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Enumeration of Viable Bacteria in the Marine Pelagic Environment

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The low percentage of living bacteria commonly obtained when comparing viable counts with total direct counts in seawater could be due more to inappropriate techniques for appreciating the growth ability of living cells than to unadapted culture conditions. The most-probable-number counts in filtered seawater cultures and the microscopic counts of 4′,6-diamidino-2-phenylindole (DAPI)-stained aggregate-forming units grown on black polycarbonate filters appeared significantly correlated to the direct counts. Both these techniques show that in the superficial and intermediate water masses, the living cells may constitute an important (frequently higher than 20%) but highly variable part of the total populations. These viable counts appear more realistic than the conventional CFU counts, which provide only 0.001 to 0.2% of the total counts.

The traditional way of assessing the number of living bacteria is based on their ability to grow in culture media. A large discrepancy between total and viable counts is a normal occurrence in these measurements. For seawater samples, total and viable counts typically differ by 2 or 3 orders of magnitude (9, 10, 17, 20). This discrepancy could be a consequence of the variety of environmental requirements and physiological adaptations of marine bacteria (23) or of the difficulty in setting up nonselective culture media (6, 8).

Most marine environments provide oligotrophic conditions. Physiological adaptation to these conditions leads to the production of ultramicrobacteria (25). Some ultramicrocells do not have sufficient DNA to be detectable by the 4′,6-diamidino-2-phenylindole (DAPI) staining procedure (24), and the majority of the dwarf cells are nonviable in nutrient-rich culture media (18).

However, even under unfavorable conditions, living bacteria may undergo a few cell divisions, but in insufficient numbers to produce bacterial colonies detectable by the unaided eye (16). In fact, the commonly observed low viable counts could be due to inappropriate observation of the cell division ability. Button et al. (4) noted that "viability is an operational term which means ability of a single cell to attain a population discernible by the observer." Diverse methods have been proposed to improve this discernment, formerly by detecting microcolonies grown on membrane filters (10, 11) or on agar slides (5, 7, 22, 25, 28) and more recently by improving the dilution culture technique (4, 19). Alternatively, counts of specifically stained metabolically active bacteria (29), elongated filamentous cell production (14), and microautoradiography (9, 17) have been used as suitable methods.

These techniques provide much higher viable counts than standard procedures, even if no one medium can offer optimal conditions for viable counts of all bacteria in all samples and on all occasions. In this study, we compared viable counts obtained under different culture conditions and with diverse counting procedures (Table 1) applied to a set of seawater samples.

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MATERIALS AND METHODS

Seawater samples. Forty-eight seawater samples were collected between 5 and 2,000 m deep during 3 different cruises (November 1993, May 1994, and November 1994) on the same sampling station located at 43°25′N and 07°52′E in the northwestern Mediterranean Sea. Samples were collected in a Niskin bottle that had been cleaned with acid (10% HCl in distilled water), sterilized with alcohol (50:50, vol/vol), and rinsed with sterile water. Samples were transferred into sterile polycarbonate flasks and processed on board the research vessel. To disrupt the naturally occurring bacterial aggregates (11), we sonicated samples for 5 min in a Bransonic 221 ultrasonic cleaner (48 kHz, 50 W) before further processing.

Total counts. To determine the total number of bacteria, we used epifluorescence microscopy. Immediately after sampling, 50 ml of seawater was preserved with 0.22-μm-pore-size filtered buffered formalin (2% actual formaldehyde final concentration). To prevent reduction of counts due to storage (26), slides were prepared aboard the research vessel as soon as possible and always within 4 h after sampling. Cells were collected onto a 25-mm black polycarbonate Nuclepore membrane with a 0.2 μm pore size and stained with DAPI (21). Slides were kept at −20°C until they were counted. Forty random fields per slide were counted in an Olympus BHA epifluorescence microscope coupled with an image analysis system (27).

Viable counts. (i) Counts of CFU. The samples were cultivated onto nutrient-rich 2216 marine agar (30). During the first cruise, we used both the spread plate technique (seeding with 0.1 ml) and the membrane filtration technique. In the latter case, 1, 5, or 10 ml was filtered through 47-mm-diameter white polycarbonate Nuclepore membranes with a 0.2-μm pore size. At the end of the incubation period (see below), CFU were counted through a ×10 magnifying glass.

