APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1987, p. 274–277
0099-2240/87/020274-04$02.00/0

Vol. 53, No. 2

Particle Counter Determination of Bacterial Biomass in Seawater

KAZUHIRO KOGURE* AND ISAO KOIKE

Ocean Research Institute, University of Tokyo, Minamidai, Nakano, Tokyo 164, Japan

Received 21 August 1986/Accepted 31 October 1986

The applicability of the Elzone particle counter to the determination of marine bacterial biomass was investigated. The biomass of bacterial pure cultures and a mixed natural population were followed by using the particle counter, a CHN analyzer, and an ATP analyzer. The particle counter showed the precise size distribution of number and volume of submicron-size particles in seawater. For the pure cultured bacterial strains, the conversion factor from volume to carbon is 0.209 mg of C per mm^3, and for natural bacterial cells of >0.6 μm in diameter, it is 0.184 mg of C per mm^3. It is recommended that 0.2 be used as the conversion factor for both pure cultured marine bacterial cells and natural bacteria from coastal and near-shore marine environments.

Bacterioplankton play a central role in degradation and turnover of organic matter in aquatic ecosystems. To clarify their activities or contributions to the ecosystem, an estimation of their biomass is prerequisite. Among the techniques used for biomass determination, the direct counting method with epifluorescent microscopy is most widely used (9, 18). This technique also enables estimation of the size and volume distribution of bacterioplankton in seawater, although it is very tedious and time-consuming (6). Recently, it has been used in combination with an image analyzer by several workers (2, 5, 10, 17). However, this approach depends critically on the quality of the primary image. A slight change in focus results in a serious error in volume estimation. For ecological arguments, it is often more valuable to estimate the bacterial biomass as carbon. Bacterial volumes have been converted to carbon by using an appropriate conversion factor and, to our knowledge, the value 0.121 mg of C per mm^3 (19) has been most widely used by aquatic microbiologists. However, the validity of this value still remains to be confirmed. Considering intercellular water, Bratbak and Dundas (4) suggested that 0.22 mg of C per mm^3 should be used for living bacterial cells. Bratbak (3) further proposed using 0.56 mg of C per mm^3 for fixed bacterial samples due to the shrinkage of cells.

The Coulter counter has often been used to obtain the size distribution of phyto- or zooplankton and detrital particles in the sea (16). However, bacterial cells in natural seawater are usually below the detection level of this instrument. Although Montesinos et al. (12) attempted to measure natural bacterioplankton, the method was limited to only large bacterial groups such as *Chromatium* spp. Paul and Jeffrey (14) used the Coulter N_4 submicron particle analyzer in conjunction with a laser. Although the instrument can estimate the size distribution of bacterial cells in natural seawater, it is not always suitable for quantitative and routine observations of natural samples. On the other hand, the Elzone particle counter, similar in principle to the Coulter counter, has not drawn the attention of oceanographic investigators. Recently, we found that this instrument is applicable to bacterial biomass determinations. The attached microcomputer calculates the bacterial number and size distribution instantaneously. Calibration and operation are much simpler than with the Coulter counter.

* Corresponding author.

The purpose of the present investigation was, first, to check the applicability of the Elzone particle counter to the determination of marine bacterial biomass and, second, to obtain the appropriate conversion factor from bacterial biovolume to carbon contents. Marine bacterial pure cultures and a mixed natural population were used for the investigation.

MATERIALS AND METHODS

Bacterial culture. Three bacterial strains were used for pure culture study in the laboratory. Strains 172 and 192 (tentatively identified as *Alcaligenes* sp. and *Pseudomonas* sp., respectively) isolated from the Indian Ocean were generous gifts of U. Simidu. Strain A-5 (tentatively identified as *Pseudomonas* sp.) was isolated by S. Achutankutty from Aburatsubo Inlet, Kanagawa, Japan. These bacteria were incubated in synthetic seawater medium, which contains the following: NaCl, 30 g; $MgSO_4 \cdot 7H_2O$, 2 g; KCl, 0.8 g; KBr, 0.1 g; $SrCl_2 \cdot 6H_2O$, 26 mg; H_3BO_3, 20 mg; Tris hydrochloride (pH 7.5 to 7.6), 1 g; yeast extract (Difco Laboratories), 0.5 g; and distilled water, 1 liter. The medium was filtered through a Nuclepore filter (pore size, 0.2 μm) just before autoclaving. The incubation was carried out in 2 liters of culture medium in 3-liter Erlenmeyer flask at 20°C.

