Applied and Environmental Microbiology, June 2009, p. 4028–4034 Vol. 75, No. 12
0099-2240/09/$08.00+0 doi:10.1128/AEM.00183-09

Geographic and Phylogenetic Variation in Bacterial Biovolume as Revealed by Protein and Nucleic Acid Staining[▽]

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Received 26 January 2009/Accepted 8 April 2009

Biovolume is an important characteristic of cells that shapes the contribution of microbes to total biomass and biogeochemical cycling. Most studies of bacterial cell volumes use DAPI (4′,6′-diamidino-2-phenylindole), which stains nucleic acids and therefore only a portion of the cell. We used SYPRO Ruby protein stain combined with fluorescence in situ hybridization to examine biovolumes of bacteria in the total community, as well in phylogenetic subgroups. Protein-based volumes varied more and were consistently larger than DNA-based volumes by 3.3-fold on average. Bacterial cells were ca. 30% larger in the Arctic Ocean and Antarctic coastal waters than in temperate regimes. We hypothesized that geographic differences in the abundance of specific bacterial groups drove the observed patterns in biovolume. In support of this hypothesis, we found that *Gammaproteobacteria* and members of the *Sphingobacteria-Flavobacteria* group were larger in higher-latitude waters and that the mean volumes of both groups were larger than the mean bacterial volume in all environments tested. The mean cell size of SAR11 bacteria was larger than the mean cell size of the total bacterial community on average, although this varied. Protein staining increases the accuracy of biovolume measurements and gives insights into how the biomass of marine microbial communities varies over time and space.

Size is an important characteristic of microbes that affects cell physiology and trophic interactions. Cell size in combination with abundance can be used to estimate microbial biomass (17, 26). The size and shape of a cell affects the efficiency of nutrient uptake by determining the ratio of surface area to volume (8). Size-dependent mortality also contributes to the structuring of microbial assemblages (18). Larger cells are grazed more, and cells too small or too large cannot be ingested by protists (16, 18). Small cells may also be less susceptible to viral lysis (45). An accurate description of microbial cell volumes is important for understanding microbial interactions and the contribution of different cells to the total microbial biomass.

In spite of its importance, the biovolume of cells in microbial communities has not been extensively examined, although some data indicate that size varies by season and among geographic locations due to variation in environmental conditions. The size of bacterial cells varied daily in an artificial lake, peaking in the afternoon (19). The mean bacterial cell volume in the Sargasso Sea was smallest in the winter, with high day-to-day variation (9). Acridine orange-stained cells in the northern Adriatic Sea were larger in June than in February for two consecutive years and varied with environmental parameters (24). Cell volume varied 20-fold, whereas cell abundance ranged 10-fold (24). Other studies are necessary to determine seasonal and geographic changes in cell volumes.

Biovolume may also vary among microbial groups. Cultured representatives of the ubiquitous SAR11 clade of *Alphaproteobacteria* measured using transmission electron microscopy have a mean size of 0.01 μm^3, among the smallest of cultured bacteria (39). The opposite is seen in the *Bacteroidetes* phylum, which in marine waters is mostly represented by the *Flavobacteria* and *Sphingobacteria* classes (including the genus *Cytophaga*), referred to as the "SF group" here (2). These cells are often larger than cells in other bacterial groups (21), although this varies. While the average size of SF group bacteria was the same as that of the total bacteria in the Delaware Bay, SF group members were smaller than the average prokaryote in the Arctic Ocean (23). Bacteria in an SF subgroup, DE cluster 2, were larger than other bacteria in both environments. Because biomass depends on cell size, not just cell abundance, the distribution of biovolume within bacterial groups is as important as abundance.

Most epifluorescence microscopic studies of natural bacteria use DAPI (4′,6′-diamidino-2-phenylindole), a stain for nucleic acids that fluoresces blue under UV excitation when bound to double-stranded DNA (38). Because DAPI is specific for DNA, DAPI images may contain only the nucleoid and not the whole cell. Cell volumes determined with DAPI are ca. 60% smaller than volumes based on acridine orange, another common nucleic acid stain (44). Acridine orange is less specific than DAPI and stains detrital particles and dead cells (7, 44). Also, when bound to RNA, acridine orange fluoresces the same color as cyanobacteria and as bacteria identified with probes commonly used in fluorescent in situ hybridization (FISH) (7). DAPI can be used to estimate cell abundances, but a different stain for measuring cell volumes is necessary.

