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Correlation of Direct Viable Counts with Heterotrophic Activity for Marine Bacteria

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Viable-bacteria counts, heterotrophic activity, and substrate responsiveness of viable bacteria have been used to measure microbial activity. However, the relationship between these parameters is not clear. Thus, the direct viable count (DVC) method was used to analyze seawater samples collected from several different geographical locations. Samples collected from offshore waters of the South China Sea and western Pacific Ocean yielded DVC that indicated the presence of surface and subsurface peaks of viable, substrate-responsive bacteria which could be correlated with turnover rates of amino acids obtained by using uniformly ^{14}C-labeled amino acids. DVC were always less than total viable counts (acridine orange direct counts), and the DVC subsurface peak occurred close to and within the chlorophyll *a* zone, suggesting algal-bacterial interactions within the layer. For comparison with the open-ocean samples, selected substrates were used to determine the response of viable bacteria present in seawater samples collected near an ocean outfall of the Barceloneta Regional Waste Treatment Plant, Barceloneta, Puerto Rico. The number of specific substrate-responsive bacteria at the outfall stations varied depending on the substrate used and the sampling location. Changes in the population size or physiological condition of the bacteria were detected and found to be associated with the presence of pharmaceutical waste.

It is now widely accepted that the epifluorescent microscopic method (9) is a reliable method for enumerating bacterial biomass in seawater. Limitations of the method include difficulty in discriminating between living and dead bacteria and in identifying bacterial species. In the latter case the fluorescent-antibody approach appears promising (4). With respect to enumeration of living bacteria, at least three methods are available at present to overcome this difficulty: microautoradiography (20), direct viable counts (DVC) (11), and measurement of respiring cells (27). The microautoradiographic method, although very promising in theory, involves complex procedures. Thus, extensive studies aimed at clarifying the vertical distribution of active bacteria in the open-ocean water column are burdensome, at best.

The purpose of the present study was to examine the vertical profile of viable bacteria as determined by DVC and measurements of amino acid (AA) turnover rates, using samples collected in the South China Sea. The DVC method is simple to use and applicable in any aquatic environment. In the studies reported here, the contribution of bacteria attached to particles was eliminated by using filtered (5-μm pore size) seawater in the heterotrophic activity measurements. The vertical profiles obtained were analyzed in relation to the phytoplankton standing stock and primary productivity data obtained at the same time. A second objective of the study was to enumerate the bacteria responsive to specific substrates. By using several different substrates, we attempted to clarify differences in bacterial populations, comparing water samples collected at several stations and measuring the physiological condition of the bacteria in the seawater samples.

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MATERIALS AND METHODS

Sample collection. Samples were collected in the South China Sea, western Pacific Ocean, and near the Barceloneta Regional Waste Water Treatment Plant, Barceloneta, Puerto Rico (Fig. 1). The first of the two cruises, during which the data reported here were obtained, was accomplished aboard the *R/V Hakuho-Maru*, Ocean Research Institute, University of Tokyo, from September to November 1981, and the second was aboard the *R/V Cape Florida*, University of Miami, November 1982. During the *R/V Cape Florida* cruise, sampling was also done at a station located near the Berry Islands of the Bahamas (Station 1, 25°56.6'N, 78°04'W). Seawater samples were collected with a Niskin bacterial sampler (General Oceanics, Miami, Fla.). Seawater of the upper layer was sampled approximately 1 m below the surface. Bacteriological examinations were done aboard ship immediately after collection of samples. For determination of chlorophyll *a* (chl *a*), samples were collected in 5-liter Niskin bottles by methods described in the preliminary report of the *R/V Hakuho-Maru* cruise KH-81-5 (19).

Enumeration of bacteria. Acridine orange direct counts (AODC) were obtained by the method of Hobbie et al. (9). For viable counts, PPES-2 agar plates (22) were used during the *R/V Hakuho-Maru* cruise. After incubation for 2 weeks at 20°C, colonies were counted. DVC were made following the method of Kogure et al. (11, 12). In brief, after nalidixic acid (0.002%, wt/vol) piromidic acid (0.001%, wt/vol), pipemidic acid (0.001%, wt/vol), and substrate were added, the seawater sample was incubated at 20°C for 8 h, followed by fixation with Formalin (final concentration, 2%). After preparation as described, the sample was observed under the fluorescent microscope. Only swollen or elongated cells were counted as viable. In the present study, the concentration of substrate was 50 mg/liter.

