

Determination of Bacterial Number and Biomass in the Marine Environment¹

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Three techniques for the measurement of bacterial numbers and biomass in the marine environment are described. Two are direct methods for counting bacteria. The first employs an epifluorescence microscope to view bacteria that have been concentrated on membrane filters and stained with acridine orange. The second uses a transmission electron microscope for observing replicas of bacteria that are concentrated on membrane filters. The other technique uses *Limulus* amoebocyte lysate, an aqueous extract from the amoebocytes of the horseshoe crab, *Limulus polyphemus*, to quantitate lipopolysaccharide (LPS) in seawater samples. The biomass of gram-negative (LPS containing) bacteria was shown to be related to the LPS content of the samples. A factor of 6.35 was determined for converting LPS to bacterial carbon.

Most previous investigators have failed to demonstrate that bacteria comprise even 1% of the biomass in the ocean. Thus, most ecologists have tended to minimize the role of bacteria in oceanic environments. The failure to show significant bacterial populations in the ocean can be primarily attributed to the inadequacy of the techniques employed.

In general, surveys employing cultural techniques have revealed only 5 to 1,000 bacteria/ml (5, 16), but it has long been known that such techniques yield only a small fraction of bacteria that are present (20). The highest bacterial counts using cultural techniques are those reported by Kriss (9), which ranged from 2.9×10^3 to 2.3×10^4 bacteria/ml. These findings, however, have never been experimentally verified by other investigators and thus have been regarded with some degree of skepticism.

Direct counts of bacteria in seawater have yielded higher values. Jannasch and Jones (8) compared the efficiency of five culture and two direct counting methods and found 13 to 9,700 times more bacteria with the direct methods than with cultural methods. Direct microscopic examination of natural material, as conducted by Jannasch and Jones (8) and by Kriss (9), however, are subject to error. The concentration of detrital material in natural samples often exceeds the concentration of bacteria. Since a large segment of the detrital material is similar in size and shape to bacteria, positive

identification of a bacterium is frequently impossible.

Fluorescence techniques have also been employed to enumerate the number of bacteria in samples of seawater. When using such techniques, the sample is either stained and then filtered onto membrane filters (7) or the cells are stained on filters (2, 15). However, many problems have been encountered using ordinary fluorescent illumination (13, 18).

Recently, fluorescence microscopes equipped with epiillumination have been employed to study the concentration and distribution of bacteria in the marine environment (1, 4, 19). Ferguson and Rublee (3), using the technique of Daley and Hobbie (1) on surface samples from coastal waters near Cape Lookout, N.C., found an average of 6.6×10^5 bacteria/ml.

Indirect techniques have also been used to estimate total microbial concentration in the marine environment. The most widely used of these is the adenosine 5'-triphosphate method which is interpreted in terms of microbial biomass (6). This technique, however, cannot discriminate between procaryotic and eucaryotic cells, thus limiting its utility for the estimation of bacterial biomass. In addition, this test depends on the physiological state of the population. At present, the physiological state of microbial populations in the ocean has not been documented. The bacteria probably exist in a variety of physiological states, with the majority of them in the dormant state.

In the present report, we describe a new indirect method for estimating bacterial biomass in

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oceanic environments and compare the results obtained with this technique to those obtained using the two direct counting methods. Bacterial biomass will be defined as cellular carbon that is associated with intact bacteria without respect to viability or metabolic activity. The indirect method employed, referred to as the lipopolysaccharide (LPS) method, is based on a technique described by Levin and Bang (11). *Limulus* amoebocyte lysate (LAL), an essential ingredient in this test, is an aqueous extract from blood cells of the horseshoe crab (*Limulus polyphemus*). LAL reacts specifically with LPS to form a turbid solution (17). The amount of turbidity is directly proportional to the LPS concentration (17) and can be quantitatively assayed with a spectrophotometer. The LPS test is a good measure of bacterial biomass in marine waters for three principal reasons: (i) the test is specific for LPS, a compound which only naturally occurs in the cell walls of gram-negative bacteria; (ii) LPS comprise a relatively constant proportion of a gram-negative bacterial cell; and (iii) finally, gram-negative bacteria account for 80 to 95% of the procaryotes found in marine waters (14, 21).

