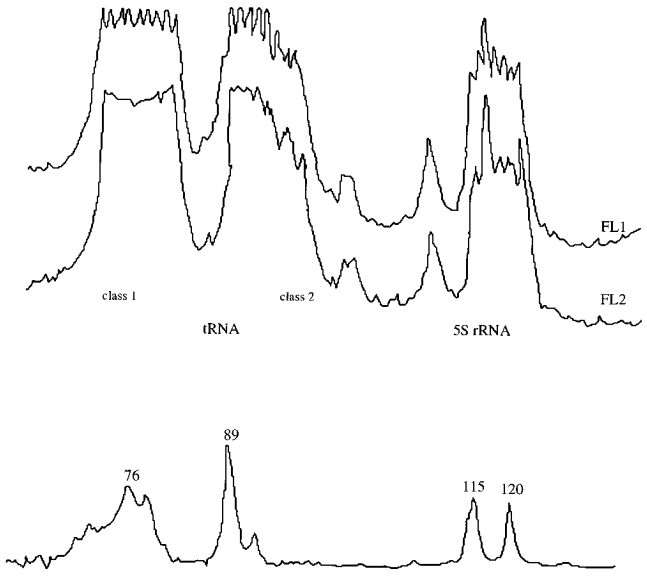

FIG. 4. Densitometry scans of LMW RNA profiles for two top water, free-living replicate samples at station 756 (FL1, Fig. 3, lane D; FL2, Fig. 3, lane E). Areas of 5S rRNA, class 2 tRNA, and class 1 tRNA molecules are indicated. Molecular size marker peaks are shown below in nucleotides.

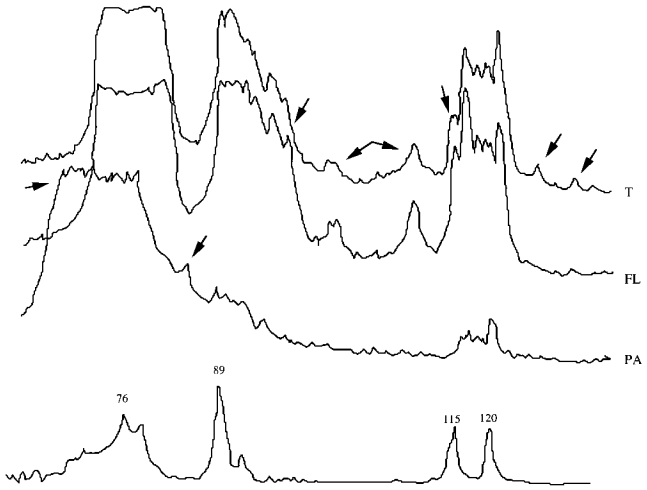

FIG. 6. Densitometry scans of LMW RNA profiles for top water communities at station 756 (T, total; FL, free living; PA, particle associated). Arrows indicate major band differences between total and particle-associated communities. Molecular size markers are shown below in nucleotides. Scans correspond to Fig. 3, lanes B (total), D (free living), and G (particle associated).

tRNAs from free-living and particle-associated communities at station 756 (arrows in Fig. 6).

Spatial distribution. Figure 7A to C shows densitometry scans of depth comparisons of free-living and particle-associated communities at station 908, as well as for total communities at station 756. During the time of year studied, community RNA composition does not differ with depth. In most cases, identical scan patterns result, with minor differences in some band intensities. A minor difference was detected between particle-associated communities at station 908 in the class 2 tRNAs (Fig. 7B). The top water community possessed a high-molecular-weight peak of tRNA class 2 which was absent in the bottom water profile (indicated by arrow). This difference is typical of those seen between very closely related organisms.