Because protein comprises ca. 60% of cell mass (41), we selected SYPRO Ruby protein stain (5) for determining cell volume by epifluorescence microscopy. SYPRO Ruby (Molecular Probes) is specific to proteins and stains most classes of proteins (5). It fluoresces at a wavelength distinguishable from cyanobacterial autofluorescence and cyanine-3-labeled FISH

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▽ Published ahead of print on 17 April 2009.

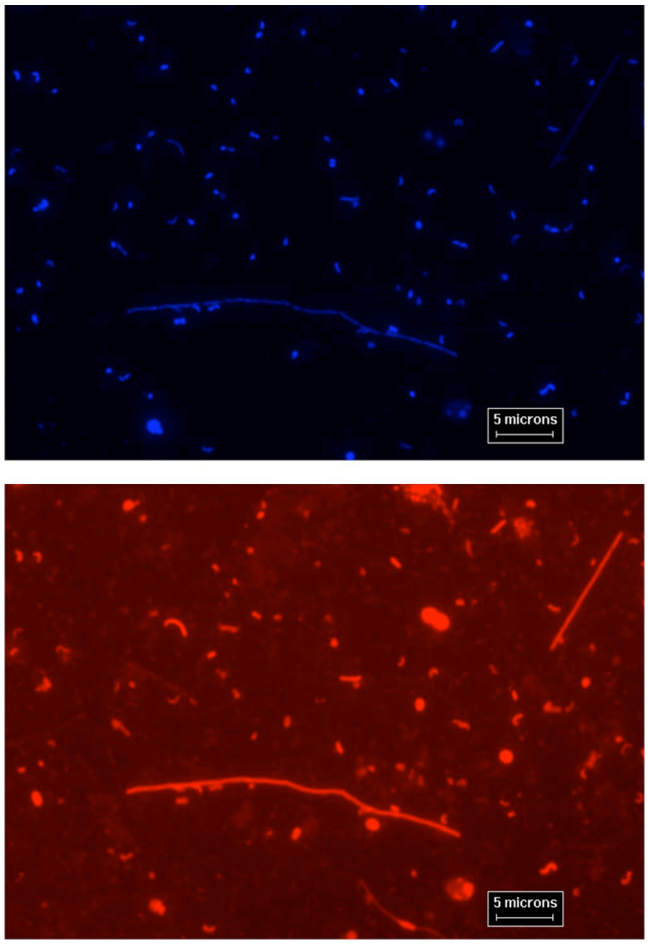

FIG. 1. Epifluorescence micrograph of cells stained with DAPI (top panel) and SYPRO Ruby (bottom panel).

probes. In the present study, we measured cell volumes with DAPI DNA staining and SYPRO Ruby protein staining of bacteria from a range of seasons and geographic locations. We observed greater variation in size based on protein staining than on nucleic acids, and we describe here the biovolumes of bacteria from several environments and bacterial groups.

MATERIALS AND METHODS

Samples were obtained from several locations to examine geographical variation in bacterial biovolume. Surface waters were sampled monthly beginning in February 2006 at the mouth of the Delaware Bay and 18.5 km offshore (http://www.ocean.udel.edu/cms/dkirchman/MOPE). Antarctic samples were collected in coastal shelf waters of the west Antarctic Peninsula in January 2007 (http://pal.lternet.edu). Arctic (Chukchi Sea) sampling was as described by Malmstrom et al. (29). Samples from the North Atlantic (32) and central North Pacific near Hawaii (11) were also examined. Briefly, water samples were fixed with paraformaldehyde (2% final concentration) and filtered onto 0.22-μm-pore-size polycarbonate filters (Millipore), rinsed with 0.22-μm-pore-size-sterilized deionized water, and then stored at −20°C.

Protein staining. For protein staining, filter pieces were dipped in 0.3% low-melting-point agarose (Metaphor) and placed cell side down on glass slides. After drying, the filter piece was wetted with 95% ethanol and then carefully peeled away, leaving the exposed cells embedded in agarose on the slide. Cells on the slides were stained with SYPRO Ruby protein stain (Invitrogen; diluted 1:1 with deionized water) for 30 min and then rinsed with deionized water. After drying, samples were mounted with DAPI (0.5 ng μl^{-1}) in a 4:1 mixture of Citifluor (Ted Pella) and Vectashield (Vector Labs) antifading mountants.