The direct methods used in this investigation to enumerate bacteria in seawater included the fluorescence technique of Hobbie et al. (Appl. Environ. Microbiol., in press) and a replica technique described in this paper. These two direct counting methods are good indicators of bacterial number; however, the considerable size variation within a bacterial population makes it difficult, if not impossible, to use them to estimate bacterial biomass.

MATERIALS AND METHODS

LPS test. Because of the ubiquitous nature of LPS, special care was taken to prevent sample contamination. Niskin sampling bags, disposable glass pipettes, plastic centrifuge tubes (Brinkmann Instruments, Inc.), and plastic tips for automatic pipettors (Brinkmann Instruments, Inc.) were used for sampling, diluting, and adding the reagents. These supplies were found to be free of LPS without any additional treatment. All other glassware and the NaCl used in this assay were made LPS-free by heating at 180°C for 3 h. LPS-free distilled water (sold as sterile water for irrigation, U.S.P.) was obtained commercially (Cutter Laboratories and Abbott Laboratories). Frozen, dried LAL (5 ml/vial) was obtained from the Associates of Cape Cod, Inc., Woods Hole, Mass. The test was performed in disposable test tubes (10 by 75 mm, Fisher Scientific Co. or Becton-Dickinson & Co.). The *Escherichia coli* 180 LPS standard was a gift from C. Milner of the Rocky Mountain Spotted Fever Laboratory. Similar LPS (endotoxin) standards are commercially available (Associates of Cape Cod, Inc.). The

LPS test was read on a Stasar II spectrophotometer (Gilford Instrument Co.) that was equipped with a rapid sampling device.

LAL was reconstituted just prior to use by adding 5 ml of LPS-free distilled water to each vial. Several vials were reconstituted and pooled to obtain enough LAL for daily use. After reconstitution, the LAL could be stored on ice without deterioration for a period of 8 h. The test was performed by adding 0.2 ml of LAL to a 1.0-ml portion of either the sample or dilution. All dilutions were made with a 3% NaCl solution. An LPS-free blank containing 1 ml of a 3% NaCl solution was included. All solutions were gently mixed and then incubated in a 37°C water bath for 1 h. After incubation, the samples were gently vortexed, and the absorbance of each was immediately read. Sample readings were corrected by subtracting the blank reading.

A standard curve was generated by diluting stock LPS (100 ng of *E. coli* standard per ml) with a 3% NaCl solution to cover a range of 1 to 100 pg/ml. This range may vary depending on the sensitivity and range of linearity of the particular LAL preparation used.

Samples of seawater or bacterial cultures contain both free and cellular-bound LPS. To distinguish free from bound LPS, samples were first assayed for their total LPS content. Sample 1-ml portions were centrifuged in a Brinkmann centrifuge (model 3200) at 8,000 × *g* for 10 min, and the supernatant was assayed for free LPS. Bound LPS was determined by subtracting the free LPS from the total LPS.

Samples were read in the spectrophotometer at 360 nm, proceeding from the most diluted to the least diluted sample. The spectrophotometer was initially zeroed with distilled water. A standard was routinely run at the beginning of each test period and with each new batch of lysate. LPS concentrations in samples were determined by comparing their absorbance readings to those found in the linear region of the standard curve.

Direct counts using epifluorescence microscopy. Direct counts of bacteria were made using the method of Hobbie et al. (Appl. Environ. Microbiol., in press) as modified below. Nuclepore polycarbonate membranes with a pore size of 0.2 μm and a diameter of 25 mm were prestained for 5 min in a 0.2% solution of Irgalan black BGL (chemical index, acid black no. 107, Union Color and Chemical Co.) in 2% (vol/vol) acetic acid. The stained membranes were rinsed by dipping in cell-free distilled water and placed wet on a cell-free, glass Millipore filter apparatus. Prestained filters could also be dried and stored after rinsing. Dried filters were wetted with cell-free distilled water prior to use.