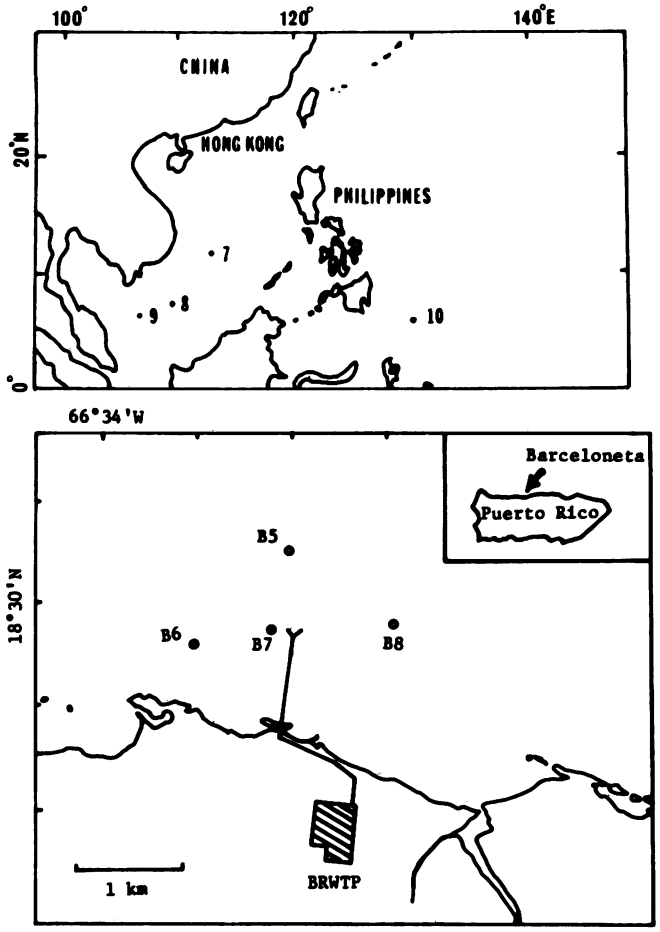

FIG. 1. Sampling locations. (Top) Stations sampled during the *R/V Hakuho-Maru* cruise; (bottom) stations sampled during the *R/V Cape Florida* cruise. BRWTP, Barceloneta Regional Wastewater Treatment Plant.

During the *R/V Cape Florida* cruise, only nalidixic acid (0.02%) was used and samples were incubated for 6 h (11). Several substrates were used for the DVC, including an AA mixture composed of equal parts (by weight) alanine, aspartic acid, glutamic acid, arginine, glutamine, lysine, leucine, cysteine, histidine, and threonine. A carbohydrate mixture was also used, containing equal amounts of glucose, maltose, ribose, fructose, mannose, and arabinose. Glutamic acid or arginine, which had proved to be suitable substrates among the several AAs tested previously, was used as a single carbon source. When combined with the AA or carbohydrate mixture, 25 mg of glutamic acid or arginine per liter and 25 mg of the mixture per liter were mixed together. The nitrogen and phosphorus sources employed were $NaNO_3$ (0.2 mg/liter) and K_2HPO_4 (0.2 mg/liter), respectively. An essential vitamin mixture (Microbiological Associates, Bethesda, Md.) was used as the vitamin source (final concentration, 1/5,000 of the original mixture). After incubation of samples prior to DVC, 30 or 40 ml of the sample was filtered through a Nuclepore filter (25-mm diameter; pore size, 0.2 μm), and a minimum of 130 fields, randomly selected, were counted under a fluorescent microscope.

