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Relationships between Biovolume and Biomass of Naturally Derived Marine Bacterioplankton†

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Microscopic estimation of bacterial biomass requires determination of both biovolume and biovolume-to-biomass conversion. Both steps have uncertainty when applied to the very small bacteria typically found in natural seawater. In the present study, natural bacterioplankton assemblages were freshly collected, passed through 0.6-μm-pore-size Nuclepore filters to remove larger particulate materials, and diluted for growth in 0.22-μm-pore-size Millipore filter-sterilized unenriched seawater. This provided cells comparable in size and morphology to those in natural seawater, but the cultures were free of the interfering particulate detritus naturally present. Cells were collected on glass-fiber GF/F filters, and biovolumes were corrected for cells passing these filters; C and N were measured with a CHN analyzer. Our criteria for size measurement by epifluorescence photomicrography were confirmed with fluorescent microspheres of known diameters. Surprisingly, in six cultures with average per-cell biovolumes ranging from 0.036 to 0.073 μm^3, the average per-cell carbon biomass was relatively constant at 20 ± 0.8 fg of C (mean ± standard error of the mean). The biovolume-to-biomass conversion factor averaged 0.38 ± 0.05 g of C cm^{-3}, which is about three times higher than the value previously estimated from *Escherichia coli*, and decreased with increasing cell volume. The C:N ratio was 3.7 ± 0.2. We conclude that natural marine bacterial biomass and production may be higher than was previously thought and that variations in bacterial size may not reflect variations in biomass per cell.

Bacterial production measurements from many pelagic marine ecosystems indicate that a major fraction of photosynthetically fixed carbon is consumed by heterotrophic bacterioplankton (11–13, 16, 21, 24). Essential elements in most of those studies are conversion factors for estimating bacterial biomass from bacterial cell abundance and cell volume (biovolume). However, there are no generally accepted values for these conversion factors, nor are there methods for determination of cell size. Because of these uncertainties, bacterial "biomass" has often been presented by citing cell abundance only.

Several experimental methods have been published for the estimation of bacterial biovolume, biomass, or both (3–7, 9, 10, 19, 22, 23, 25, 27). In these studies cell size was usually measured by epifluorescence or electron microscopy or both, and the latter was proven to underestimate cell size if not corrected for cell shrinkage during sample preparation (10, 22). Epifluorescence microscopy has its own drawback, namely, "fuzzy" edges of cell images, but this is less severe than the drastic shrinkage in electron microscopy. A computerized image analysis method showed that the fuzziness can be corrected for by calibration with fluorescent microspheres of known size (4). Such controls for the size measurement criteria are critical to biovolume estimation, but have not generally been incorporated in the conversion factor calculation in previous studies. Biovolume-to-biomass conversion factors reported in the recent literature (4, 6, 7, 23) vary by up to fivefold from the value most commonly cited, 0.121 g of C cm^{-3} (27). Recently, Norland et al. (25) have found that smaller bacterial cells tend to have higher dry-matter-to-volume ratios, which may partly explain the wide variation of the reported conversion factors. However, these conversion factors were generally determined from cultures grown in supplemented media (6, 7) or by using nonmarine bacteria (4, 6, 7). Consequently, they may not be applicable to natural marine bacteria, which are usually much smaller than cultured forms and are probably taxonomically different.

Our study was designed to estimate the conversion factor and C:N ratio of natural marine bacteria from cultures grown in particle-free unenriched seawater. Novel aspects of this study include corrections for bacterial cells passing through glass-fiber GF/F filters and calibration of biovolume measurement with fluorescent microspheres of known diameters. Results from a range of bacterial cell sizes yielded the unexpected conclusion that the biomass-to-biovolume ratio varied significantly with cell size.