LMW profiles of free-living community RNA differ markedly between communities in the upper and middle regions of the bay (Fig. 8A and B). Free-living bacteria at station 908

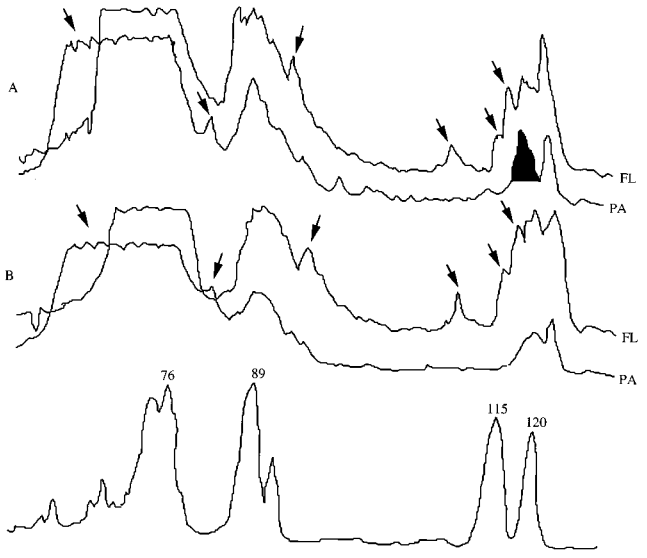

FIG. 5. Densitometry scans of LMW RNA profiles for top (A) and bottom (B) water communities at station 908 (FL, free living; PA, particle associated). Arrows indicate major band differences between free-living and particle-associated communities. The shaded 5S rRNA peak in panel A identifies a major peak in the particle-associated community which correlates closely with a trough in the free-living community. Molecular size markers are shown below in nucleotides. Top water scans correspond to Fig. 2, lanes B (free living) and E (particle associated), while bottom water scans correspond to Fig. 2, lanes L (free living) and K (particle associated).

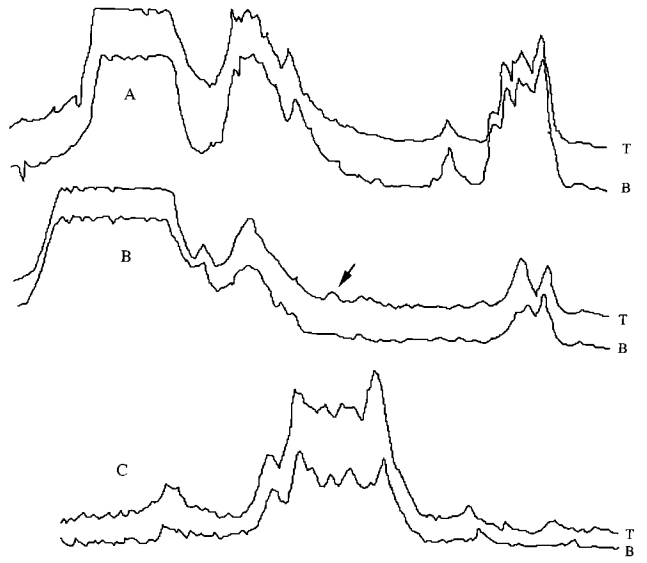

FIG. 7. Community depth comparisons showing densitometry scan LMW RNA profiles for free-living bacteria at station 908 (A), particle-associated bacteria at station 908 (B), and total bacteria at station 756 (C). Panels A and B show the full LMW RNA range, while panel C shows only the 5S rRNA range (T, top water; B, bottom water). The arrow indicates the slight difference with depth between particle-associated bacterial communities. Scans: A, Fig. 2, lanes B and L; B, Fig. 2, lanes E and K; C, Fig. 3, lanes B and I.