Microbial uptake of AAs. To establish a vertical profile in the water column for AA turnover rate, the method of Williams and Askew (26) was applied to seawater samples collected in the South China Sea. After passage through a 5.0-μm Nuclepore filter, 30-ml portions of seawater samples run in duplicate were transferred to 50-ml glass bottles fitted with rubber stoppers. Uniformly ^{14}C-labeled (0.1 μCi of ^{14}C) AA mixture (Amersham International Limited) was added to each bottle, followed by incubation in the dark for 3 h at a temperature within 2°C of the ambient temperature of the seawater at the time of sample collection (gross uptake). The concentration of AAs and time period of incubation were determined from the results of preliminary experiments accomplished on board ship. The uptake rate was linear for ca. 4 h at the time of the *R/V Hakuho-Maru* cruise. Incubation was halted by filtering the sample with a 25-mm Millipore GS filter (pore size, 0.22 μm), and radioactivity was measured with an LKB Wallack RackBeta 1215 scintillation counter, available aboard ship. An aquasol-2 scintillation cocktail (New England Nuclear Corp., Boston, Mass.) was used. Formalin-fixed seawater samples run in duplicate were treated in parallel, serving as controls. The amount of CO_2 released (respired fraction) was measured by the method of Hobbie and Crawford (8). Duplicate determinations were made for each sample. A 25-mm Millipore GS filter was suspended in an incubation bottle. At the end of the incubation period, 0.1 ml of phenethylamine was added to each filter, followed by 0.2 ml of 2 N H_2SO_4 injected into the seawater sample. The flask, which was shaken by hand every 30 min, was placed at room temperature for at least 5 h. Recovery efficiency of $^{14}CO_2$ was determined by using an $Na_2{}^{14}CO_2$ solution. Net uptake was obtained by subtracting the respired fraction from the gross uptake.

RESULTS

Vertical profiles for bacteria present in seawater samples collected in the western Pacific Ocean are shown in Fig. 2. These profiles are typical of those obtained by the indicated method for all stations in this study. The concentration of nutrients at station 10 was published by Maeda et al. (16). Relevant to this study, it is important to point out that the subsurface chl *a* maximum layer occurred at 92 m. Below 80 m, temperature decreased rapidly with depth. Total counts also, in general, decreased with depth, although a small peak in abundance occurred at 75 m at station 10. Rapid decreases were frequently observed between 60 and 80 m, at the point where subsurface chl *a* peaks also usually occurred and the nutrient concentration showed a rapid increase. With one exception, viable counts measured during the *R/V Hakuho-Maru* cruise were highest at the surface and decreased with depth, although small subsurface peaks occasionally occurred below 100 m. DVC also showed surface and subsurface peaks in abundance. The former were, in general, larger than the latter. The depth of the subsurface peak usually occurred close to the chl *a* subsurface peak.

It should be pointed out that filtered seawater samples were examined because incubation of seawater without filtration, for AA uptake, was also done. Even though particle-associated bacteria may have higher activity, the values obtained are not always satisfactory because of large variations from sample to sample due to patchiness. For the purposes of this study, larger amounts of seawater and triplicate samples were employed. Since most of the DVC cells were free-living and no significant differences were noted when both filtered and unfiltered samples were examined, all of the samples thereafter were filtered.

In Table 1 are given the turnover rates for the mixed AA substrate measured at stations 7 and 8 in the South China