Natural bacterial population. Surface seawater was collected at the pier in Otsuchi Bay, Iwate Prefecture, Japan, on 11 October (experiment 1) and 17 October (experiment 2) 1985. The sample seawater was immediately brought back to Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo. Subsequent treatment was initiated within 15 min after sampling. Another two seawater samples were obtained at the pier (experiment 3) and from the sea grass shore (experiment 4) in Aburatsubo Inlet on 29 April 1986. Samples were brought back to the Ocean Research Institute, where treatment and incubation were performed. The seawater samples were filtered through a Nuclepore filter (pore size, 1.0 μm) to remove most of the phyto- or zooplankton. At most, 750 ml was filtered per one filter. Care was taken not to exceed 150 mm Hg (ca. 20 kPa) for the suction. Incubation was performed at ambient temperature (18 and 16°C for Otsuchi and Aburatsubo samples, respectively) in glass bottles. Subsamples were taken at 4-h intervals.

Bacterial growth. Bacterial growth was followed by three methods. The increase in particle numbers and size was calculated by using the Elzone particle counter, that of particulate carbon and nitrogen was calculated with the

TABLE 1. Cellular volume and carbon and nitrogen contents of pure cultured bacterial strains

Bacteria	Time (h)	Particle counter		CHN analyzer			ATP (× 250) (mg of C per liter)[a]	B/A (mg of C per mm³)[b]
		No. of cells (10^9/liter)	Cell vol (A) (mm^3/liter)	mg of C per liter (B)	mg of N per liter	C/N		
Strain 172	0	2.17	4.17	0.984	0.273	3.60	2.66	0.236
	1.5	6.07	11.2	2.37	0.720	3.29	5.84	0.212
	2.5	11.6	21.8	5.46	1.85	2.96	13.4	0.250
	3.5	24.4	45.1	10.1	3.37	3.01	23.3	0.224
	4.5	31.3	63.3	14.7	5.21	2.82	24.9	0.232
	5.5	46.8	76.1	21.5	7.16	3.00	39.0	0.282
Strain 192	0	1.10	1.48	0.283	0.0477	5.90	0.254	0.191
	1.5	4.79	6.01	1.20	0.275	4.37	0.811	0.200
	2.5	14.2	17.3	3.65	0.877	4.16	3.24	0.211
	3.5	57.9	50.5	7.95	2.82	2.82	4.00	0.157
	4.2	82.5	65.6	11.9	4.02	2.96	11.1	0.181
Strain A5	0	4.49	3.80	0.590	0.153	3.86	0.325	0.155
	1.5	7.36	7.63	1.26	0.356	3.54	0.975	0.165
	2.5	13.4	12.8	2.24	0.721	3.11	2.76	0.175
	3.5	21.4	16.9	4.93	1.49	3.32	2.28	0.292
	4.5	40.5	21.2	4.68	1.34	3.49	2.50	0.221
	5.5	53.4	27.7	4.84	1.53	3.16	2.59	0.175

[a] From Hamilton and Holm-Hansen (8).
[b] Mean, 0.209 ± 0.041.

CHN analyzer, and that of carbon was calculated by ATP analyzer. As for natural samples, however, ATP determination was omitted. The Elzone particle counter 80XY (Particle Data, Inc.) was equipped with a 12-μm orifice tube and 10-μl volumetric tube. The current and gain were set at 4.5 and 1.0, respectively. The calibration was carried out with two kinds of latex spheres (1.09 and 2.02 μm in diameter). The blank value was obtained with filtered 3.5% NaCl solution (Nuclepore filter; pore size, 0.2 μm), which was prepared with freshly distilled water. This solution was also used for the dilution of samples when required. The conductivity of the sample was normalized for each one. Immediately after sampling, the seawater samples were fixed with 0.2 μm of filtered Formalin (final concentration, 2%). The pure culture samples were then analyzed within a few hours. The natural seawater samples were stored for, at most, 1 week until analyzed. The preliminary observation clarified that there was no appreciable change in volume of pure cultured bacteria after fixation. For natural samples, the fixation caused a decrease in volume of 6.3 ± 3.5% (average of five samples). The 1-week storage did not affect biomass determination. A CHN analyzer (Yanagimoto MT-2) was used for analysis of bacterial carbon and nitrogen contents. An appropriate amount of sample seawater was filtered through double-layered Whatman GF/F glass-fiber filters which had been precombusted at 400°C for several hours. The bottom filter was treated as a blank, and the value was subtracted from the top (15). Because the GF/F filter collected the particles of >0.6 μm in diameter (data not shown), the present investigation only concerns such particles. ATP was determined by the method of Holm-Hansen and Booth (10). A membrane filter (type HA, 24 mm; Millipore Corp.) was used to collect bacterial cells.

