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Total Counts of Marine Bacteria Include a Large Fraction of Non-Nucleoid-Containing Bacteria (Ghosts)

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Counts of heterotrophic bacteria in marine waters are usually in the order of 5×10^5 to 3×10^6 bacteria ml^{-1}. These numbers are derived from unspecific fluorescent staining techniques (J. E. Hobbie, R. J. Daley, and S. Jasper, Appl. Environ. Microbiol. 33:1225–1228, 1977; K. G. Porter and Y. S. Feig, Limnol. Oceanogr. 25:943–948, 1980) and are subsequently defined as total counts of bacteria. In samples from the Baltic Sea, the North Sea (Skagerrak), and the northeastern Mediterranean Sea, we found that only a minor fraction (2 to 32%) of total counts can be scored as bacteria with nucleoids. Lack of DNA no doubt means inactive cells; therefore, a much lower number of bacteria that grow at rates higher than those previously estimated must be responsible for the measured bacterial production in these seas. The remaining bacterium-sized and/or -shaped particles included in total counts may be cell residues of virus-lysed bacteria (ghosts) or remains of protozoan grazing.

Several methods, in addition to plate counts, have been used to detect the number of active bacteria in natural samples. These have included microautoradiography (11) with various radioactive tracers, measurements of respiratory activity by the INT-formazan technique (29), and most probable numbers of marine bacteria in liquid media (2). The outcomes of these methods have one common feature: the numbers of active bacteria detected are always lower than the total counts determined by fluorescent staining. Numbers as low as 1% of total counts have been observed, while numbers above 60% are rarely found (2, 4, 12, 14, 17, 19, 23, 24, 26, 29). This observation has been taken as evidence for the presence of a large fraction of dormant or nonactive marine bacteria (13). An alternative interpretation is that only a small fraction of bacteria is alive (31). Perhaps the most obvious criterion of a bacterial cell that is able to respire and reproduce is that it possess DNA. In a bacterium, DNA is organized in a condensed structure (nucleoid) that is located at the center of the cell (3, 5, 18). In the cell, the nucleoid can be observed with transmission electron microscopy when it is thin sectioned or stained with a DNA-specific fluorochrome such as DAPI (4′, 6-diamidino-2-phenylindole). For this purpose, DAPI-stained cells are fixed to glass slides and observed under combined epifluorescence and phase-contrast microscopy (1). To allow quantitative measurements of the natural abundance of nucleoid-containing (NUCC) cells, we have developed a method by which bacteria are retained on polycarbonate filters. The results indicate that only a fraction of the particles that are enumerated as bacteria by traditional staining protocols are truly NUCC cells.

MATERIALS AND METHODS

Enumeration of bacteria. Traditional total counts of bacteria were determined by fluorescent staining (DAPI or acridine orange) as described elsewhere (10, 20), with the exception that the final concentration used was 2 μg of DAPI per ml of sample. To quantify the number of NUCC bacteria in seawater samples, we stained bacteria with DAPI and collected them on polycarbonate filters. Low-saline samples (<12‰) were incubated for 1 h with DAPI and Triton X-100 (final concentrations, 2 μg of DAPI per ml of sample and 0.1% [vol/vol] Triton X-100). Samples with salinity above 12‰ were killed with sodium azide (final concentration, 0.5 M) and then diluted with 0.2-μm-pore-size-filtered Millipore Milli-Q water. DAPI and Triton X-100 were added to the final concentrations stated above, and samples were incubated for 2 h. After incubation, samples were filtered, at 100 mm of Hg, onto 0.2-μm-pore-size black-stained polycarbonate filters (Micron Separations, Inc.), placed on GF/C filters (Whatman), and until filtered dry, with a metal grid as support. Three to ten milliliters of proanalysis grade 2-propanol was added and filtered through at 100 mm of Hg, and the filter was air dried for a few minutes before being mounted on microscopic slides. Immersion oil (type A; Cargille) was applied both under and on the filter. After being washed with 2-propanol to remove unspecifically bound DAPI stain, bacterial nucleoids became clearly visible by epifluorescence (Zeiss Axioplan) with magnification of ×1,250 and filter set 450-490 FT 510 LP 520 (Fig. 1). We caution against using sintered glass as a support when samples are filtered since in our hands this has resulted in less sharp images. In addition, we have noticed that epifluorescence units with a faded UV filter set decrease the ability to detect nucleoid structures.

CFU were determined by colony counting on Zobell agar plates (30).