(ii) Counts AFU. The technique of aggregate-forming unit (AFU) counting combines the membrane culture procedure with epifluorescence microscopy counts. We selected a nutrient-rich culture medium (2216 marine agar) and two low-nutrient culture media. During the first cruise, the low-nutrient medium (MEW) was prepared from a slurry of marine mud in seawater (20:180, wt/vol). The slurry was boiled for 1 h, sieved through a GF/C Whatman filter, and then filtered through a 0.2-μm-pore-size Nuclepore filter. The material was transferred into 500-ml flasks and then autoclaved for 20 min at 120°C. We used this slurry (total organic carbon concentration, 32.5 ± 2.5 mg liter^{-1} [final concentration]) as a liquid culture medium. During the second cruise, we used a 10-fold dilution of the low-nutrient medium described by Akagi et al. (1). Aged deep seawater was amended with (per liter) polypeptone, 1.0 mg; proteose peptone, 0.5 mg; Bacto Soytone, 0.5 mg; Bacto Yeast Extract, 0.5 mg; sodium glycolate, 0.5 mg; sodium malate, 0.5 mg; D-mannitol, 0.5 mg; sucrose, 0.5 mg; and ferric citrate, 0.05 mg. The organic carbon final concentration was 1.8 mg liter^{-1}.

To seed these culture media, seawater subsamples (1, 5, and 10 ml) were filtered in duplicate onto 25-mm black polycarbonate membranes (Nuclepore) with a 0.2-μm pore size. The membranes with the associated cells were transferred to the surface of 2216 marine agar or onto cellulose pads soaked with 1.5 ml of MEW or with 1.5 ml of 10-fold-diluted Akagi culture medium. Petri dishes (diameter, 5 cm) were used for these cultures.

The cultures were incubated in the dark at 14 ± 1°C. This temperature is comparable to that of the water column in the Mediterranean Sea under spring and fall conditions. Cultures on 2216 marine agar were stopped after 48 h of incubation to prevent colony overlapping under nutrient-rich conditions. Under

TABLE 1. Sample processing under different culture conditions and viability detection methods and percentages of viable bacteria in relation to the total direct counts

Parameter	Results for sonicated samples by the following method:					
	Direct count	Membrane culture	Membrane culture	Membrane culture	Membrane culture	MPN procedure
Cell harvesting	0.2-μm black filter	0.2-μm white filter	0.2-μm black filter	0.2-μm black filter	0.2-μm black filter	Serial decimal dilution
Culture medium	None	2216 marine agar	2216 marine agar	Mud extract seawater	10-fold-diluted Akagi medium	Unamended 0.2-μm-filtered seawater
Solidifying agent	None	Agar	Agar	Cellulose pad	Cellulose pad	Liquid culture
Incubation	None	15 days	15 days	2–4 days	2–4 days	60 days
Staining	DAPI	None	DAPI	DAPI	DAPI	DAPI
Counting procedure	Microscope	×10 glass	Microscope	Microscope	Microscope	Microscope
Bacterial density calculation	Direct	Direct	Direct	Direct	Direct	MPN calculation tables (2)
Minimum (ml^{-1})	4.0×10^4	0.4×10^1	3.3×10^2	1.1×10^3	1.3×10^2	0.1×10^1
Maximum (ml^{-1})	2.2×10^6	3.3×10^2	3.8×10^5	4.2×10^4	1.4×10^5	4.5×10^5
Mean (ml^{-1})	3.8×10^5	6.3×10^1	6.8×10^4	1.7×10^4	3.9×10^4	62.7×10^4
n	48	32	25	7	14	47
Range (% of direct counts)	100	0.001–1.3	0.4–96.0	0.8–66.1	0.05–96.8	0.003–106.8

oligotrophic conditions (MEW and diluted Akagi medium), incubation was maintained for 10 days without colony overlapping (Fig. 1).