MATERIALS AND METHODS

Sampling and culture. Seawater for experiments (experiment I, January; experiment II, May; experiment III, July; 1985) was collected at a pebble beach at Crane Neck, Long Island (40°58′ N, 73°09′ W, salinity approximately 28‰). Containers for sample collection or for culturing were acid washed and rinsed with unfiltered or filtered (0.22-μm pore size, type GS; Millipore Corp., Bedford, Mass.) seawater. Culture medium was prepared by filtering freshly collected seawater through a presoaked and rinsed (with deionized water) GS filter in a polycarbonate filter unit (Millipore Corp.) to remove particulate material. This method was improved in experiment III by placing a glass-fiber GF/F filter (Whatman) underneath the GS filter to remove organic particles shed from the GS filter. The GS filter was replaced after every 450 to 500 ml of seawater was filtered. Vacuum pressure for all filtration was maintained at 20 to 24 kPa (15 to 18 cm Hg). Culture medium for each batch (culture bottle) of experiment III was dispensed from one pooled filtrate (4,500 ml), whereas those of the other two experiments were

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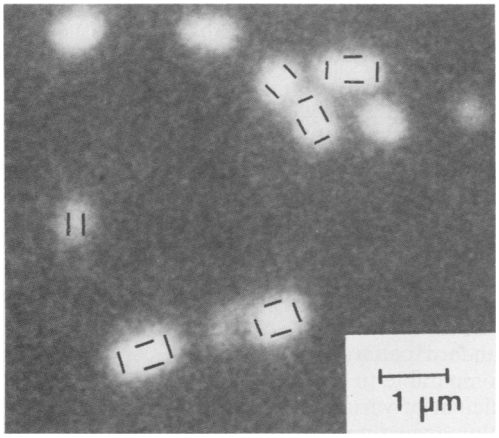

FIG. 1. Cell image by AO epifluorescence photomicrography and our size-measurement criteria (black bars).

prepared separately. Part of the collected seawater was filtered through a polycarbonate Nuclepore filter (0.6-μm pore size), and the filtrate was used to inoculate the medium. The volume ratio of inoculum to medium was 0.02 to 0.03, with final culture volumes of 900 to 1,000 ml. Cultures were grown at room temperature (ca. 20°C) in the dark. We monitored cell abundance with the acridine orange (AO) direct count method (18) by aseptically subsampling from each batch at 6- to 10-h intervals. Cells were harvested when log cell abundance leveled off or distinctively deviated from the log-linear increase (i.e., at the end of logarithmic growth phase). This was usually 48 to 72 h after the inoculation.

Cell collection and preparation of CHN samples. Cultures were filtered in a clean acid-washed and rinsed glass filter unit (for a 2.4-cm-diameter GF/F filter, experiment I) or stainless-steel filter unit (for a 1.3-cm-diameter GF/F filter, experiments II and III). Each culture batch was filtered onto a precombusted (450°C, >4 h) GF/F filter (sample GF/F). Batches were filtered separately except in experiment I, when two batches were mixed before filtration and the sample GF/F was cut in half before CHN analysis. Filters for CHN analysis were oven dried at 60°C for >4 h to prevent possible loss of elements due to decay. Samples were assayed on a Perkin-Elmer model 240B CHN analyzer.

To estimate the C and N contribution from organic particles shed from the Millipore filters, culture medium alone was filtered onto a precombusted GF/F filter. Volumes filtered were 1,700, 500, and 900 ml, respectively, in experiments I, II, and III (note that it was the second GF/F filtration for experiment III, since the medium had been prepared with the double filter). At each experiment, two blank precombusted GF/F (no water filtered) samples and two precombusted GF/F samples soaked with a known amount of 10 mM β-alanine were made. To check for carbon and nitrogen contributed by substances dissolved in seawater such as bicarbonate, two precombusted GF/F samples soaked with culture medium were prepared in experiment III.

Biovolume determination. Since some of the bacterial cells passed through the precombusted GF/F filters (see Results), final subsamples for epifluorescence microscope slides were made from the unfiltered culture and the GF/F filtrate, respectively, just before and after the cell collection. Subsamples were immediately fixed with buffered Formalin (final volume ratio, 5%), and 3 to 6 ml was filtered onto a prestained (Irgalan black) Nuclepore filter (0.2-μm pore size). We used the AO direct count method (18) and AO epifluorescence photomicrography to determine cell abundance and cell size. Photographs (Kodak Ektachrome ASA 400) were taken within at least 4 h after preparation of the microscope slide, except in experiment II, for which photographing was delayed ca. 2 weeks. An Olympus model BH microscope with a 100× SPlan objective, mercury lamp (100 W), and PM-10AD photomicrography system was used. Photographs were taken from four or five randomly selected fields of each microscope slide. Photographic images were projected onto a screen (final magnification, $\times 10^4$) and cell dimensions were recorded to an accuracy of 0.1 μm of the original scale. We included every small particle that looked like a bacterial cell (distinct, smooth edge), using the same criterion as when we enumerated cells by microscopy. The criterion we employed to measure the size was to strictly cut off the dim fluorescent "halo" (including film grain) around the cell image (Fig. 1). This criterion of subjectivity was tested by applying the same criterion to measuring fluorescent microspheres of known diameters (see below).