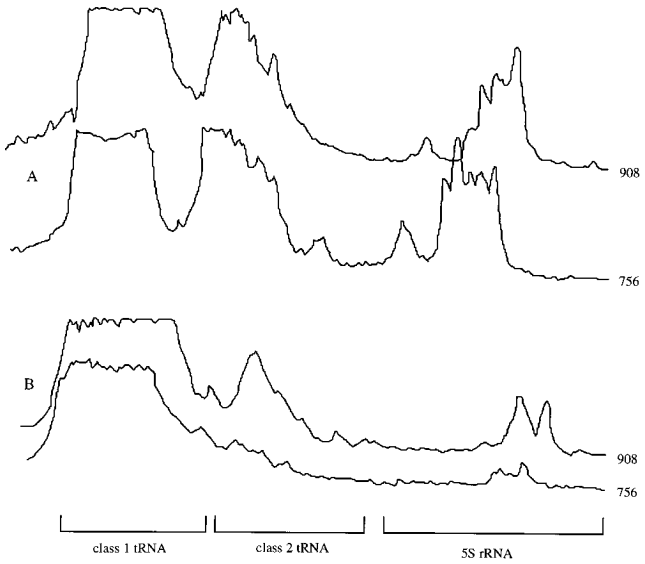

FIG. 8. Geographic community comparisons showing densitometry scans of LMW RNA profiles for free-living (A) and particle-associated (B) bacteria. Results from stations 908 and 756 are given for top water communities. Scans: A, Fig. 2, lane B, and Fig. 3, lane D; B, Fig. 2, lane E, and Fig. 3, lane G.

possess higher-molecular-weight 5S rRNAs than those at station 756, suggesting that community members differ greatly in relatedness (Fig. 8A). Particle-associated community RNAs, however, were similar in samples from these two stations. 5S rRNA and tRNA band migrations differed only slightly (~1 to 3 nt) (Fig. 8B).

Isolate comparisons. There was a wide range of profiles for the isolates at each station, with some 5S rRNA molecules differing markedly. Densitometry scans of the 5S rRNA region (Fig. 9A and B) demonstrate how RNAs from pure culture isolates compare with those from environmental samples. In several cases, isolates have 5S rRNA peaks that cannot be detected in the community range (indicated by arrows).

DISCUSSION

The aims of this study were (i) to determine whether free-living and particle-associated bacterial communities in the Chesapeake Bay were substantially different, (ii) to compare communities at different depths in the water column and at different geographical locations, and (iii) to determine whether organisms isolated onto growth media were representative of community members. LMW RNA electrophoretic analysis was the technique of choice because it enables comparison of gross community similarities and/or differences relatively quickly. This technique focuses on stable molecules that are directly involved in protein synthesis and cell maintenance. Such molecules have the ability to serve as markers of active cells in the community. In addition, stable LMW RNA molecules in bacterial cells remain unchanged regardless of their physiological state and/or the growth medium used (13).

The dominance of free-living bacteria over particle-associated bacteria, in terms of numbers, in natural environments has been well documented. Free-living bacteria usually make up large percentages of total bacterial communities, while numbers of bacteria associated with particles are lower. In this study, proportions of particle-associated bacteria were higher in samples with the greatest particle load. The higher number of particle-associated bacteria at station 908 than at station 756 was probably related to the suspended particulate material in the upper bay brought in by the Susquehanna River. At both stations, particle-associated bacteria were more common in bottom water than in surface water. Once particles are introduced into the bay, they are subjected to different settling rates depending on the size and composition of the particles, as well as on prevailing currents. As particles slowly settle out of the water column, they become more concentrated in bottom waters and are reintroduced into the water by sediment disturbance.

The ability to produce identical LMW RNA profiles for separately extracted environmental samples provided confidence in the consistency and utility of the technique for comparing natural communities. Pure culture studies in our laboratory have consistently yielded the same profile patterns between separately extracted RNAs for the same organism. Similar reproducibility was achieved when community structure in separate microcosms was analyzed (17). Moreover, normalized relative migration distances ensured accurate comparisons between samples run on different gels. Studies in our laboratory with both pure culture and environmental samples have shown that after normalization of profiles with respect to the 120-nt 5S rRNA molecular weight marker, there is no detectable run-to-run variation for identical samples run on different gels.

In pure culture and noncomplex communities, the entire profile can be used for tentative identification of organisms to the species level if profiles of constituent species are available. In these cases, particular emphasis is placed on 5S rRNAs and class 2 tRNAs (15, 16). However, for complex communities, species identity is difficult to determine and the emphasis shifts to the first-order molecule of comparison, namely, the 5S rRNA (107 to 131 nt). Distantly related bacterial organisms differ markedly in 5S rRNA molecule size (15). If natural samples share no 5S rRNA molecular sizes, their communities are made up of very different organisms. If communities have the same 5S rRNA molecular sizes, one must compare tRNA molecules for more specific clues to community structure. In addition, one can completely or partially sequence 5S rRNAs of the same size to identify whether the 5S rRNA molecules are homogeneous (i.e., from the same organisms).