At the end of the incubation period, the membranes were stained with DAPI as described above. With a microscope, we counted separately the individual cells and the bacterial aggregates composed of at least two cells (Fig. 1). Since naturally occurring bacterial aggregates were disrupted by sonication before sample filtration (Fig. 1), the bacterial aggregates we observed after incubation supposedly are derived from the division of individual cells. Bacterial aggregate background was determined for the sonicated sample prior to incubation and was deducted from AFU counts. To avoid operator-dependent bias (12), background and counts were always determined by the same operator.

The AFU method appears as a modification of the microlony counts on agar slides proposed by Postgate et al. (22) and Fry and Zia (7). However, because the centrifugation step in their method is a possible source of error, it was eliminated from our protocol. A second change in the protocol was the use of epifluorescence microscopy as the detection method, since it gives much more accurate results than phase-contrast microscopy. The AFU method is also an improvement on the membrane culture technique described by Jannasch and Jones (10) because of the introduction of a preliminary sonication step, which allows disruption of the naturally occurring microaggregates, and because of the use of epifluorescence microscopy counts.

(iii) MPN enumeration. The most-probable-number (MPN) technique combines the dilution experimental procedure to cultivate the bacterial cells (3, 4, 15), epifluorescence microscopy counts to detect their viability (21), and the MPN calculation tables to estimate the bacterial density (2). We used unamended 0.2-μm-pore-size-filtered seawater as the diluent as well as the culture medium (3, 4). During the first cruise, for each sample, we used a part of the sampled seawater to prepare the culture medium aboard the research vessel. By using a Whatman Clyde (pore size, 0.2 μm), disposable filter device, seawater was filtered and aseptically distributed into gamma-sterilized polyethylene culture tubes. During the second and the third cruises, we used aged seawater collected from 1,100 m deep in the same sampling area during a previous cruise. Seawater was autoclaved for 20 min at 120°C, filtered through a 0.2-μm-pore-size membrane, and aseptically distributed into the culture tubes as mentioned above.

Serial decimal dilutions of the sample were prepared with 0.2-μm-pore-size-filtered seawater as the diluent, prepared exactly in the same manner as above for the culture tubes. Three tubes were inoculated with decimal dilutions to 10^{-8}, as is usual for MPN determinations (2). Cultures were incubated for 2 months in the dark at 14°C (the sample temperature) ± 1°C. At the end of the incubation period, 1 ml of culture was aseptically collected from each tube and filtered onto a 25-mm-diameter black polycarbonate Nuclepore membrane with a 0.2-μm pore size and stained with DAPI (21). Thirty microscope fields were examined. Tubes were scored positive when the number of cells per field appeared larger than 4, i.e., the average background determined by counting the cells into the culture tubes prior to incubation.

RESULTS AND DISCUSSION

Direct counts ranged between 6.4×10^4 and 5.8×10^5 cells ml^{-1} under fall conditions and between 4.0×10^4 and 9.6×10^5 cells ml^{-1} under spring conditions (Table 2). Variability