RESULTS

Table 1 shows the results of the pure culture experiments. All parameters indicate that each strain increased exponentially during incubation. During the mid-exponential growth phase, the average cell diameters (assumed to be spheres) were, 1.47, 1.17, and 1.83 μm for strains 172, 192, and A5, respectively. Therefore, it was assumed that virtually all cells were retained on the GF/F filter. The carbon content obtained by particulate carbon measurements and the ATP method differed by a factor of, at most, 2.5. Since the carbon numbers from the ATP method are indirect, we used carbon numbers from the CHN analyzer for calculation of bacterial carbon/volume ratios. An average value of 0.209 mg of C per mm^3 was obtained (Table 1). The average carbon/volume ratios obtained from three strains agreed well and were 0.205 ± 0.093 for strain 172, 0.188 ± 0.021 for strain 192, and 0.197 ± 0.052 for strain A5.

Figure 1 shows a typical example of particle size-volume distributions in the incubated seawater (experiment 4, 20 h) obtained with the Elzone particle counter. In most cases, the increase in total volume is mainly due to particles of >0.6 μm in diameter (manuscript in preparation). Table 2 shows the results of natural seawater incubation. Increases in numbers and volumes of particles of >0.6 μm are shown. Average volumes of particles for each time course are also reproduced in Table 1. Both particle volumes and carbon contents increased with time. It is possible, however, that a considerable part of the particles in natural seawater are not bacterial cells but are nonliving particles. We therefore calculated the carbon/volume ratios of natural bacterial cells by using the increased portion of measured components during the incubation. Nonliving particles were rather stable as shown by using Formalin-treated seawater. The average ratio of 20 samples was 0.184 ± 0.06, close to the value of 0.209 obtained for pure cultures (Table 1).

DISCUSSION

The Elzone particle counter proved to be very useful for the measurement of bacterial biomass and size distribution. With the conventional epifluorescent microscopic technique,

TABLE 2. Increase in number and volume and carbon and nitrogen contents of particles in natural seawater during the course of incubation[a]

Expt	Time (h)	Increase in particles				C/N	B/A (mg of C per mm^3)[b]	Avg vol (μm^3)
		No. (10^5/ml)	Vol (mm^3/liter) (A)	Carbon (μg/liter) (B)	Nitrogen (μg/liter)			
1	0	0 (4.97)	0 (0.113)	0 (42.0)	0 (3.34)	(12.6)	(0.372)	(0.226)
	4	4.30	0.122	19.5	2.78	7.01	0.160	0.284
	8	16.2	0.494	64.0	8.16	7.84	0.130	0.305
	12	29.2	1.08	149	14.4	10.3	0.138	0.370
	16	46.5	1.61	214	17.6	12.2	0.133	0.346
	20	56.8	1.89	255	25.7	9.92	0.135	0.333
2	0	0 (4.37)	0 (0.0827)	0 (21.1)	0 (4.09)	(5.16)	(0.255)	(0.189)
	4	1.24	0.0333	6.60	1.54	4.29	0.198	0.269
	8	1.95	0.0423	8.30	1.59	5.22	0.196	0.217
	12	11.6	0.243	20.3	4.31	4.71	0.083	0.209
	16	20.1	0.445	57.1	10.5	5.43	0.128	0.221
	20	23.7	0.572	119	14.0	8.49	0.208	0.241
3	0	0 (30.7)	0 (0.650)	0 (146)			(0.225)	(0.212)
	4	25.2	0.420	79			0.188	0.167
	8	78.3	1.35	282			0.209	0.172
	12	107	2.29	650			0.284	0.213
	16	134	3.19	904			0.283	0.238
	20	130	3.68	1,300			0.354	0.282
4	0	0 (38.9)	0 (0.865)	0 (111)	0 (23.0)	(4.83)	(0.128)	(0.222)
	4	50.6	2.70	452	132	3.42	0.168	0.533
	8	273	9.34	1,320	368	3.58	0.141	0.342
	12	256	10.1	1,810	448	4.04	0.178	0.396
	16	319	12.1	2,210	538	4.11	0.182	0.380
	20	439	12.5	2,890	623	4.64	0.230	0.285