The cells were catagorized as rods when lengths of two perpendicular axes (the longest and the shortest) differed by ≥0.1 μm; otherwise they were classified as spheres. Since cells of "rod" shape are not always true rod forms with two spherical ends on each side of a cylinder, nor oblate spheroids with circular cross-sections, volumes of "rods" were obtained by averaging the two volumes calculated based on the two shapes, a rod and a spheroid. Biovolume estimation by this method needs a very large number of cells, partly because of the resolution of the photographs as well as because of visual problems in differentiating cell size to 0.1 μm. We measured 200 to 250 cells per photographic slide, or 800 to 1,000 cells per AO microscope slide. The size frequency distributions of cells in both culture and filtrate were measured. Total biovolume in culture or in filtrate was calculated by multiplying average per-cell volume, cell abundance, and volume filtered. Total biovolume retained by the sample GF/F was calculated by subtracting total biovolume in the filtrate from that in the culture.

Fluorescent microsphere measurement. Suspensions of green fluorescent microspheres (Covalent Technology Corp., Ann Arbor, Mich.; Polysciences, Inc., Warrington, Pa.) were sonicated (Bransonic 12, Branson) for >30 min and diluted with deionized water. The fluorescent spheres were collected onto a prestained (Irgalan black) 0.2-μm-pore-size Nuclepore filter. Spheres of three different diameters (0.21, 0.63, and 0.7 μm; size data supplied by the manufacturers) were photographed under the same camera setting conditions used for bacteria to verify our measuring criteria as well as to calibrate our results. We also tested the effect of exposure variations on size measurement of the photographic images. The brightly green-fluorescent 0.7-μm spheres (Covalent Technology Corp.) were photographed under illumination conditions utilizing a UV exciter filter (transmits 300 to 400 nm) plus a O-515 barrier filter (transmits >500 nm) to reduce the fluorescence to levels more like those of AO-stained bacterial cells. The moderately bright 0.63- and 0.21-μm green-fluorescent spheres (Polysciences, Inc.) were photographed with illumination utilizing the same filters (blue exciter [transmits 360 to 500 nm] and O-515 barrier) as AO bacteria slides.

We selected photographic slides of the spheres which looked similar to those of the bacterial cells with regard to background darkness, strength of fluorescence, general

TABLE 1. CHN analysis results

Expt	Batch	Sample (μg)		Shedding control (μg)		Filter blank[a] (μg)	
		C	N	C	N	C	N
Expt. I[b] (Jan.)	A	22.12	3.51	8.30	−0.13	1.63	0.20
	B	26.12	4.59	8.24	0.81	2.19	−0.47
Expt. II (May)	A	35.45	14.71	9.90	4.61	1.65	0.39
	B	31.49	7.25	4.54	1.05		
Expt III (July)	A	54.69	15.94				
	B	59.49	14.32	6.18[c]	1.08[c]	2.81	0.76
	C	60.43	14.32				

[a] Mean of two replicates
[b] A and B of experiment I are from the same filter cut in half (see Materials and Methods).
[c] From pooled culture medium of A, B, and C. Mean of two replicates.

color appearance, and camera settings. A total of 400 to 1,000 spheres were measured from each size class.

Biomass determination. To estimate the bacterial biomass retained on the GF/F filters, we first calculated blank-corrected alanine-standard calibration factors (F_{ala}) for carbon and nitrogen. *P* (the C or N contributed by particles shed from GS filters and retained on the GF/F filters) was estimated based on the assumption that the shed particles in the subsample were of the same concentration as those in the whole culture (see below for discussion). Finally, net bacterial biomass (*B*) in the sample GF/F was estimated as follows:

$$B = (M - b - P) \cdot F_{ala} \quad (1)$$

where *B* is the biomass in micrograms of C or of N; *M* is the particulate C or N on the sample GF/F; and *b* is the filter blank value.