It was our objective to determine whether the communities found associated with particles ($\geq$3.0 μm in size) were unlike those that are free in the water column at the same location. Comigration of some 5S rRNA molecules in free-living and particle-associated community RNAs was evident, suggesting possible similarities. However, LMW RNA profiles indicated significant differences between the free-living and particle-associated bacteria. For example, free-living communities at both stations possessed unique 5S rRNA molecules (Fig. 2 and 3; arrows in Fig. 5 and 6). The presence of lower-molecular-weight (~110-nt) 5S rRNA bands at station 756 may indicate the presence of bacteria other than proteobacteria living free in the water column instead of attached to particles (15, 17). Significant differences were seen in the tRNAs, as well, where both free-living and particle-associated communities possessed some unique molecules (Fig. 2 and 3; arrows in Fig. 5 and 6).

tRNAs are far more numerous than the 5S rRNAs in bacterial cells. For a typical bacterium, about 26 tRNA molecules (of the theoretically possible 60) can be expected, and they account for about 70% of cellular tRNAs (19). Usually, each is the dominant isoacceptor tRNA molecule for a single amino acid (19, 20). Moreover, the tRNAs are unevenly distributed between the two size classes, with most belonging to class 1. Therefore, class 1 tRNAs make up the bulk of the protein-synthesizing machinery examined in LMW RNA profile stud-