Most probable numbers (MPN) of bacteria were obtained with autoclaved seawater as the growth substrate. This water was collected from the same areas as the samples tested. Dilutions ranged from 1 to 100,000 bacteria per tube in respect to traditional counts of bacteria (acridine orange direct count or DAPI staining), and at least 5 replicas of each dilution step were prepared. Sample volumes were 1 to 5 ml. Tritiated thymidine or tritiated amino acid mixture (Amersham) was added to a final concentration of 10 nM, and samples were incubated for 1 to 3 weeks. Incubation was terminated as described by Smith and Azam (25). Uptake of radioactivity was counted with a Beckman scintillation counter. At times, growth was also detected by scoring the tubes with increases in bacterial cells by microscopic enumeration of the total number of bacteria. Detection of growth by uptake of radiolabelled substrates and increased bacterial counts matched perfectly.

Destaining-staining test. Cells were stained and destained with DAPI, Triton X-100, and 2-propanol as described in the NUCC bacterium staining protocol above. The filter was washed with 5 ml of sterile seawater and then stained once again with acridine orange or DAPI. The numbers of bacteria recorded after this procedure were compared with the total counts of bacteria in untreated samples.

Cultures. Samples were collected at sampling station NB1 (63°30.5′N, 19°48.0′E) in the northern Baltic Sea with a polycarbonate water sampler and transferred to acid-rinsed polycarbonate bottles. Sampled water was filtered through 0.2-μm-pore-size filters (Gelman Supor) at <200 mm of Hg with acid-washed filtration equipment (Nalgene polysulfone unit or Millipore Sterifile unit). Predator-free inoculum of natural bacterial communities was prepared by gravity filtration three times through 0.6-μm-pore-size polycarbonate filters (Micron Separations, Inc.) and added to cultures to give a 20-fold dilution of inoculum. When isolates of marine bacteria were used, colonies were picked from Zobell plates and grown in Zobell broth. Then a fraction of cultures was diluted in autoclaved seawater three times over 2 days in order to exhaust any nutrient supply from Zobell broth before the experiment started. Cultures was incubated in the dark at in situ temperatures. At regular time intervals, cultures

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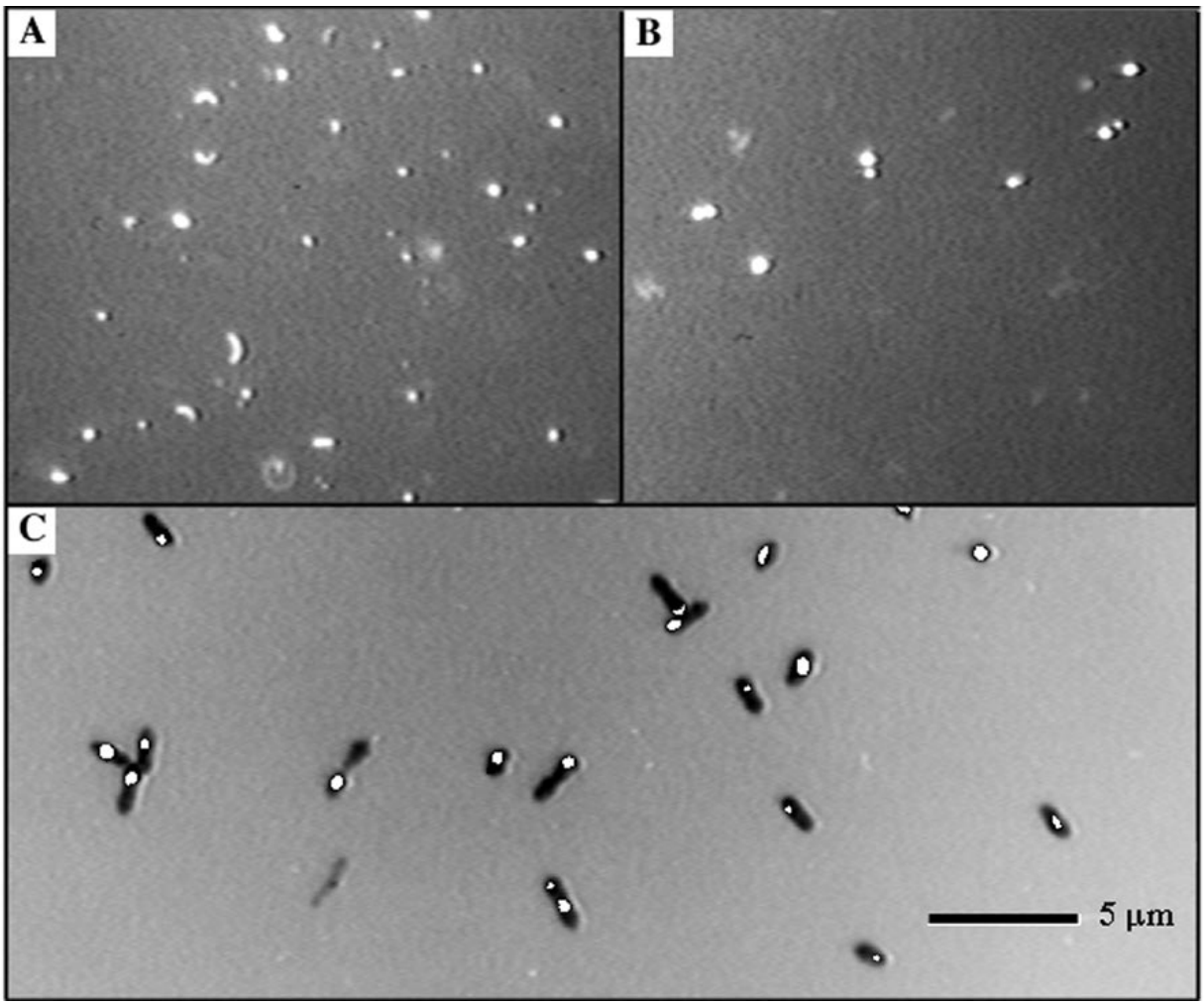