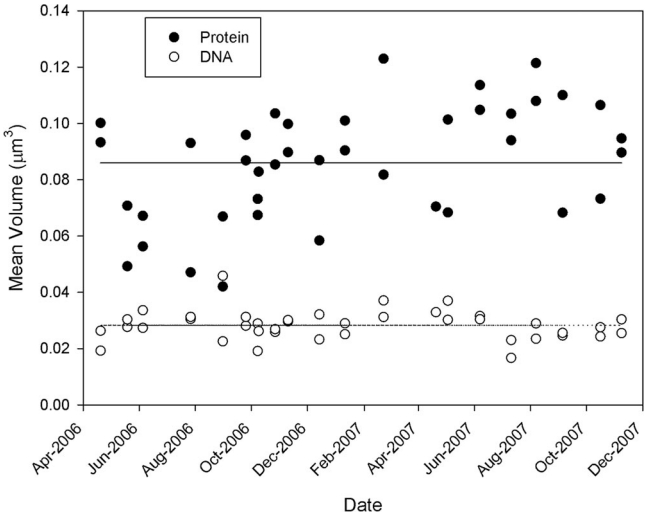

FIG. 2. Seasonal variation in biovolume based on protein and DNA stains in Delaware Bay. Lines are means over the entire period.

Abundance of bacterial groups. To estimate the abundance and biomass of specific bacterial groups, samples were analyzed by catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH [36]) prior to SYPRO Ruby staining. Probes for *Alphaproteobacteria* (Alf968), *Gammaproteobacteria* (Gam42a), the SF group (CF319a), the SAR11 clade (SAR11-441r) of *Alphaproteobacteria*, all bacteria (EUB338), and a negative control were used (1, 15, 20, 30, 31, 34). Cells were embedded on the filter using 0.1% agarose and then treated with lysozyme to increase permeability. Filter pieces were hybridized with horseradish peroxidase-labeled oligonucleotide probes, and subsequently stained with cyanine 3 (Cy3)-labeled tyramides (TSA kit; Perkin-Elmer). After this staining, the filter pieces were transferred onto slides and stained with SYPRO Ruby and DAPI as described above.

To test the impact of the protocols on biovolume measurements, we compared cell volumes from the same sample with only protein staining, FISH with protein staining, and CARD-FISH with protein staining. There was no significant impact of the protocol on the size of the protein-stained image (independent Student *t* test, $n = 6$, $P > 0.05$). The mean cell volumes (± the standard error) based on the protein were 0.08 ± 0.01 μm^3 for SYPRO Ruby staining only, 0.10 ± 0.02 μm^3 after FISH, and 0.09 ± 0.02 μm^3 after CARD-FISH.

Microscopic and data analyses. Samples were examined by using a modification of the semiautomated microscopy system described by Cottrell and Kirchman (10). Ten fields of view were counted per sample, using a constant exposure time of 300 ms for SYPRO Ruby images and 75 to 100 ms for DAPI images (Fig. 1). The Cy3 image exposure time for CARD-FISH analyses was set using the negative control, and ranged 200 to 300 ms. Objects in the SYPRO Ruby and Cy3 images were only considered to be cells if there was a corresponding object in the DAPI image. Group abundances from CARD-FISH were determined as the percentage of the total DAPI objects appearing in both the Cy3 and DAPI images. Nonspecific probe binding was below 5% for all samples.

The volumes of the DNA- and protein-stained images were calculated assuming cells were cylinders with hemispherical caps (4) and were compared by using a paired Student *t* test. Objects with volume less than 0.0042 μm^3 (equivalent to the pore size of the filter) or greater than 0.344 μm^3 (equivalent to 0.87 μm in diameter) in any stain image were not considered to be prokaryotic cells and were excluded from all calculations (14). Volume data were log-transformed and community percentage data were arcsin-transformed for all statistical tests.

RESULTS

We tested the application of SYPRO Ruby protein stain for measuring the biovolume of environmental bacteria and compared protein volumes to DNA volumes. In the Delaware Bay from 2006 to 2007, the protein-stained cell images were always larger than the DNA-stained images (Fig. 2). The mean protein/DNA ratio was always greater than 1 and ranged from 1.5