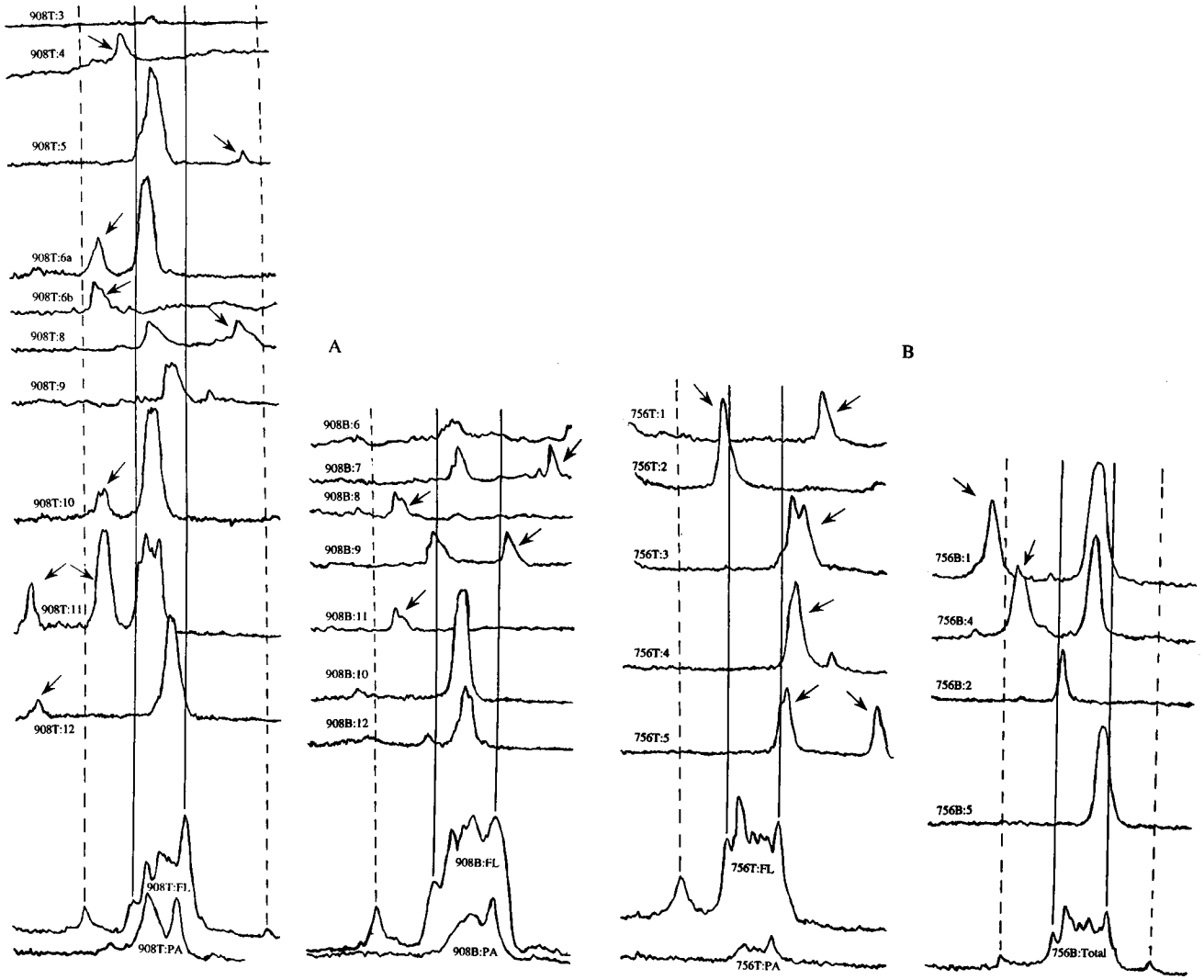

FIG. 9. Densitometry scans of 5S rRNA regions for bacterial isolates and their native communities. Scans for stations 908 (A) and 756 (B) are given for top (T) and bottom (B) waters. Natural communities are shown below the bacterial isolates. Community 5S rRNA ranges are indicated with solid lines, while individual peaks are indicated with dashed lines (FL, free living; PA, particle associated). Arrows identify peaks from bacterial isolate RNAs that do not coincide with peaks from their respective community RNAs. Scans for station 908 correspond to Fig. 2, lanes B, E, L, and K. Scans for station 756 correspond to Fig. 3, lanes D, G, and I.

ies. Class 1 tRNAs have far less resolution of individual molecules than do class 2 tRNAs. This is supported by the differing resolutions of class 2 and 1 tRNAs in published LMW RNA profiles (13–18, 34), as well as those in this study. In addition, tRNA patterns in LMW RNA profiles are distinctly different between species and reproducible within different samples of the same species (13). Species differences within a genus of bacteria are seen as species-specific bands in class 2 tRNAs, while class 1 tRNA molecules are highly conserved between closely related organisms. A major group of *Pseudomonas* species, for example, has characteristic LMW class 1 tRNA bands ($\ll$75 nt) that are separated from the bulk of the class 1 tRNAs (13, 15, 16, 18).

Very substantial differences between LMW RNA profiles for free-living and particle-associated community RNAs were in the class 1 tRNAs. There were distinctive peak differences in high- and low-molecular-weight areas, as shown by densitometry scans of the tRNA region (arrows in Fig. 5 and 6). The fact that these differences appear in the class 1 tRNAs (the bulk of the RNA) suggests that the dominant members of free-living and particle-associated communities are different.

Seasonal circulation patterns in the Chesapeake Bay are characterized by stratification of the water column in the warmer months (late spring, summer, and early fall) and thermal mixing of the water column during the cooler months (late fall, winter, and early spring). When the samples described here were collected (December), the water column should have been well mixed, with relatively even distribution of organisms down the water column. Hydrographic data were consistent with depth for most of the parameters measured at stations 908 and 756, although some variations were seen at bottom depths (Table 3). Vertical mixing of the communities is supported by identical LMW RNA profile scans for top and bottom water free-living community samples at station 908, as well as for top and bottom water communities at station 756. There was a small difference in particle-associated communities with depth. Such a difference is probably indicative of very closely related organisms and was seen at only one location in

TABLE 3. Water column data for stations 908 and 756 on 2 December 1992[a]