FIG. 1. DAPI-stained marine bacteria; total numbers compared with NUCC bacteria as viewed by epifluorescence microscopy. (A) Conventional DAPI-stained (14) sample (site NB1). (B) Bright NUCC bacteria in the same sample used in panel A after destaining (overexposure creates images that are larger than the original cell). (C) Nucleoids in a marine isolate (ZS2) visualized by superimposing a binary image of bright nucleoids onto the original negative image to compensate for color information that was lost in transforming to gray scale.

were sampled for total counts, NUCC bacteria, and CFU. Cultures were not amended with nutrients at any time. All of the filters that were used in preparing cultures and inocula were soaked in 1 M HCl and thereafter extensively rinsed in ultrapure water (Millipore Milli-Q) prior to use.

Bacterial production. Bacterial production was measured by the uptake of tritiated thymidine, as described by Smith and Azam (25). The uptake of thymidine was converted to the number of produced cells by using a conversion factor of 1.0×10^{18} cells per mol of incorporated thymidine. This factor was experimentally determined for the Baltic Sea and Mediterranean Sea samples. It was also applied to data from the North Sea (Skagerrak) samples.

Hybridization. Natural seawater samples were prefiltered through a 2-μm-pore-size polycarbonate filter (Micron Separations, Inc.). Samples were filtered onto 0.45-μm hybridization membranes (Hybond-N; Amersham) by using a 96-well dot blot (GIBCO BRL) with 6-mm-diameter slots. Ten-milliliter reservoirs were connected to wells to allow 10 to 20 ml of filtering volume, and samples were filtered at 200 to 300 mm of Hg. Less than 0.1% of bacteria were found to pass through the filter. Samples were lysed in situ during slot blot analysis. Slots were covered with 100 μl of 0.5 M NaOH for 3 min, the solution was filtered through, and the procedure was repeated. Slots were covered with 100 μl of 1 M Tris-HCl (pH 7.4) for 5 min, the solution was filtered through, and the procedure was repeated. Finally, slots were covered with 100 μl of 1.5 M NaCl–0.5 M Tris-HCl (pH 7.4) for 5 min and the solution was filtered through. Each filter was placed on Whatman 3MM filter paper and air dried for 1 h. Membranes were wrapped in Saran Wrap, and DNA was covalently linked to membranes by UV exposure for 3 min. Filters were stored at 4°C prior to hybridization. The natural DNA collected on filters was hybridized against a universal deoxyoligonucleotide 16S rRNA probe (*Escherichia coli* positions 519 to 536), as described in detail by Rehnstam et al. (22).

Centrifugation test. NUCC and nucleoid-devoid cells were separated by centrifugation (Beckman J2-21). A natural sample from the northern Baltic Sea was prefiltered through a 2-μm-pore-size filter; divided into four tubes, each with 30 ml; and centrifuged with a swingout rotor (Beckman JS-13.1) at 13,000 rpm ($26,900 \times g$). Two tubes were withdrawn after 10 min, and the other two tubes were withdrawn after 30 min. Supernatants were carefully withdrawn in 10-ml aliquots with a syringe. All three layers were stained, and the total bacterium counts and NUCC bacterium counts were determined for all three layers. Sedimented cells were calculated by subtracting the number of cells found in these three upper layers from that in the water originally.