[a] Numbers in parentheses represent the initial value of measured components. Number and volume of particles are the fractions bigger than >0.6 μm.
[b] Mean, 0.184 ± 0.064.

these measurements were very tedious and the results were more or less subjective with each worker. The particle counter gave reproducible and consistent results regardless of the operator. Because this counter has 128 channels, it is possible to follow the details of change in bacterial size distribution throughout the incubation. This information can be especially important in clarifying the behavior of bacterial cells in natural seawater (1). Our results clearly indicated that the increase in bacterial number and volume were not parallel. To obtain the production rate of a natural bacterial population, it is necessary to follow the change in bacterial volume. This instrument can be a useful tool for the measurement of bacterial growth rate in the sea.

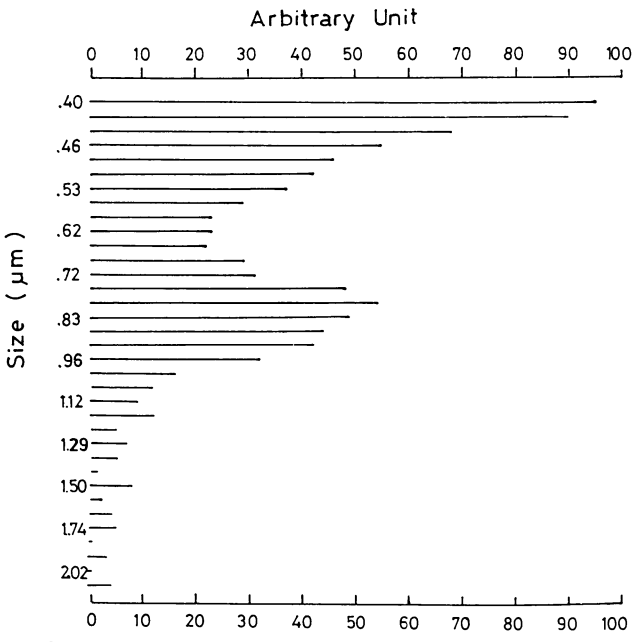

FIG. 1. Particle size-volume distribution (experiment 4) after 20 h of incubation.

Bratbak (3) stated that fixation caused the shrinkage of cells, which results in an underestimation of biovolume. As was stated in Materials and Methods, we checked the effect of fixation for both bacterial pure culture and natural bacterial populations. From our observations, the shrinkage of cells due to fixation should not cause as serious an effect as was stated by Bratbak (3).

The carbon/volume ratios (0.08 to 0.35) for natural samples showed much more variation than those (0.16 to 0.29) for pure cultures. The large variation with natural samples may reflect the physiological conditions of natural bacterial populations. But the average ratio (0.184) for natural populations agreed well with those for (0.209) pure cultures. Taking the average, we recommend using 0.2 as the conversion factor for both natural and pure cultured marine bacteria. Our factor is nearly double the value of 0.121 (19) widely used by aquatic microbiologists. The validity of the value recommended by Watson et al. (19), however, has not yet been seriously checked. Since the conversion factor is used for the calculation of bacterial production rate from thymidine uptake rate (7), a change in the factor is of considerable importance in quantifying the bacterial contribution to aquatic ecosystems. Our carbon/volume ratios in the natural

bacterial population were obtained by using fractions of >0.6 μm. Except for a few extremes, the average cell volume ranged from 0.2 to 0.4 μm^3, which was several times larger than the average size of open-sea bacteria (19). The marine bacterial population, however, increases its size in the nutrient-rich coastal and estuary waters (D. J. W. Moriarty, personal communication). Our conversion factor can apply at least to the microbial population of coastal and near-shore environments.

Compared with the direct count method that uses epifluorescent microscopy, the number of particles counted by the Elzone particle counter is much higher, usually by an order of magnitude (K. Kogure, unpublished data). Most are <0.45 μm in diameter. It seems that these particles, at least in part, increased rather slowly with time during the incubation. Since they passed through GF/F glass-fiber filter, we could not quantitatively estimate their carbon content. It is possible that these particles are not stained with the fluorochromes commonly used for marine bacteria. It is not certain whether these particles includes any viruses, as was suggested by Sieracki et al. (17). Investigations to clarify the nature and behavior of these particles are now being undertaken.