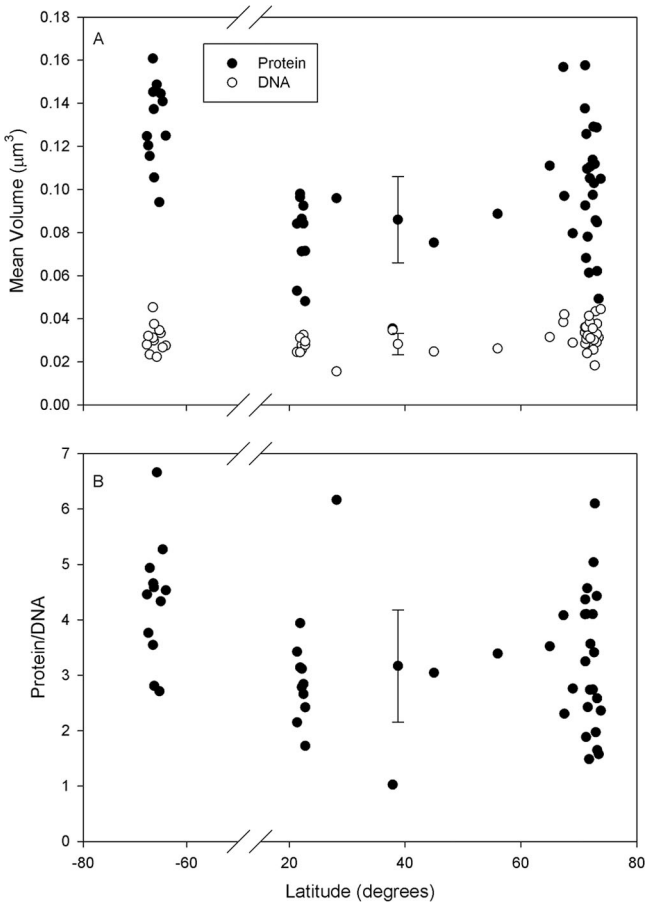

FIG. 3. Geographic variation in biovolume based on protein and DNA stains (A) and protein/DNA ratio (B). Delaware Bay samples are represented by the mean ± the standard deviation.

to 5.6 (mean 3.2 ± 1.0). Protein volume (coefficient of variation [CV] = 0.74) varied more than DNA volume (CV = 0.58), but there was no clear seasonal trend (Fig. 2). No significant correlations of cell volume or protein/DNA ratio were observed with other parameters, such as chlorophyll *a* concentrations, [^{3}H]leucine incorporation, temperature, and photosynthetically active radiation (data not shown).

We analyzed geographic variation in cell volume in "high" (Arctic and Antarctic) and "low" latitudes (Delaware Bay, North Atlantic, and North Pacific). Cells were significantly larger in high-latitude waters than in low-latitude waters based on protein (independent *t* test, $P = 0.00001$) and DNA ($P = 0.007$) volumes, as well as on the protein/DNA ratio ($P = 0.01$) (Fig. 3). The mean difference for protein-based volume was 0.02 μm^3 or 32%, whereas for DNA the difference was only 0.003 μm^3 (16%). The protein/DNA ratio in these samples ranged from 1 to 6.6, with a mean of 3.3 ± 1.2. The biovolume based on both DAPI (CV = 0.58) and SYPRO Ruby (CV = 0.74) staining varied more than the cell abundance (CV = 0.18).

To further explore this geographic difference in volume, we examined the average cell size of major bacterial groups in the Arctic, Antarctica, and Delaware Bay by using CARD-FISH combined with SYPRO Ruby staining. *Alpha-* and *Gammaproteobacteria* were less abundant in Delaware Bay than in the high latitude samples (*t* test, $P = 0.02$ and 0.0002, respectively), whereas the abundance of the SF group was not significantly different (Table 1). Protein volumes within the bacterial groups across the latitudes varied for some groups (Table 2). SF cells were significantly smaller (mean difference 0.04 μm^3, or 25%) in the Delaware Bay than in the Arctic and Antarctica, which did not significantly differ (analysis of variance [ANOVA], $P \geq 0.05$) from each other (Fig. 4). The *Gammaproteobacteria* were also smaller in the Delaware Bay than in the Arctic (40% larger in the Arctic) and largest in Antarctic waters (64% larger than in the Delaware Bay). The volume of *Alphaproteobacteria* did not significantly differ among the three locations (ANOVA, $P > 0.05$).