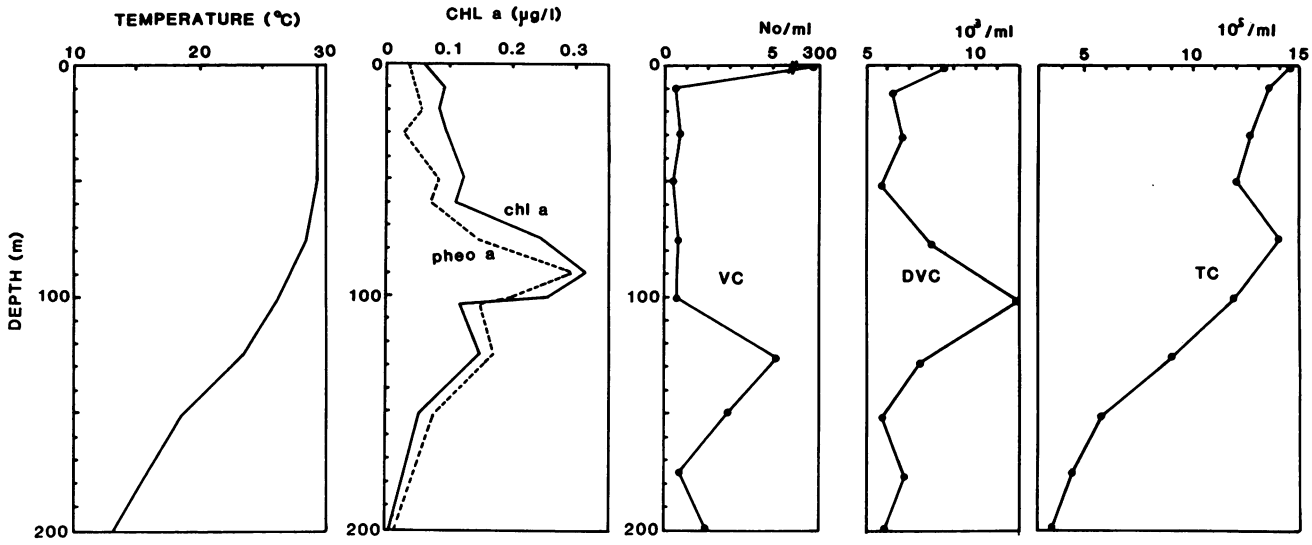

FIG. 2. Vertical profiles for temperature, chl *a* and pheopigment *a* (pheo *a*), and bacterial numbers for station 10 in the western Pacific Ocean, sampled during the *R/V Hakuho-Maru* cruise. VC, Viable count; TC, total count (AODC).

Sea. At both stations, rapid turnover rates were measured for surface water samples (26). For comparison, Fig. 3 and 4 show vertical profiles of the relative uptake rate of AAs [$A \times (f/t)$, where A is the amount of added substrate, f is the fraction taken up, and t is the time (in hours)], bacterial numbers, and chl *a* at stations 8 and 7, respectively. At both stations, DVC and uptake rate profiles were similar but differed significantly from the total counts. At both stations, thermoclines were detected between 50 and 75 m and chl *a* peaks at 65 m, and nutrient concentrations increased below the thermocline, but the particulate organic carbon (POC) showed broad peaks near the thermocline (16).

The DVC obtained with several substrates for seawater samples collected at Barceloneta, Puerto Rico, are shown in Table 2. The results were analyzed by two-way analysis of variance, showing significant differences among substrates used and stations sampled. Paired comparisons of stations and substrates are given in Table 3. Although the distance from shore was approximately the same for stations B6, B7, and B8, the DVC for station B6 samples was lower than for those collected at the outfall itself (station B7) or east of the outfall (station B8).

To clarify the activities of larger-sized cells in the samples, seawater collected at the station located at 19°12.1'N, 60°49.0'W, north of Puerto Rico, was filtered through a 1.0-μm Nuclepore filter to remove large particles and attached bacteria and incubated in bottles with a small amount of glutamic acid added (2.5 to 20 μM) at ambient temperature (Fig. 5). During the 24-h incubation period, no apparent increase in total count occurred. On the other hand, enlarged cells, counted in the DVC procedure, increased exponentially after 8 h. The enlarged cells, as a group, comprised a mixture of larger-sized cells which had enlarged and dividing cells.

TABLE 1. Turnover rates with the AA mixture as substrate[a]

Depth (m)	Turnover rate (h)	
	Station 7	Station 8
0	760	150.6
10	—[b]	1,127
30	—	3,762
50	2,030	1,370
75	1,427	1,634
100	5,825	2,575
200	7,433	—

[a] Seawater samples were collected at stations 7 and 8 in the South China Sea.
[b] —, Not done.

DISCUSSION

From the results obtained in this study, the DVC is concluded to be related to the substrate used, the sampling station, and/or the depth from which the seawater sample is collected. Among the substrates tested, yeast extract yielded maximum DVC values.