Seawater samples (2 ml or greater) that had been fixed at a final concentration of 0.1% glutaraldehyde (neutralized with BaCO₃) were added to the filter. Acridine orange (40% dye content, Sigma Chemical Co.) at a 0.1% concentration in 0.02 M tris-(hydroxymethyl)aminomethane (Sigma), pH 7.2, at 20°C was added to make a final stain concentration of 0.01%. After staining for 3 min, the seawater-acridine orange solution was drawn through the filter by suction (125 mm of Hg). The membrane was

removed from the filter apparatus and placed over a drop of Cargille type LF or A immersion oil on a microscope slide. Another drop of immersion oil was placed on top of the filter, followed by a glass cover slip. The steps after filtration must be performed rapidly enough to insure that the membrane does not become dry.

Slides were viewed and counted with a Zeiss standard microscope equipped with an epifluorescent illumination system containing a 100-W halogen lamp, a BG12 excitation filter, a LP510 barrier filter, and an FT510 beam splitter.

For counting bacteria, a 20-mm² reticule with a scored grid (400 squares) was used. An appropriate amount of seawater (2 to 10 ml) was filtered to give approximately 100 bacteria per grid field. At least 1,000 bacteria were counted per sample by counting one-half of a grid (200 squares) for each field. Representative fields which extended from the membrane center to the edge were counted (approximately 20 fields per sample).

Replica technique for size determination and direct counts of bacteria with TEM. Seawater samples (2 ml or greater) that had been fixed as described above were filtered through Nuclepore membranes (0.2- μ m pore size). The membranes were first washed by filtration (5-ml volumes) with prefiltered seawater (2 \times) and then washed by a graded series of prefiltered seawater-distilled water (75, 50, 25, and 10% seawater), which was followed by two washes with cell-free, distilled water. The bacteria on the membrane were then dehydrated through a graded ethanol series (5-ml volumes of 10, 25, 50, 75, 95% and twice in 2 \times absolute) and air-dried. The membrane was removed from the filter, placed in a holder designed to keep the membrane flat, and shadowed with Pt-C at an incident angle of 30°. The membrane was then coated with a layer of evaporated carbon. Membranes were removed from the replica by floating small squares (5 by 5 mm), which had been cut from the coated membrane, on 6 N NaOH for 12 h. Membrane-free replicas were rinsed by floating on distilled water, followed by a 35% chromic acid treatment for 6 h. Replicas were washed with distilled water several times and picked up on 300-mesh copper grids. Replicas were viewed, counted, and photographed with a Philips 300 transmission electron microscope (TEM). At least 400 bacteria on each of three grids were counted, and an average was obtained. The total area counted was computed by multiplying the area of an average grid square by the number of squares counted. Differential counts were also done by assigning observed bacteria into the various size and shape categories as listed in Table 2. In this manner, integrated total volumes could be determined for each sample.

Culture techniques. A preculture of *E. coli* strain Y-10 was prepared by inoculating 100 ml of 50% strength antibiotic medium 3 (Difco Laboratories) in a 250-ml Erlenmeyer flask and incubating it with shaking at 37°C overnight. A portion of this preculture was used as an inoculum to obtain an initial concentration of 5×10^5 cells/ml in 10 liters of the above medium in a 20-liter carboy. The culture was

sparged with air and mixed with a magnetic stirring bar at 37°C. Samples were taken aseptically at 1-h intervals for the first 9 h and at 24-h intervals thereafter.

Carbon analysis. Carbon analysis was performed on a Perkin-Elmer elemental analyzer. Appropriately sized portions were filtered through a stack of three carbon-free glass fiber filters (Whatman GF/C) to insure the trapping of all cellular carbon and a minimum of 10 μ g of carbon on the top filter. A blank that consisted of cell-free medium was also filtered. Each filter was separately assayed for its carbon content, and the total carbon content (sum of three filters) for each sample was determined. All samples were corrected for blank readings. Direct counts were also done on filtrates to determine the percentage of cells that had been collected.