In conclusion, the Elzone particle counter can be used for bacterial biomass determination in the sea. As a conversion factor for volume to carbon conversions, we recommend using 0.2 mg of C per mm^3 for both pure cultured bacterial cells and natural bacteria of coastal and near-shore marine environments. This instrument is also applicable to oceanographic investigations of other planktonic organisms and detrital particles.

ACKNOWLEDGMENT

We thank T. Shiba for his help during the work at Otsuchi Marine Research Center.

LITERATURE CITED

1. **Ammerman, J. W., J. A. Fuhrman, A. Hagstrom, and F. Azam.** 1984. Bacterioplankton growth in seawater. I. Growth kinetics and cellular characteristics in seawater cultures. Mar. Ecol. Prog. Ser. **18**:31–39.
2. **Bjornsen, P. K.** 1986. Automatic determination of bacterioplankton biomass by image analysis. Appl. Environ. Microbiol. **51**:1199–1204.
3. **Bratbak, G.** 1985. Bacterial biovolume and biomass estimations. Appl. Environ. Microbiol. **49**:1488–1493.
4. **Bratbak, G., and I. Dundas.** 1984. Bacterial dry matter content and biomass estimations. Appl. Environ. Microbiol. **48**:755–757.
5. **Fry, J. C., and A. R. Davies.** 1985. An assessment of methods for measuring volumes of planktonic bacteria, with particular reference to television image analysis. J. Appl. Bacteriol. **58**:105–112.
6. **Fuhrman, J. A.** 1981. Influence of method on the apparent size distribution of bacterioplankton cells: epifluorescence microscopy compared to scanning electron microscopy. Mar. Ecol. Prog. Ser. **5**:103–106.
7. **Fuhrman, J. A., and F. Azam.** 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. **66**:109–120.
8. **Hamilton, R. D., and O. Holm-Hansen.** 1967. Adenosine triphosphate content of marine bacteria. Limnol. Oceanogr. **12**:319–324.
9. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
10. **Holm-Hansen, O., and C. R. Booth.** 1966. The measurement of adenosine triphosphate in the ocean and its ecological significance. Limnol. Oceanogr. **11**:510–519.
11. **Krambeck, C., H.-J. Krambeck, and J. Overbeck.** 1981. Microcomputer-assisted biomass determination of plankton bacteria on scanning electron micrographs. Appl. Environ. Microbiol. **42**:142–149.
12. **Montesinos, E., I. Esteve, and R. Guerrero.** 1983. Comparison between direct methods for determination of microbial cell volume: electron microscopy and electronic particle sizing. Appl. Environ. Microbiol. **45**:1651–1658.
13. **Novitsky, J. A., and R. Y. Morita.** 1976. Morphological characterization of small cells resulting from nutrient starvation of a psychrophilic marine vibrio. Appl. Environ. Microbiol. **32**:617–622.
14. **Paul, J. H., and W. H. Jeffrey.** 1984. Measurement of diameters of estuarine bacteria and particulates in natural water samples by use of submicron particle analyzer. Curr. Microbiol. **10**:7–12.
15. **Sharp, J. H.** 1974. Improved analysis for "particulate" organic carbon and nitrogen from seawater. Limnol. Oceanogr. **19**:984–989.
16. **Sheldon, R. W., and T. R. Parsons.** 1967. A continuous size spectrum for particulate matter in the sea. J. Fish. Res. Board Can. **24**:909–915.
17. **Sieracki, M. E., P. W. Johnson, and J. M. Sieburth.** 1985. Detection, enumeration, and sizing of planktonic bacteria by image-analyzed epifluorescence microscopy. Appl. Environ. Microbiol. **49**:799–810.
18. **van Es, F. B., and L.-A. Meyer-Reil.** 1982. Biomass and metabolic activity of heterotrophic marine bacteria, p. 111–170. *In* K. C. Marshall (ed.), Advances in microbial ecology, vol. 6. Plenum Publishing Corp., New York.
19. **Watson, S. W., T. J. Novitsky, H. L. Quinby, and F. W. Valois.** 1977. Determination of bacterial number and biomass in the marine environment. Appl. Environ. Microbiol. **33**:940–946.