A preliminary study, performed with seawater samples collected from the same geographical area, also revealed that yeast extract is better than an AA or a carbohydrate mixture for DVC. The results also showed that an AA mixture yielded higher DVC values. Therefore, yeast extract is concluded to be the best substrate but equal to the AA mixture, since no significant difference between yeast extract and the AA mixture could be detected. On the other hand, the carbohydrate mixture clearly was not as effective as the AA mixture. Addition of vitamins and a nitrogen and

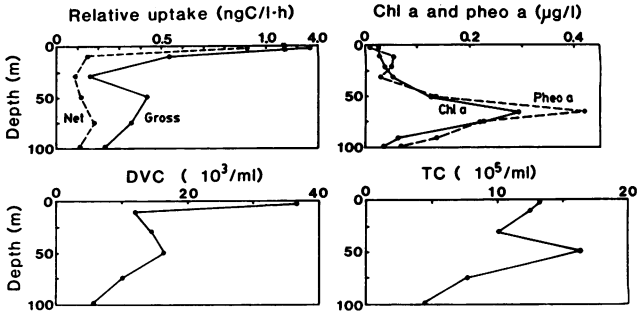

FIG. 3. Vertical profiles of relative uptake of AAs, bacterial numbers, and chl *a* and pheopigment *a* (pheo *a*) at station 8 in the South China Sea. Abbreviations are as in Fig. 2. For definition of relative uptake, see Materials and Methods.

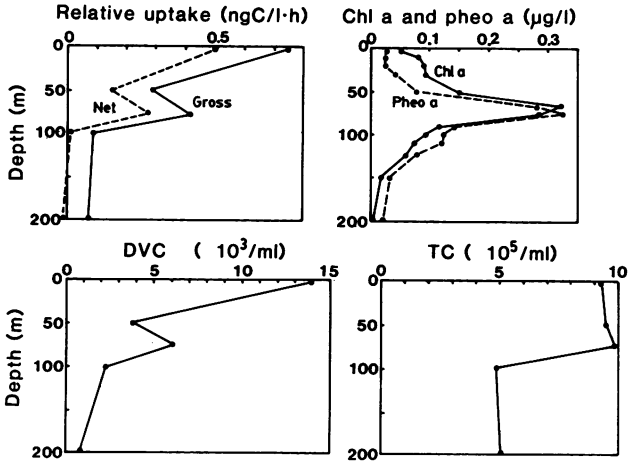

FIG. 4. Vertical profiles of relative uptake of AAs, bacterial numbers, and chl *a* and pheopigment *a* at station 7 in the South China Sea. Abbreviations are the same as in Fig. 2. For definition of relative uptake, see Materials and Methods.

phosphorus source resulted in significant improvement in glutamic acid as a substrate, but not in the case of arginine.

The results suggest that several bacterial groups respond to different substrates simultaneously and/or that bacterial cells take up several AAs and nitrogen and phosphorus sources simultaneously (14), an efficient mechanism since the cells can conserve energy for new metabolic pathways by simultaneous uptake. The majority of the bacterial cells in seawater offshore can be considered to have lower activity because of nutrient limitation (18, 21). Therefore, such a mechanism would be very important for rapid response and efficient nutrient uptake, with subsequent increase in biomass.

Most AAs are readily converted to intermediates of the tricarboxylic acid cycle or other important metabolic pathways. The observation that low DVC values were obtained from samples with carbohydrates added is curious, and the reason for this result not clearly understood. Carbohydrates may not be utilized rapidly for biosynthesis, or only a small portion of the total bacterial population may be able to utilize those carbohydrates found in seawater (3).

The clear difference observed in DVC between the westernmost station 6 and eastern station 8 at the outfall area in Puerto Rico suggests that the spreading of wastes towards the east or north can be monitored bacteriologically, a conclusion supported by data from previous work done by using current meters (7). Interestingly, the total count was relatively constant, whereas the viable count was sporadic, suggesting a local response to available nutrients.

Vertical profiles of DVC, relative uptake rates for AAs, and chl *a* concentrations showed good agreement for seawater samples collected in the South China Sea. Peaks of primary productivity occurred at 65 to 75 m and 50 to 70 m at stations 7 and 8, respectively (M. Takahashi, personal communication). Thus, the results obtained in this study suggest a close relationship between bacterial and algal populations in subsurface layers. The characteristics of the phytoplankton populations in the subsurface layers that were measured during the cruise have been reported elsewhere (23).

A clear diurnal vertical migration of zooplankton from 500 m to the surface was also observed (16). Exudates of the phytoplankton, i.e., dissolved organic matter released, during grazing by zooplankton can quickly be utilized by bacteria occurring in the layer (13). At the surface, however, the high bacterial activity and DVC cannot be explained by the presence or activity of the phytoplankton populations. An accumulation of organic matter, especially that which can be degraded easily and used to sustain high bacterial activity, is required (25).