With data from all latitudes pooled together, SF group cells were larger than cells of all other groups (Table 2). *Gammaproteobacteria* were 10% smaller than the SF group but ca. 15% larger than *Alphaproteobacteria* and the average bacterial (EUB338-positive) cell. The *Alphaproteobacteria* did not differ from the average bacterial cell. This was the pattern observed in Arctic samples. In Antarctica, the cell volumes of SF group organisms and *Gammaproteobacteria* were not significantly different, and both were larger than *Alphaproteobacteria* and the average bacterial cell, which were the same. In the Delaware Bay, SF cells were the largest of all of the groups by ca. 15% (ANOVA, $P \leq 0.05$), and cell sizes of *Gammaproteobacteria*, *Alphaproteobacteria*, and average bacteria were not significantly different from each other (ANOVA, $P \geq 0.05$).

To determine whether it is possible to account for the mean bacterial biovolume based on the examined bacterial groups, we calculated the sum of the biovolumes of the groups weighted by their abundances. In the Arctic and Antarctica, the sum of the measured group volumes was similar to the average volume of the total community. The sum of the Arctic group volumes was 0.15 ± 0.02 μm^3 versus the EUB338 cell mean of 0.10 ± 0.01 μm^3, while the sum of Antarctic group volumes was 0.11 ± 0.02 μm^3 versus 0.09 ± 0.01 μm^3 for the

TABLE 1. Abundance of total prokaryotes and bacterial groups

Area	Mean no. ± SE					
	Total abundance (10^5 cells ml^{-1})	Bacteria[a]	*Alphaproteobacteria*	*Gammaproteobacteria* (% of total prokaryotes)	SF group	SAR11
Arctic	9.5 ± 2.2	74 ± 4	11 ± 4	71 ± 8	39 ± 11	30 ± 12
Antarctica	7.2 ± 1.9	81 ± 4	12 ± 3	50 ± 12	20 ± 5	10 ± 4
Delaware Bay	4.8 ± 0.69	50 ± 9	3 ± 1	11 ± 2	16 ± 10	5 ± 4

[a] Cells identified with EUB338 CARD-FISH probe.

TABLE 2. Mean protein volume of bacterial groups

Region	Mean vol (μm^3) ± SE				
	Bacteria[a]	*Alphaproteobacteria*	*Gammaproteobacteria*	SF group	SAR11
Arctic	0.100 ± 0.04	0.123 ± 0.06	0.120 ± 0.04	0.138 ± 0.03	0.107 ± 0.05
Antarctica	0.106 ± 0.04	0.094 ± 0.06	0.142 ± 0.03	0.152 ± 0.05	0.082 ± 0.07
Delaware Bay	0.087 ± 0.06	0.090 ± 0.03	0.086 ± 0.04	0.109 ± 0.04	0.114 ± 0.06
All	0.097 ± 0.04	0.103 ± 0.05	0.118 ± 0.04	0.134 ± 0.04	0.100 ± 0.06

[a] Based on cells identified with EUB338 CARD-FISH probe.