RESULTS

Laboratory studies. To determine if the LPS test could be used to estimate bacterial biomass, initial studies were carried out using cultures of *E. coli*. Growth of *E. coli* as a function of increase in bacterial number and LPS is shown in Fig. 1. Bound LPS increased and was directly proportional to the bacterial number throughout the logarithmic phase of growth. After the culture entered stationary phase, the concentration of total LPS and bacteria neither increased nor decreased (data not shown); however, bound LPS began to decline. After 9 h, the *E. coli* became too fragile to directly measure bound LPS. Table 1 shows the LPS and cell carbon content and the size of *E. coli* during growth. Cell carbon and bound LPS were fairly uniform throughout the logarithmic phase of growth. During stationary phase, LPS and carbon contents were lower than during the logarithmic phase. However, the average C/LPS

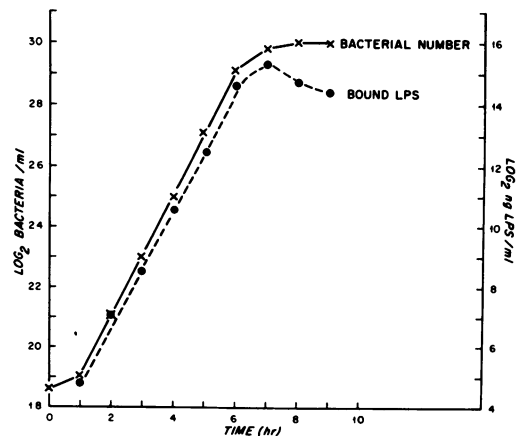


FIG. 1. Growth curve of *E. coli* as followed with the LPS and direct counting (epifluorescence) techniques.

TABLE 1. LPS and carbon concentrations in *E. coli* during growth

Time (h)	No. of cells/ml ^a	fg of C/cell	fg of LPS/cell	C/LPS	Cell vol (μm ³)
2	2.22 × 10 ⁶		62.1		2.4
3	8.13 × 10 ⁶		44.5		2.4
4	3.32 × 10 ⁷	323	42.5	7.6	1.9
5	1.44 × 10 ⁸	305	40.9	7.5	1.9
6	5.68 × 10 ⁸	165	44.0	3.7	1.5
7	9.70 × 10 ⁸	146	42.1	3.5	1.4
8	1.11 × 10 ⁹	163	24.7	6.7	1.2
9	1.06 × 10 ⁹	186	20.0	9.0	1.4
25	1.84 × 10 ⁹	109			0.9
Average log phase (2-6 h)		264	49.9	6.3	2.0
Average stationary phase (7-25 h)		151	28.9	6.4	1.2
Average total		207.5	39.40	6.35	1.60

^a Determined by epifluorescence technique.

ratio remained relatively the same, 6.3 and 6.4, during the logarithmic and stationary phases of growth. Cell size (volume), as based on 300 direct measurements, decreased from 2.0 μm³ during logarithmic growth phase to 1.2 μm³ during stationary phase.

Field studies. A total of 188 seawater samples were assayed by the epifluorescence counting technique and the LAL test. These samples were collected from local waters (Woods Hole, Mass.) during the past year, during cruise no. 8 of the R/V Oceanus (Sargasso Sea), and during cruise no. 93, leg 3 of the R/V Atlantis II (Southwest African coast). A scatter diagram of the natural logarithms of the values for bacterial number and bound LPS concentration is shown in Fig. 2. Direct counts ranged from 1.5 × 10⁴ to 6.29 × 10⁶ cells/ml, and LPS concentrations ranged from 0.04 to 17.8 ng/ml. The correlation coefficient (γ) calculated from this regression plot was 0.948. The average concentration of LPS per cell was found to be 2.78 ± 1.42 fg.

Verification of direct counts was made using a replica technique for TEM viewing of bacteria that had been collected on the membranes. It should be noted, however, that this technique is not applicable for routine analysis. Counts which had been obtained with the replica technique versus the epifluorescence technique were 6.59 × 10⁵ versus 1.15 × 10⁶ cells/ml and 6.20 × 10⁵ cells/ml versus 1.00 × 10⁶ cells/ml on March 19 and March 25, respectively. The size distribution on these samples was also made using the replica technique. Although similar in number, the bacterial populations on March 19 and March 25 were quite different in regard to size (hence, biomass) and morphology (Table 2). LPS determinations on these occasions were found to be 5.20 and 1.8 ng/ml for March 19 and