Since a variety of factors act to control bacterial populations at different depths in the open-ocean water column, simple statistical analysis is of dubious value.

Vertical profiles of DVC and relative AA uptake rates showed good agreement. The vertical concentration of dissolved AAs in these water columns was relatively constant (M. Amano, personal communication). The correlation between the number of active bacteria detected by autoradiography and heterotrophic activity has already been reported for near-shore areas (17). However, because of the technical complexity and cost of the autoradiographic method, it has not been extensively applied to open-ocean water columns, especially subsurface layers. Among the data reported in the literature, the autoradiographic method usually yields larger numbers than the DVC, the active bacteria detected by the former method constituting, at most, 5 to 10% of the total bacteria in offshore water that are enumerated by AODC. Neither the DVC nor the autoradiographic method provides information about the smaller bacteria (<0.4 μm) dominant in offshore areas (5, 6, 11).

To clarify metabolic activity associated with bacteria of different size ranges, AA uptake, combined with size fractionation, was measured at station 9 in the South China Sea during the *R/V Hakuho-Maru* cruise. Seawater collected at the surface was filtered through different sizes of Nuclepore filters and amended with a uniformly ^{14}C-labeled AA mixture. The procedures followed were as described above. Although filtration itself can stress bacterial cells, the results

TABLE 2. Bacterial counts of seawater samples collected off Puerto Rico

Station	No. of bacteria (10^3 organisms/ml)											
	Total count (AODC)	DVC with substrate(s)[a]:										
		YE	Glu	Glu, V	Glu, V, NP	Glu, CH, V, NP	Glu, AA, V, NP	Glu, Arg, V, NP	Arg	Arg, V	Arg, V, NP	Arg, CH, V, NP	Arg, AA, V, NP
1	220	7.2	1.5	1.7	2.3	2.5	8.5	6.2	1.3	1.3	1.4	1.6	5.8
B5	210	13	2.7	2.4	5.1	5.2	7.0	6.5	3.1	2.5	3.5	4.4	4.8
B6	280	5.3	2.0	2.0	2.2	2.5	2.7	2.9	2.3	2.5	2.7	2.4	2.5
B7	290	10	4.3	5.2	6.6	5.1	8.6	6.1	3.5	4.8	5.8	5.1	8.0
B8	290	12	5.9	5.8	7.5	8.1	10	9.1	6.5	—[b]	5.7	9.0	11

[a] Abbreviations: YE, yeast extract; Glu, glutamic acid; Arg, arginine; CH, carbohydrate mixture; V, vitamin mixture; NP, nitrogen and phosphorus source.
[b] —, Not done.

TABLE 3. Paired comparisons of DVC obtained during the *R/V Cape Florida* cruise off Puerto Rico

Substrate or station	*P*[a]										
	1 (1)	2 (B5)	3 (B6)	4 (B7)	5 (B8)	6	7	8	9	10	11	12
Substrates[b]												
1. YE		+	+	+	+	−	+	+	+	+	+	−
2. Glu			−	+	+	+	+	−	−	−	−	+
3. Glu + V				+	−	+	+	−	−	−	−	+
4. Glu + V + NP					−	−	−	−	−	−	−	−
5. Glu + V + NP + CH						−	−	+	−	−	−	−
6. Glu + V + NP + AA							−	+	+	+	−	−
7. Glu + V + NP + Arg								+	+	−	−	−
8. Arg									−	−	−	+
9. Arg + V										−	−	−
10. Arg + V + NP											−	−
11. Arg + CH + V + NP												−
12. Arg + AA + V + NP												
Stations												
1		+	−	+	+							
B5			+	−	+							
B6				+	+							
B7					+							
B8												

[a] Significant (+) or not significant (−) at the 0.05 level. Designations in parentheses refer to stations, for lower part of table.
[b] See Table 2, footnote *a*.

show that the smaller cells retained uptake activity in response to several nutrients (Table 4). Percent respiration, however, was relatively high compared with larger cells. It is possible that the smaller cells use these substrates as an energy source rather than as a carbon source for biosynthesis. These results are in good agreement with observations reported for glutamic acid uptake by starved cells under laboratory conditions (1). Larger cells, on the other hand, account for a considerable portion of the total activity. Based on rough calculations of data obtained in the study reported here, cells in seawater are more or less equally active on a per unit volume basis (10). Therefore, although they may be metabolically active and dominant in number, the relative contribution of smaller cells to the total heterotrophic activity appears to be very small.