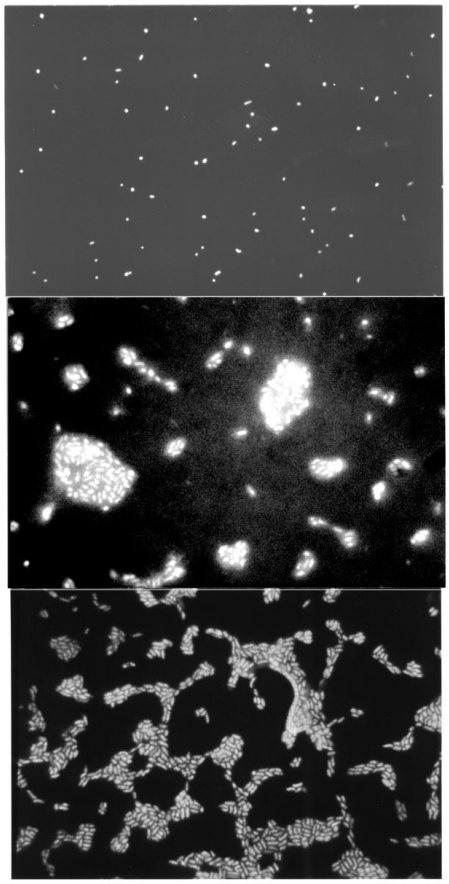

FIG. 1. Examples of microscope fields after DAPI staining on black Nuclepore filters. After sonication and before incubation, most of the bacteria are isolated cells (upper panel). After a 3-day incubation onto the 10-fold-diluted Akagi medium, living bacteria give rise to paired cells and to microcolony aggregates (middle panel). After a 3-day incubation on the 2216 marine agar, colony overlapping prevents accurate enumeration (lower panel). The images were created with Adobe Photoshop.

TABLE 2. Comparison of total and viable counts as determined by diverse enumeration procedures on seawater samples collected in the Ligurian Sea

Depth (m)	Nov 1993				May 1993					Nov 1994	
	DC[a] (10^3 cells/ml)	AFU (ME)[b] (10^3 cells/ml)	MPN[c] (10^3 cells/ml)	CFU[d] (ml^{-1})	DC (10^3 cells/ml)	AFU (MA) (10^3 cells/ml)	AFU (Ak) (10^3 cells/ml)	MPN (10^3 cells/ml)	CFU (ml^{-1})	DC (10^3 cells/ml)	MPN (10^3 cells/ml)
5	584	ND[e]	250.00	4	963	200.0	ND	140.00	30	1,346	300.00
10	472	9.45	250.00	5	728	25.0	ND	3.50	10	1,216	150.00
25	349	ND	95.00	5	860	380.0	4.3	0.01	335	1,104	150.00
45	163	ND	0.20	4	595	100.0	ND	3.50	275	2,281	200.00
60	143	1.10	1.50	20	590	280.0	0.3	140.00	15	996	200.00
80	213	ND	0.02	5	399	89.0	140.0	110.00	30	649	450.00
100	239	12.20	0.01	55	490	240.0	130.0	140.00	170	572	20.00
150	119	29.00	0.15	126	279	67.0	95.0	140.00	265	263	15.00
200	ND	ND	ND	ND	222	ND	6.6	1.10	55	181	45.00
300	121	24.60	0.09	50	131	24.0	34.0	140.00	25	293	2.00
400	ND	ND	ND	ND	172	22.0	ND	0.02	100	ND	ND
500	134	ND	0.95	6	101	15.0	7.6	0.01	15	203	0.01
600	ND	ND	ND	ND	154	36.0	ND	0.01	65	ND	ND
700	64	42.40	0.95	44	123	36.0	18.0	0.11	ND	127	0.05
800	ND	ND	ND	ND	131	ND	ND	0.08	25	ND	ND
900	ND	ND	ND	ND	70	8.7	40.0	0.01	5	ND	ND
1,000	70	5.98	0.35	32	71	5.6	0.1	0.01	30	41	0.45
1,200	ND	ND	ND	ND	75	ND	8.0	0.01	10	ND	ND
1,500	ND	ND	ND	ND	40	5.9	ND	0.03	15	54	0.02
1,800	ND	ND	ND	ND	63	0.3	61.0	0.05	105	ND	ND
2,000	ND	ND	ND	ND	50	48.0	5.3	ND	100	51	0.01

[a] DC, direct counts determined by epifluorescence microscopy.
[b] AFU on black polycarbonate Nuclepore filters (optical microscope counts). (ME), cultures onto mud extract seawater; (MA), cultures onto 2216 marine agar; (Ak), cultures onto 10-fold-diluted Akagi et al. (1) culture medium.
[c] MPN culture in unamended aged deep-seawater (0.2-μm filtered) (optical microscope counts).
[d] CFU on white polycarbonate Nuclepore filters incubated onto 2216 marine agar culture medium (×10 magnifying glass counts).
[e] ND, not determined.

has been estimated from triplicate counts carried on three samples concomitantly collected at 994, 997, and 1,000 m deep. The overall comparison of these counts leads to 82,333 cells ml^{-1} (SE ± 2,947; $n = 9$).