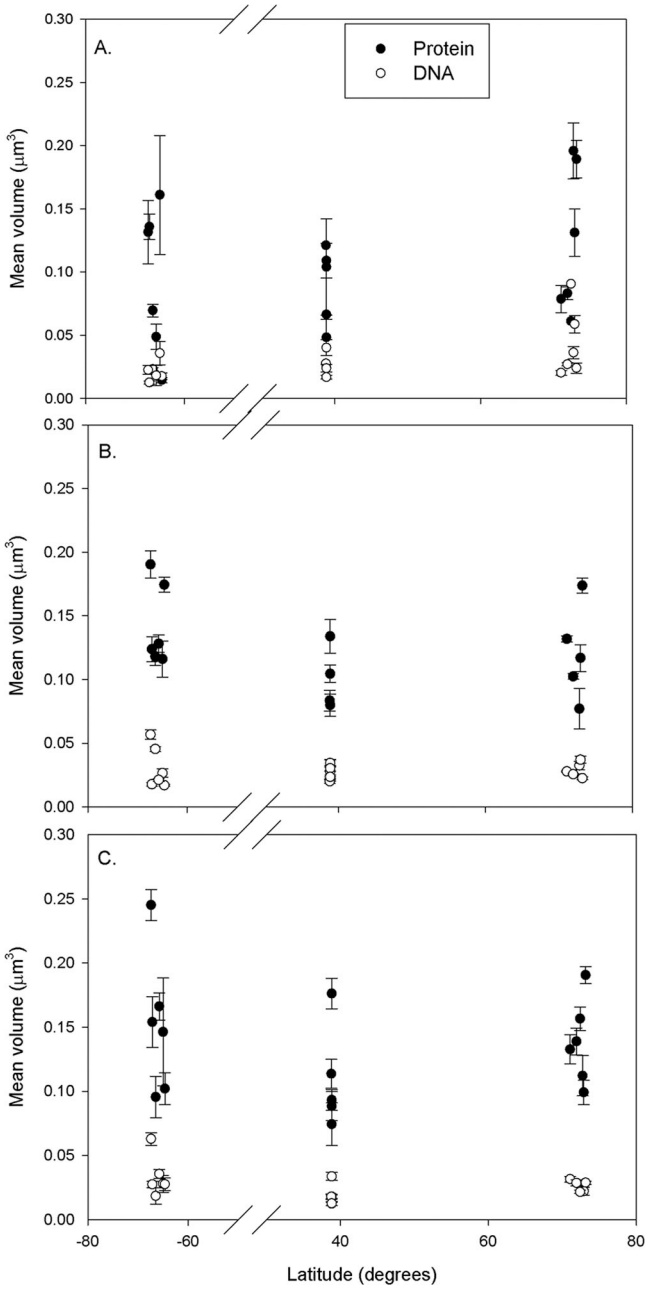

FIG. 4. Geographic variation in biovolumes of *Alphaproteobacteria* (A), *Gammaproteobacteria* (B), and the SF group (C). Error bars indicate one standard error.

EUB338 cells (Table 2). However, in the Delaware Bay this calculation differed greatly from measured EUB338 cell volumes. The sum of group biovolumes was 0.03 ± 0.01 μm^3, which is substantially less than the EUB338 cell mean volume of 0.11 ± 0.01 μm^3, suggesting that another abundant group is driving the average cell volume in the estuary. The presence of another abundant group is implied by the low percentage (29% ± 10%) of the community accounted for by *Alpha-* and *Gammaproteobacteria* along with the SF group (Table 1).

Because of the suggestion that SAR11 cells may be smaller than other bacteria (39), we examined this clade in the same regions discussed above. When all samples were pooled, the protein biovolumes of SAR11 cells were larger than the mean of all bacteria (EUB338-positive), while there was no significant difference between DNA-based volumes (ANOVA; Fig. 5). The protein/DNA ratio was also higher for SAR11 than for all bacteria. However, the mean difference in protein volume between SAR11 and total bacterial cells was only 0.003 μm^3 or 3%. In the Arctic samples, SAR11 cells were larger than cells in the total bacterial community and in total *Alphaproteobacteria* (Table 2). In the Delaware Bay, SAR11 cells were larger than the bacterial mean, as well as the mean sizes of the *Alpha-* and *Gammaproteobacteria*. However, in Antarctica, SAR11 cells were smaller than cells in the total assemblage, although they were not different from cells in the alphaproteobacterial group (ANOVA, $P > 0.05$).

DISCUSSION

Bacterial abundance and biovolume are commonly examined by using DAPI staining for DNA. When nucleic acid stains such as DAPI are used, the localization of the fluorescent signal due to concentration of DNA in the nucleoid can lead to the underestimation of cell sizes (44, 48). We tested the application of the SYPRO Ruby protein stain for epifluorescence microscopy to measure bacterial biovolumes. Biovolumes based on protein-stained cells were larger and varied more than DNA-based biovolume estimates in the marine systems examined here.

Proteins are a large portion of bacterial cells but may also be part of marine detritus (27). Long and Azam (27) used Coomassie blue staining to show that noncellular protein particles may be highly abundant in coastal systems, ranging in abundance from 10^6 to 10^8 liter^{-1} in coastal waters with 20 to 40% colonized by bacteria. In the present study, the objects we considered as cells were present in DAPI-stained images, with a volume within a set size range (0.0042 to 0.344 μm^3). This excludes objects such as Coomassie blue-stained particles,