RESULTS

CHN results and biomass. CHN results are presented in Table 1. The average CHN reading of the "dissolved C and N" control filter, 3.96 μg of C or 1.15 μg of N, was slightly higher than the filter blank reading from experiment III. These values would account for <5% of the particulate carbon and nitrogen measured on the sample GF/F filters. Both carbon and nitrogen levels of the filter blank were low compared to those of the samples, varying from 3 to 8% of the sample level. Particulate carbon and nitrogen values from the second GF/F filtration of culture medium in experiment III caught shed particles amounting to about half of those of the first filtration, inferred from other shedding controls. The third GF/F filtration tested in experiment III caught an amount not significantly different from the blanks (2.30 μg of C and 0.67 μg of N; mean of two replicates).

The alanine-standard samples indicated that variation of ca. 2 μg of C or ca. 1 μg of N could be attributed to instrument error, since replicates of the standard sample showed variation of that much. Therefore, another method (method 2) was employed to incorporate the control/blank samples into estimating bacterial biomass from the sample CHN readings. Method 2 is somewhat conservative compared to method 1 described earlier with equation 1, in the sense that not all sample readings were corrected with the blanks. This was compensated for by recalculating F_{ala} with the blank-uncorrected standard readings. Thus:

$$B = (M - P) \cdot F_{ala} \quad (2)$$

where all symbols are the same as defined before. Though the biomasses estimated with the two methods are slightly different from each other, each method generated the alanine-standard calibration factors for carbon and nitrogen fairly comparable to each other within an experiment (mean of coefficient of variation, 3%).

TABLE 2. Fluorescent-microsphere measurement

Diameter[a] (μm)	No. of spheres measured	Mean (SD) of diameter	% Measured[b]
0.21	1,034	0.192 (0.051)	91
0.63	714	0.580 (0.052)	92
0.70	374	0.643 (0.078)	92

[a] Nominal diameter; data supplied by the manufacturers.
[b] (Measured diameter/actual diameter) × 100 (%).

Biovolume measurement. The criteria we used for size measurement underestimated slightly the real linear dimensions of fluorescent microspheres, consistently for all three size classes, by a factor of ca. 8% (Table 2). Thus biovolumes were corrected from original estimates with the factor 1.28 (cube of 100/92). It is significant that the size of the sphere image was closely related to the background darkness of the photographic slide and the brightness of the fluorescent spheres, which were in turn related to the exposure time and the darkness of the microscopic field. Longer exposure time or brighter fluorescence made cells appear larger; this tendency was reversed when the fluorescence faded out due to excessively long exposure time or too-bright excitation light (data not shown). This must be considered in size calibration.

Almost half (average, 47%) of the bacteria passed through the GF/F filters (Table 3), and the filters retained cells of all size classes, slightly preferring the larger ones. Thus, total biovolume collected on the filters had to be calculated from bacterial abundance and per-cell biovolume measurements of both original cultures and filtrates. Total biovolume in either the culture or the filtrate was calculated as abundance times average per-cell biovolume and culture volume filtered, and total biovolume caught on the filter was calculated as the difference between the total biovolumes of the culture and filtrate. Finally, the average per-cell biovolume of the cells retained by the filter was calculated by dividing the total biovolume by the total number of retained cells (Table 3). The average cellular biovolumes ranged from 0.036 to 0.073 μm^3, corresponding to average equivalent spherical diameters ranging from ca. 0.4 to ca. 0.5 μm.

Relationships between biovolume, biomass, and cell abundance. The average biovolume-to-biomass conversion factor from the two methods was 0.38 ± 0.05 g of C cm^{-3} (Table 4). Our results suggested that smaller cells tend to have more

TABLE 3. Biovolume measurement

Expt	Cell abundance (10^6 ml^{-1}) of:		Avg per-cell volume (μm^3) of:			Vol (ml) filtered[b]
	Culture	Filtrate	Culture	Filtrate	Sample[a]	
I	1.56	0.68	0.055	0.044	0.063	1,800
IIA	2.25	1.01	0.037	0.029	0.043	730
IIB	2.62	0.91	0.032	0.023	0.036	715
IIIA	7.02	3.62	0.059	0.046	0.073	808
IIIB	7.03	4.04	0.053	0.052	0.054	830
IIIC	7.47	3.83	0.062	0.056	0.067	827

[a] Average per-cell volume of cells retained by a sample GF/F, measured by biovolume difference (see Results).
[b] Volume of culture filtered onto sample GF/F.