From the results of the experiments done to clarify activities of the larger cells in the seawater samples collected north of Puerto Rico, it is concluded that most of the bacteria in offshore seawater increase in volume before cell division (2, 15), and one seldom observes "dividing" smaller cells in incubated seawater samples (24). Therefore, after addition of organic matter, only a part of the total bacterial population will actively take up nutrient and increase in volume and number. Although their relative proportion initially will be small, the larger cells contribute significantly to the biovolume and heterotrophic activity of the bacterial populations in seawater, and those cells enumerated by the DVC represent the active bacteria, rapidly converting nutrients to cell constituents. This can therefore explain the excellent correlation for water column samples collected in the South China Sea. This view is supported by recent observations made with the Elzone particle counter. The results obtained with the particle counter clearly showed that a small number of the larger bacteria (>0.6 μm in diameter) grow much faster than the smaller cells (<0.6 μm), on both a per cell

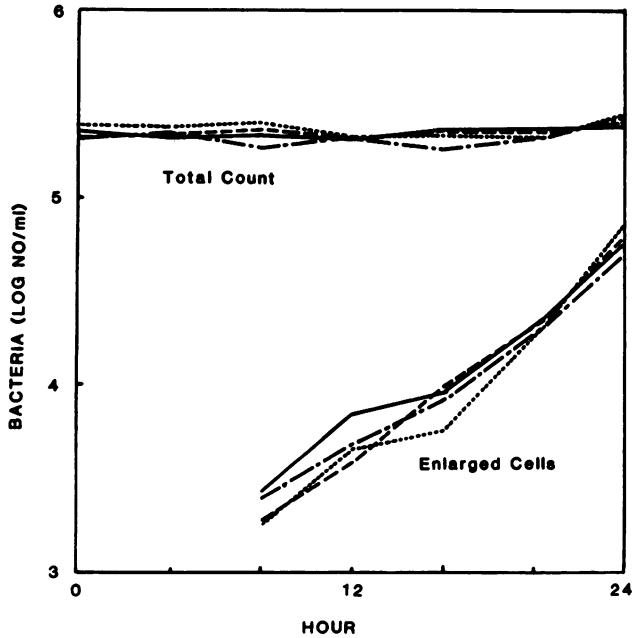

FIG. 5. Number of bacteria measured during incubation experiments aboard ship in seawater samples collected off Puerto Rico during the *R/V Cape Florida* cruise. Symbols: ———, amended with 2.5 μM glutamic acid; –·–·–, amended with 5 μM glutamic acid; – – –, amended with 10 μM glutamic acid; · · · ·, amended with 20 μM glutamic acid.

TABLE 4. Relative uptake rate of AAs by microorganisms of different size fractions at station 9 in the South China Sea

Size fraction (μm)	Relative AA uptake (ng/liter per h)			Total count (10^5 cells/ml)
	Respiration	Net	Gross	
0.4	0.527	1.16	1.69	7.3
0.6	1.09	3.78	4.87	8.7
0.8	1.23	3.19	4.42	8.9
1.0	1.20	3.71	4.91	9.1
5.0	1.29	4.23	5.52	9.4
Control[a]	1.53	6.78	8.31	9.5

[a] Untreated seawater control.

basis and per cell volume. The larger cells appear to contribute mainly to turnover of organic matter by the bacterial populations in seawater (K. Kogure, manuscript in preparation).

In conclusion, the DVC method provides a robust correlation with the heterotrophic activity observed for seawater samples collected from the water column of the open ocean. It also is unique in that it offers the means to determine the specific characteristics of substrate utilization by natural bacterial populations by direct microscopic observation. Obviously, a variety of substrates and environments, as well as many more samples collected from environments of wider geographic distribution, when combined with activity measurements, should yield further clarification of the role of the bacterioplankton in nutrient cycling and mineralization throughout the world oceans.

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