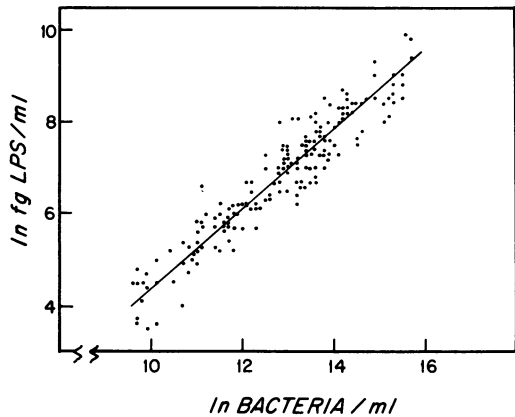


FIG. 2. Scatter diagram of bacterial numbers versus LPS concentrations from 188 seawater samples.

March 25, respectively. The LPS was more closely correlated with bacterial biomass than with bacterial number. The ratio of cell volumes on March 19 and March 25 was 3.21:1, and the ratio of bound LPS was 2.89:1.

Operator error involved in the direct counting procedure was estimated by analyzing the results of nine different laboratory personnel who counted the same sample. The error involved in the LPS test was estimated by comparing the results of four operators using two different samples. (Since the LPS test involves more skill compared to the direct counting techniques, only four qualified personnel were available for this comparison.) The relative error between operators with these two techniques are shown in Table 3. The LPS test has the higher operator error, possibly because of the greater number of steps that are necessary to perform this test. Error between duplicate samples examined by the same operator, how-

TABLE 2. Cell volumes of bacteria from Woods Hole seawater calculated from replicas photographed with a transmission electron microscope

Cell type	Avg vol (μm^3)	% Total count March 19 sam- ple	Avg vol \times %- count	% Total count March 25 sam- ple	Avg vol \times %- count
Bacilli ^a	0.4182	11.75	0.049	5.25	0.022
Small bacilli ^a	0.0751	11.75	0.009	27.13	0.020
Coccibacilli ^a	0.4144	10.1	0.042		0.000
Cocci ^b	0.1102	7.1	0.008	5.26	0.006
Lobular ^a	0.8685	6.3	0.055		0.000
Small vibrio ^a	0.0830		0.000	5.47	0.005
Vibrio ^a	0.3339	32.0	0.107	1.75	0.006
Minibacteria ^c	0.0415	21.0	0.009	54.71	0.023
Hyphomicrobium ^a	1.10			0.44	0.005
Weighted average volume (μm^3)			0.279		0.087

^a Volume = $1/4(\pi d^2 h)$.

^b Volume = $1/6(\pi d^3)$.

^c Minibacteria were composed of bacilli, cocci, and vibrio, all with a diameter $<0.3 \mu\text{m}$ and a volume as calculated for cell type.

TABLE 3. Comparison of operator results with the direct counting (epifluorescence) technique and the LPS test

Operator	Direct count (bacteria/ml) in sample A	Bound LPS (ng of LPS/ml) in:	
		Sample B	Sample C
A	3.49×10^6	3.55	2.34
B	3.68×10^6	3.15	2.74
C	3.89×10^6	3.85	2.38
D	3.90×10^6	2.75	1.90
E	3.76×10^6		
F	3.65×10^6		
G	3.19×10^6		
H	3.75×10^6		
I	3.11×10^6		
\bar{Y}	3.60×10^6	3.28	2.34
s	$\pm 2.68 \times 10^5$	± 0.42	± 0.30
C.V. (%)	7.5	12.8	12.8

ever, was low: 3.1% for the direct counting method (operator G) and 8.0 and 8.9% for the LPS test (operators A and B, respectively).

DISCUSSION

From results presented here, bacterial biomass (from volume determinations) can vary by a factor of three. Our lower value of $0.087 \mu\text{m}^3/\text{cell}$ favorably compares with the value of $0.090 \mu\text{m}^3/\text{cell}$, which was obtained by Ferguson and Rublee (3) and determined by measuring marine bacteria using an epifluorescence technique. Our results indicate a variation with time and space in the size of individual members of the bacterial population within the marine ecosystem. In addition, these investiga-