TABLE 4. Conversion factors and C:N ratios

Method[a]	Expt	Biovolume-to-biomass conversion factor (g cm^{-3})		Biomass per cell (fg)		C:N ratio
		C	N	C	N	
1	I	0.33	0.08	21.09	5.02	4.20
	IIA	0.57	0.22	24.83	9.48	2.62
	IIB	0.60	0.14	21.72	5.09	4.27
	IIIA	0.26	0.08	19.11	5.61	3.41
	IIIB	0.43	0.10	23.24	5.53	4.20
	IIIC	0.29	0.07	19.50	4.56	4.27
2	I	0.29	0.08	18.41	5.36	3.43
	IIA	0.49	0.20	21.09	8.55	2.47
	IIB	0.51	0.13	18.59	4.56	4.07
	IIIA	0.21	0.07	15.40	4.82	3.19
	IIIB	0.35	0.09	18.71	4.76	3.93
	IIIC	0.23	0.06	15.70	3.92	4.00
Mean ± SEM		0.38 ± 0.05	0.11 ± 0.02	19.8 ± 0.8	5.6 ± 0.6	3.7 ± 0.2

[a] See the text for the two methods employed to incorporate the controls into conversion factor estimation.

carbon and nitrogen per biovolume than larger cells (Table 4; Fig. 2). This trend is more clearly demonstrated by the fairly constant cell-number-to-biomass conversion factor (Fig. 3), which is unrelated to biovolume estimation. This result was contrary to expectation, as one would expect biovolume and biomass to be positively correlated. The data in Fig. 3A even suggest a decrease of cellular carbon biomass with increase of average per-cell biovolume, yielding a correlation coefficient of −0.79 (method 2; significance, $0.05 > P > 0.01$) or −0.75 (method 1, $P = 0.05$). We found a C:N ratio of marine bacteria of 3.7, the same as that found by Seiderer et al. (26) with isolates of cultured marine bacteria and higher than the 3.4 ratio found by Heldal et al. (17) with *Escherichia coli*. Our average C:N ratio increases slightly, to 3.9, if we exclude the N measurement from experiment IIA, which appears unusually high compared to the other samples (Fig. 2 and 3) and may have been contaminated.

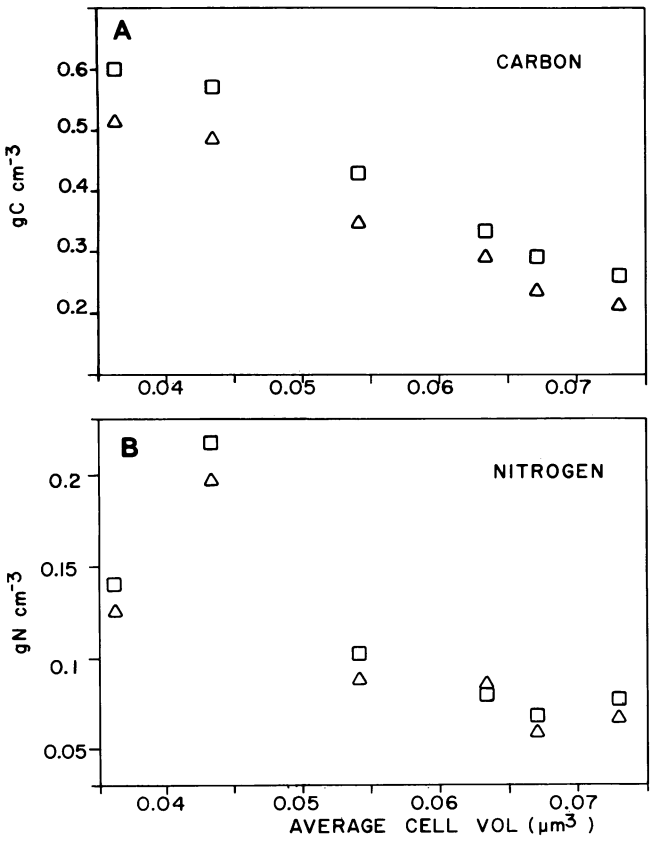

FIG. 2. Biovolume-to-biomass conversion factor. (A) Carbon, 0.38 ± 0.05 g of C cm^{-3} (mean ± standard error of the mean); (B) nitrogen, 0.11 ± 0.02 g of N cm^{-3}. Symbols indicate different methods, 1 (□) and 2 (△), for bacterial biomass estimation from CHN results (see the text).