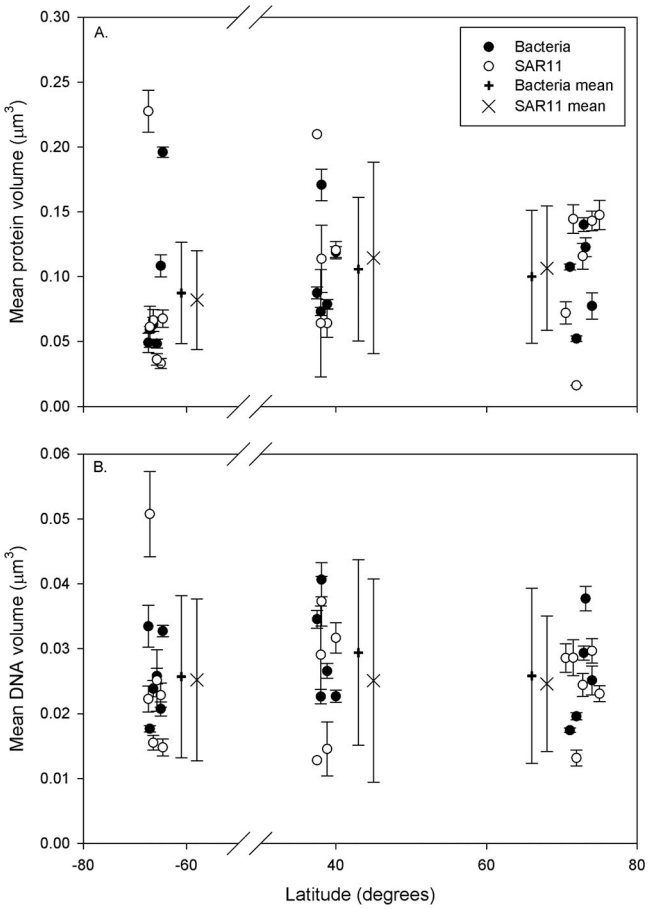

FIG. 5. Biovolumes of SAR11 and total bacteria (EUB338) based on protein (A) and DNA (B). Within each site, the latitude for the means were offset slightly to clarify. Error bars indicate one standard error.

which would be present only in the SYPRO Ruby-stained image. In addition, using the two images together allows us to compare protein- and DNA-based volumes.

We observed a mean protein/DNA volume ratio of 3.3 but with much variation, perhaps due to the dependence of the cellular protein/DNA ratio on growth rates and phases during growth cycles. The expected ratio of protein to DNA based on weight is on the order of 4.8 to 5.8 (41), larger than what we observed. Because DAPI staining may be nonspecific (48), the observed DAPI volume may be larger than the actual nucleoid size, leading to the slightly lower protein/DNA ratio that we observed.

Classic studies of pure cultures found that rapidly growing cells in high-nutrient conditions have a high protein content and large volumes (3, 25, 35, 40). These same cells when starved can decrease their total size and may condense their nucleoids into a small portion of the cell (25). Greater plasticity in protein content explains why we observed more variability in protein-based volumes than in DNA-based estimates, although there were no significant relationships with environmental factors. Zubkov et al. (47) compared Hoechst DNA-stained cells with SYPRO Red protein-stained cells using flow cytometry and found that protein biomass varies with growth conditions for cells with similar DNA content. Our observations of low variation in DAPI-based volumes agree with this consistency in DNA content, which may be explained by the presence of cells growing at different rates in a complex microbial community responding to different environmental pressures. We observed no relationship of mean cell size with mean community growth rates, suggesting that factors other than growth rate alone control the cell volume in natural microbial communities.

We observed larger cells at higher latitudes, even with DNA staining, but especially using the protein stain, where cell volumes differed by 32% between high and low latitudes. The most obvious difference between the high- and low-latitude sites is temperature. The cell volumes determined using acridine orange staining of four facultatively psychrophilic strains grown by Wiebe et al. (46) were largest at the lowest temperatures at a given substrate concentration. The mechanism is unknown but was suggested to be related to generation time, i.e., cells were largest when growing slowly at low temperatures (46). However, an increase in the cell volume with a slower growth rate is opposite to the classic model (40). A different mechanism must be applicable in these environmental conditions.

In addition to temperature and other bottom-up controls of the bacterial cell volume, the size distribution of natural assemblages is also affected by top-down factors of cell mortality. Perhaps the prevalence of large cells at high latitudes is a result of changes in grazing pressure or viral mortality, as has been proposed to explain the larger volumes of bacteria in sea ice than in the surrounding water (33). Viral mortality in the Chukchi Sea varies; however, the measured grazing and viral lysis in Arctic waters leaves much cell mortality unexplained (42, 43). Either changes in rates of mortality or differences in the functional response could alter the size structure of bacterial communities. The observed variation in size suggests that the pressures or responses differ between high- and low-latitude environments.