tors as well as others (18) point out the presence of very small ($<0.3\text{-}\mu\text{m}$ diameter) "coccoidal" forms in seawater when viewed with epifluorescence. Our findings using the replica technique and viewing with TEM show that these small forms (minibacteria) are actually small rods and vibrios (T. J. Novitsky and S. W. Watson, unpublished observations). It is likely that these small rods and vibrios appear as small "cocci" when viewed with epifluorescence because of the lower resolution and the fluorescent "halo" that are characteristic of this technique. Furthermore, based on the TEM measurements, the majority of these small rods and vibrios have actual diameters of $\leq 0.2 \mu\text{m}$, although their lengths may reach $0.5 \mu\text{m}$. It is important, therefore, that filters that measured $>0.2 \mu\text{m}$ not be used when attempting to accurately quantitate bacterial number with the direct counting technique.

Although the respective counts obtained with the replica technique were 43 and 38% lower for each of two samplings than the counts that were obtained with the epifluorescence technique, we believe that the counts obtained are comparable. These are two reasons for this conclusion: (i) the two-sigma confidence (96% confidence level) for the number of counts is higher for the epifluorescence technique, 6.32% (1,000 counts) versus 10.0% (400 counts) for the replica technique, and (ii) the number of manipulative steps (washes, etc.) for the replica technique greatly increases the chance of specimen loss. The most important result of this comparison is the demonstration that most of the small ($<0.3 \mu\text{m}$) points of fluorescence which were observed with the epifluorescence technique are actually bacteria.

Although the epifluorescence technique can be used for estimating bacterial biomass, it is tedious and subject to errors due to size fluctuations in bacterial populations. The LPS test, on the other hand, appears to be a more accurate indicator of bacterial biomass and is relatively simple to perform. We have used the LPS technique successfully aboard ship as well as in the laboratory. Regression analysis indicates a close relationship between bacterial number and LPS content of seawater samples. This regression was generated from data gathered from local waters and two cruises (Watson et al., unpublished data; Fig. 2). Although the regression indicates a close correlation, the mean LPS/bacteria ratio obtained from the same data is subject to a 50% error (as indicated by the coefficient of variation of the mean). It is obvious that predicting bacterial number from LPS data, or vice versa, would be subject to the same error. However, from experiments where bound LPS and biomass (from measured total volumes) had been determined, LPS was closely proportional to biomass. Therefore, the deviation obtained in the regression analysis is probably due to fluctuations in bacterial size.

Since most microbial ecologists refer to biomass in terms of carbon content, we have attempted to convert LPS values to bacterial carbon. Experimentally, the C/LPS ratio is impossible to determine on natural seawater samples due to the presence of organic detritus and eucaryotic microorganisms. In culture experiments with *E. coli* the C/LPS ratios were similar in both logarithmic and stationary growth phases, resulting in an average C/LPS ratio of 6.35. It appears that during stationary phase, the organism loses LPS to the medium while decreasing in size so that the bound LPS/cell volume, and ultimately the bound LPS/cell carbon, remains constant. A C/LPS ratio can also be estimated for a natural population by making the following assumptions: bacterial density of 1.1 g/cm^3 (10), a dry weight/wet weight ratio of 0.22 (12), and a carbon/dry weight ratio of 0.50 (12). Based on these assumptions, a bacterium with a volume of $0.279 \mu\text{m}^3$ would have a carbon content of 33.8 fg. A bacterium with a volume of $0.087 \mu\text{m}^3$ would contain 10.5 fg of C. By dividing these carbon volumes by the experimentally determined LPS values of 4.52 and 1.80 fg of LPS per cell for bacteria with volumes of 0.279 and $0.087 \mu\text{m}^3$, respectively, C/LPS ratios of 7.48 and 5.83 are obtained. Since the average of these values approximates the experimentally determined C/LPS ratio of 6.35, this value has been selected for converting LPS to bacterial biomass carbon.

In this paper we have described and compared three methods for measuring the concentration, distribution, and biomass of bacteria in oceanic environments. The LPS test is of special interest because of its extreme specificity and sensitivity. The test is specific for LPS, compounds that are only naturally occurring in the cell walls of gram-negative bacteria, and is capable of detecting picogram quantities, thus eliminating the necessity of concentrating samples. The use of these techniques should further our understanding of the role bacteria play in the world's oceans.

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