DISCUSSION

An important consequence of our results is that, in general agreement with the conclusions of Bjørnsen (4), Bratbak (6), and Bratbak and Dundas (7), bacterial biomass may have been underestimated in previous reports. The average cell-number-to-biomass conversion factor we found is two to three times larger than the values used in several previous studies (13, 28, 29). The biovolume-to-biomass conversion factor we found is also larger, by ca. three times, than the commonly cited value, 0.121 g of C cm^{-3}. The use of a constant biovolume-to-biomass conversion factor for field data regardless of bacterial size may cause incorrect estimation of bacterial biomass. Our observation of a higher biovolume-to-biomass conversion factor for smaller cells may imply that a conversion factor on a cell-number basis, rather than a biovolume basis, would be more practical and accurate for biomass estimation, as long as the cells are in the size range of those reported here. Why should the biomass per unit volume change? Heldal et al. (17) reported that *E. coli* cells of late exponential growth phase have a dry-matter content about twice that of cells of early stationary phase, while cell sizes of the two phases are almost the same. Norland et al. (25) also reported an inverse relationship between cellular dry matter content-to-volume ratio and cell volume. Such a difference in dry matter (or water) content may explain the trend we found.

Although our values were obtained from cultured cells, they are probably not much different from those of naturally growing cells, because our samples were freshly collected and grown on the dissolved substrate present in unenriched natural seawater. The size distribution of bacteria in our

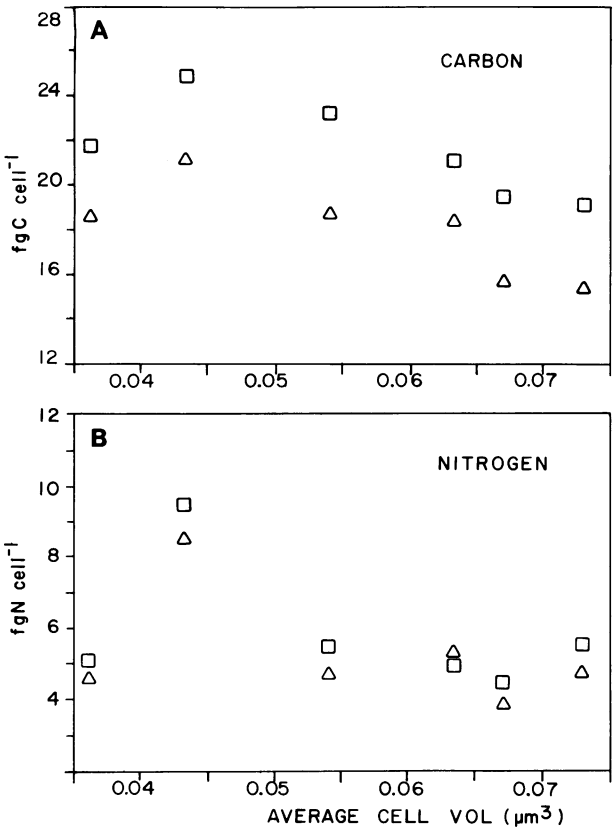

FIG. 3. Cell-number-to-biomass conversion factor (per-cell biomass). (A) Carbon, 19.8 ± 0.8 fg of C cell^{-1} (mean ± standard error of the mean); (B) nitrogen, 5.6 ± 0.6 fg of N cell^{-1}. Symbols indicate different methods, 1 (□) and 2 (△), for bacterial biomass estimation from CHN results (see the text).

cultures was similar to that in many marine environments, but naturally growing bacteria may sometimes have cell sizes outside the range of our cultured cells. For example, we have measured the size of natural marine bacteria collected at our sampling site in February, 1986, and average biovolume per cell was ca. 0.02 μm^3. Extrapolation to cells much larger or smaller than those reported here probably should be avoided.