We hypothesized that differences in size among latitudes may be explained by shifts in community structure, which are known to occur among aquatic regimes (6, 15, 37). The groups targeted by our FISH probes are phylogenetically diverse but have been shown to have distinct biogeographical patterns (22). There were no differences in the abundance of the SF group among the latitudes, but both *Alpha-* and *Gammaproteobacteria* were more abundant in the high-latitude samples. In addition, in the Arctic and Antarctica, *Gammaproteobacteria* and the SF group cells were on average significantly larger than their counterparts in the Delaware Bay. The high numbers of large *Gammaproteobacteria* in high-latitude waters may drive the changes observed in the mean biovolume of the total bacterial community.

The group-level probes used here were designed based on a smaller database of bacterial sequences than is available now and as such may miss some bacteria (2). However, the probes appear to match to a sufficiently large fraction of the targeted groups for the purpose of the present study. The probes GAM42a and ALF968 cover 76 to 80% of *Gamma-* and *Alphaproteobacteria*, respectively, with few mismatches (2). CF319a matches 90% of both *Flavobacteria* and *Sphingobacteria* (including *Cytophaga*) (2). Better group-level probes that

capture the true diversity of the groups, without being compromised by mismatches, have not yet been developed (2). However, problems with the probes do not affect our general conclusion that variation in bacterial biovolume is related to the abundance and sizes of cells in different bacterial groups.

The broad phylogenetic groups examined here can be further divided into subgroups. We found no difference in SAR11 abundance among regions, indicating that another alphaproteobacterial subgroup contributed to the higher total alphaproteobacterial abundance in the higher latitudes. The mean size of SAR11 cells was the same as the mean size of alphaproteobacterial cells in Antarctic waters, but SAR11 cells were larger in the Delaware Bay and Arctic waters. Similarly, using DAPI, Kirchman et al. (23) found that the biovolumes of the DE cluster 2 SF subgroup were greater than the biovolumes of total SF bacteria in both the Delaware Bay and the Arctic. Variation in the average cell size of a bacterial group may be due to variation in the cell size of its subgroups and the relative abundance of those subgroups. The use of a single biovolume for a microbial group can hide ecologically relevant variation within that group.

Bacteria of the SAR11 subgroup of *Alphaproteobacteria* are ubiquitous in marine environments and are hypothesized to have a compressed genome and small cell size (39). In contrast to expectations (39), SAR11 cells were not always smaller than the mean volume of bacteria in our samples, and with all samples pooled together the SAR11 cells were actually slightly larger than the average bacterium. Malmstrom et al. (28) obtained similar results using DAPI and found that members of the SAR11 clade in the North Atlantic and Sargasso Sea were at least as large as other bacteria. The small size of cultured SAR11 strains may have been influenced by the fixation and transmission electron microscopy preparation, which can cause cell shrinkage (39). In addition, SAR11 is a very diverse clade (13), with some members perhaps smaller than others. A better understanding of SAR11 biovolumes will improve our measure of their contributions to the biomass and size structure of the total assemblage.

Cell volume can be used along with conversion factors of carbon per unit biovolume to estimate total bacterial biomass (17, 26). If the size of the DAPI image is used to calculate both the volume and the conversion factor, then the inaccuracy in the resulting volume estimate could be canceled out for the average bacterium. However, the use of DAPI may lead to a perceived lack of variation in biovolume and biomass. While the abundance of bacterial cells usually does not vary greatly (12), cell size can vary substantially even using DAPI (9, 24), but especially using a protein-based biovolume measurement (the present study). Using DNA-stained images to calculate biomass masks the true variation in total biomass of microbial assemblages.

In the present study, we described seasonal and geographic variation in cell biovolumes of bacterial groups. Importantly, we observed large variations in cell-specific biovolumes based on protein staining that were not apparent with DNA staining. Bacterial groups differed both in abundance and in biovolume for different geographic regions. Some, such as the SF group, displayed biovolume characteristics that we expected while others, such as the SAR11 clade, did not. A simple calculation showed that mean cell volume does depend on the groups present. Larger mean cell volumes in higher latitudes may be explained by the presence of more *Gammaproteobacteria*. The combination of biovolume measurements such as SYPRO Ruby staining with group-specific identification furthers our understanding of variability in complex natural assemblages.

ACKNOWLEDGMENTS

This study was supported by NSF grants MCB 0453993 and OPP 0632233 to D.L.K. and OPP 0217282 to H.W.D.

G. Christman, V. Michelou, A. Sundberg, L. Yu, and the crew of the R/V *Sharp* helped with sample collection. We thank R. Dale for assistance with Python and MySQL and two anonymous reviewers for their comments.

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