Station	Depth (m)	Temp (°C)	Salinity (‰)	Oxygen (ml/liter)	Attenuation (*A*)[b]	Fluorescence (F)[c]
908	0.1	8.1	6.6	9.4	4.4	25.3
	1.0	8.1	6.6	9.4	4.4	25.5
	2.0	8.1	6.6	9.4	4.3	26.5
	3.0	8.1	6.6	9.3	4.3	26.7
	4.0	8.3	7.4	9.2	4.2	24.4
	5.0	9.7	11.5	8.4	4.4	19.1
	6.0	11.1	15.4	7.5	7.6	11.5
756	0.8	9.8	16.1	8.7	2.1	10.2
	2.0	9.8	16.1	8.8	2.1	10.0
	3.0	9.8	16.1	8.8	2.1	10.2
	4.0	9.8	16.2	8.8	2.1	10.5
	5.0	9.8	16.2	8.8	2.1	10.8
	6.0	9.9	16.2	8.8	2.1	10.5
	7.0	9.9	16.3	8.8	2.1	10.7
	8.0	9.9	16.4	8.8	2.1	10.9
	9.0	10.0	16.4	8.8	2.1	11.7
	10.0	10.1	16.8	8.7	2.1	11.5
	11.0	10.7	19.6	8.3	2.2	11.9
	12.0	11.7	22.0	7.9	2.3	11.1
	13.0	11.9	22.8	7.5	2.3	9.4
	14.0	12.0	23.4	7.3	3.0	7.3

[a] Data were obtained by CTD measurement aboard the research vessel *Cape Henlopen* as described in reference 41.
[b] *A*, absorbance units.
[c] F, Turner units.

the class 2 tRNAs. Since station 908 typically contains high concentrations of particulate material of various shapes, sizes, and compositions, it is possible that community differences could be due to differential settling of different particle types. However, differences in parameters at bottom depths cannot be ruled out. Our data are consistent with vertical mixing and demonstrate that bacterial communities are distributed accordingly. They also indicate that communities are distributed throughout the water column in areas where water is somewhat stratified.

The Chesapeake Bay is variable in many parameters over its geographical range. These include particulate material, salinity, dissolved gases, dissolved nutrients, and temperature, and all are able to have an impact on community structure. The two stations of our study were nearly 75 nautical miles (about 139 km) apart and are located in two very different regions. Station 908 is an upper-bay station quite near Baltimore and other urban centers. It is exposed to a heavy influx of freshwater often loaded with nutrients (N and P) and other anthropogenic substances. With its proximity to the Susquehanna River, a river that provides nearly 97% of the total freshwater reaching the bay north of latitude 39°12′N, particulate material is very highly concentrated and quite variable in size and makeup (31). Salinity generally ranges between 5 and 10 ppt during autumn and winter months. Station 756, a mid-bay mesohaline station, receives a much lower freshwater input. It is situated in a region of very low urban development, and as a result, particulate material and dissolved nutrient levels are generally lower. Salinity ranges between 15 and 20 ppt during autumn and winter months.

Free-living and particle-associated community compositions are clearly different over geographical distance. Free-living community RNA differences are considerable in the 5S rRNA range, with station 908 possessing a considerable portion of higher-molecular-weight 5S rRNAs than station 756. This suggests that the differences might be due to the presence of different species, as well as the presence of distantly related organisms. Differences between particle-associated community RNAs were small compared with those seen for the free-living communities. This suggests that the environmental factors that select, or enrich, for particle-associated bacteria are consistent along the length of the bay. There may be a characteristic assemblage of microorganisms that is adapted to growth on particles, e.g., by their substrate utilization capabilities or attachment characteristics.

A majority of viable bacterioplankton in natural environments resist cultivation onto standard laboratory medium (22). For this reason, traditional methods in microbial ecology have been restricted and emphasis has shifted towards nonculturing techniques (4, 7, 17). We wanted to determine whether cultured bacterial isolates from the Chesapeake Bay were representative of in situ communities. If any of the isolated bacterial organisms were dominant members of the communities, their 5S rRNA peaks should have been detectable within the community profiles. Since several of the isolated bacterial 5S rRNA peaks lie outside the community ranges, they are probably not significant members of the communities examined. Therefore, with Bay communities, as with other aquatic environments, laboratory isolates may not be characteristic of the total community and great care must be taken when extrapolating from laboratory studies to ecosystem structure and function.

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