Statistical errors may have arisen in collecting the final subsamples for the microscope slides and counting bacterial cells (20). However, our sample size (800 to 1,000 cells per one population) was large enough to minimize such error and is considerably larger than those reported in the literature. Two other possible artifacts could arise, first, from the criteria of the size-measuring method and its accuracy, and second, from the degree of cell shrinkage due to the preservative. Though we concluded that our criteria measured ca. 92% of the real linear dimensions, this figure may not apply to all conditions. Since we tested with only three discrete size classes, our calibration factor might not be applicable to the whole range of size readings, 0.1 to 2 μm. If the volume-measurement calibration factor considerably deviates from the factor 1.28 beyond the size range we tested, our results should be corrected correspondingly. However, this would have little effect on the overall volume estimation, because most of the size readings fell in the range of 0.2 to 0.8 μm and the few readings larger than 1 μm were exclusively from the long axes of very elongated rods. Cells with both axes longer than 0.9 μm were not found in our cultures. Thus the error would not be cubically exaggerated.

We checked published data concerning mean volume of marine bacteria determined by epifluorescence microscopy, and found considerable variability. It is possible that this variability is partly due to different size-measurement criteria. Appropriate care should be taken to verify one's criteria.

Epifluorescence microscopy is thought to be the method least affecting the cell size (6, 10, 22), although heterotrophic bacteria generally shrink less during sample preparation for electron microscopy than do phototrophic ones (22). Even for epifluorescence microscopy, shrinkage is still possible when cells are fixed with preservatives. The degree of the shrinkage may depend on concentration as well as type of preservative. It is also currently unclear whether the shrinkage is constant or varies with cell size.

An additional contribution to error was that the GS filter shed particles containing C (mostly) and N. The amount of shed particles varied from filter to filter, and the shed particles were not totally filtrable by the precombusted GF/F filters. However, corrections for this artifact minimized its effect in the final result. When we reduced the shed-particle release into culture medium in experiment III, we obtained the same results as when we corrected the results of the other two experiments with their own controls (Fig. 3).

In this study extracellular particulate organic materials (3) were regarded as part of the bacterial cells. The distinction between the intracellular and the extracellular may not be straightforward in an ecological food-web context. Interestingly, while per-cell carbon biomass was rather constant over a twofold size range, neither constancy nor correlation with cell size could be found in per-cell nitrogen biomass (Fig. 3). Variation of cellular N content, unlike the rather stable C content, may be controlled by factors other than cell size. It may also possibly be related to stress imposed during filtration (14).

There are several reasons why our results may be more appropriate than previously published ones for the estimation of natural bacterial biomass. Our culture method provided a natural, unenriched, particle-free culture medium (1) and would have reduced possible interference from nonbacterial organic materials because of heterotrophic (mostly bacterial) growth (2). Our method screened out larger (>0.6 μm) living or nonliving particles and extremely diluted those which passed through 0.6-μm pores. Interference by detritus would of course overestimate cellular C and N content as well as C:N ratio, because detritus probably contains more carbon than nitrogen compared with bacterial cells. The possible presence of unfiltrable small (<0.6 μm) bacterivores has been suggested recently (15), but any grazers would have had difficulty in grazing on a diluted bacterial suspension, and no evidence of proliferation of non-bacterial organisms could be found throughout the culture period. In addition to the culture method, the biovolume correction we made for the cells passing through the GF/F filters (see Table 3) and the verification of size-measurement criteria are also critical to this kind of study. However, as far as we know no previous study has included all of these considerations. Our conversion factor would probably be the most suitable one for marine bacterioplankton and may be applicable to other studies if cells are fixed with Formalin (5%, vol/vol) and measured appropriately.

In conclusion, our results yielded a rather constant per-cell carbon biomass. Previously, small bacterioplankton

have been regarded as contributing little to biomass in spite of their high abundance. Our results suggest that this may not be so. Concerning the activity, not biomass, Azam and Hodson (2) and Fuhrman (10) concluded that small bacteria have higher per-biovolume activity (i.e., activity is proportional to cell number, not to cell size). A ramification of the higher biomass conversion factor we reported here is that organic material flow through heterotrophic bacterioplankton (8, 13, 28) should be reviewed. Moreover, the low C:N ratio of bacteria and their high nitrogen content compared to other plankton profoundly emphasize their role in nutrient dynamics in the marine ecosystem, where usually N is limiting.

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