The conventional counts for viable bacteria obtained on 2216 marine agar cultures varied between 4 and 850 CFU ml^{-1} in fall and between 10 and 355 CFU ml^{-1} in spring. As usually observed, the percentages of bacteria able to produce visible colonies on this peptone-rich culture medium were rather low, ranging between 0.001 and 0.2% of the microscope counts.

Counts of AFU onto black Nuclepore membranes varied between 1.1×10^3 and 4.2×10^4 AFU ml^{-1} when incubated on marine mud extract (Table 2), i.e., in a range of 0.7 to 66% of the total direct counts. When incubated onto 2216 marine agar, aggregate counts varied between 3.3×10^2 and 3.8×10^5 AFU ml^{-1} and between 1.3×10^2 and 1.4×10^5 AFU ml^{-1} when incubated onto the 10-fold-diluted Akagi culture medium (Table 2). For both these culture media, the percentages of viable bacteria were in the range of 0.4 to 96% of the total direct counts (Table 1).

MPN counts appeared highly correlated to total direct counts (Table 3). An overall comparison of AFU counts (including counts on mud extract seawater, on the nutrient-rich marine agar, and on the 10-fold-diluted oligotrophic Akagi culture medium) showed a significant correlation with total direct counts. Surprisingly, the highest correlation was not observed under the most oligotrophic culture conditions but with the nutrient-rich marine agar (Table 3). Furthermore, there was no significant correlation between AFU counts obtained on nutrient-rich and nutrient-depleted culture media (Table 3). On the other hand, we pinpointed the importance of the method used to detect bacterial viability: with the same culture medium (2216 marine agar), AFU counts were 3 orders of magnitude higher than CFU counts (Table 1).

Samples collected in the superficial waters showed MPN counts higher than AFU counts and, for some of them, slightly higher than the direct DAPI counts (Tables 1 and 2). These results could be due to the following. (i) The direct DAPI counts could be underestimated. Because of their low DNA content, some naturally occurring marine bacteria could escape from DAPI detection in the natural samples (24). It is usual that sample dilution provokes a physiological stress to the bacterial cells, resulting in a drastic production of nucleic acid, making possible their detection after cultivation even in unenriched seawater (15). (ii) The MPN data could be overestimated. Uncertainty of MPN counts is well known (13), particularly with the most commonly used approach (i.e. 10-fold serial dilutions, with three tubes inoculated per dilution).

TABLE 3. Spearman rank correlation coefficients for variables

Parameters[a]	r	P	n
DC versus MPN	0.703	0.0001	47
DC versus AFU	0.609	0.0029	25
DC versus CFU	0.035	0.8439	32
AFU versus MPN	0.387	0.0632	24
AFU versus CFU	0.421	0.0433	24
MPN versus CFU	−0.109	0.5517	31
DC versus AFUma	0.767	0.0016	18
DC versus AFUak	0.051	0.8554	14
AFUak versus AFUma	−0.009	0.9769	12

[a] DC, direct counts; AFUma, AFU on nutrient-rich marine agar; AFUak, AFU on oligotrophic Akagi diluted culture medium.

Furthermore, MPN calculation tables have been elaborated by using the visual observation of pH indicator change to detect the positive tubes. Changing the detection method (microscopic observation of bacterial proliferation) could provoke an overestimation of the microbial density.

The diverse cultivation and enumeration techniques we used suggest that viable bacteria could be highly correlated to the total counts and frequently form a high percentage of the pelagic bacterial population. The highest contributions were frequently observed in the superficial water masses, and the proportion of living cells varied irregularly and to a very large extent with increasing depth in the water column.

In conclusion, the cultures in unenriched filtered seawater lead to MPN viable counts that are highly and significantly correlated with the total counts. However, this technique could appear too expensive and time-consuming for routine use, with each sample requiring coloration and microscope counting of 24 membranes. The microscopic count of AFU grown on two black polycarbonate membranes appears an interesting alternative when large series of samples have to be processed. This technique take into account all the cells as long as they are able to divide at least once under the laboratory conditions. Basically, the use of such a high-sensitivity technique could be extended to enumerate viable bacteria in a large diversity of environments.

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