RESULTS

Natural samples. Depth profiles of samples from four locations and from one sampling occasion in the northeastern Mediterranean Sea showed low numbers of NUCC cells (Table 1). In samples from these sites, the fraction of NUCC cells was 2 to 32% of the traditional total count of bacteria. The upper water layer harbored the highest number of NUCC cells, concurrent with the highest production rates (Fig. 2). The fraction of NUCC cells, compared with traditional total counts, was

TABLE 1. Measurements of bacterial abundance by various enumeration techniques in depth profiles

Sea, sampling site, date (mo/day/yr) and depth (m)	Temp (°C) (salinity [‰])	Cell count (ml^{-1})				% Total count	
		Total count (10^6)	NUCC (10^5)	MPN (10^4)	CFU (10^2)	NUCC	MPN
Baltic Sea, NB1, 10/18/93, 0–30	7–8 (4)	2.5–3.2	1.1–2.3	0.4–1.0	1.8–7.8	4	0.1
						6	0.3
Baltic Sea, SR5, 5/6/94, 0–100	0.8–2.5 (5–7)	0.7–1.2	1.5–2.8	4.5–15	2.5–160	17	7
						27	14
Baltic Sea, US5b, 5/5/94, 0–200	0.9–3.2 (5–7)	0.6–2.7	1.4–3.5	4.7–19	0.7–21	12	6
						27	15
North Sea, Skagerrak, 8/10/94, 0–25	11–20 (31)	1.1–1.4	0.3–0.7	0.7–0.8	1–44	2	0.5
						5	0.6
North Sea, Sakgerrak, 8/10/94, 25–340	5–11 (35)	0.2–0.8	0.1–0.5	0.1–0.2	0.1–4	4	0.2
						32	0.8
Mediterranean Sea, Point B,[a] 10/15/94	19 (35)	0.5	1.4	7.5	10	20	16

[a] Villefranche-sur-Mer, France.

lowest in surface water, implying that bacterial "ghosts" accumulate during high-level bacterial production. NUCC bacteria were measured in parallel with total counts of bacteria, most probable number (MPN), CFU, and bacterial production (25). NUCC bacterium measurements yielded numbers that were 1.25 to 40 times higher than those yielded by the MPN technique and 14 to 1,200 times higher than those yielded by CFU.

Accuracy of the NUCC staining protocol. In order to test if the NUCC staining procedure gave rise to loss of small and fragile cells, we compared the total counts of bacteria in original samples with the total counts of bacteria after cells had been treated according to the NUCC staining protocol. The acridine orange direct count was $(1.23 \pm 0.09) \times 10^6$ cells ml^{-1}, while the NUCC staining protocol and subsequent acridine orange protocol yielded $(1.16 \pm 0.08) \times 10^6$ cells ml^{-1}. The DAPI staining protocol produced a count of $(1.21 \pm 0.09) \times 10^6$ cells ml^{-1}, while the NUCC staining protocol and subsequent DAPI protocol yielded $(1.21 \pm 0.08) \times 10^6$ cells ml^{-1}. We did not find any significant loss in cell number nor did we observe any morphological differences due to the treatment of samples.

We tested a total of 16 randomly picked marine strains with different colony morphologies and the gram-positive bacterium *Bacillus subtilis*, and all of these strains were susceptible to staining and produced bright nucleoids (Fig. 1). In addition, both mixed marine bacterial communities and marine isolates were grown in culture, with seawater as the growth medium, in order to demonstrate any differences in staining ability during various growth phases. At regular time intervals, cultures were sampled for total counts of bacteria, NUCC bacteria, and CFU. Marine isolates showed perfect matches among total counts of bacteria, NUCC bacterium counts, and CFU throughout the growth cycle (Fig. 3). When a natural mixed bacterial community (predator-free seawater) was used as the inoculum, the counts by these three methods also increased identically in magnitude (Fig. 4). However, in this inoculum, 96% of total counts were presumably dead cells (ghosts) that lacked nucleoids, thus rendering surpluses in total counts. These surpluses remained constant throughout the growth cycle. Two weeks after stationary phase had been reached and no bacterial production was measurable, NUCC bacteria were still detectable (note that Fig. 3 and 4 have discontinued timescales). Mixed cultures were sampled until senescence, and NUCC cells and CFU decreased concomitantly, leaving total counts unchanged. The same experiment, plotted in logarithmic scale, also revealed that viable cells initially have to double

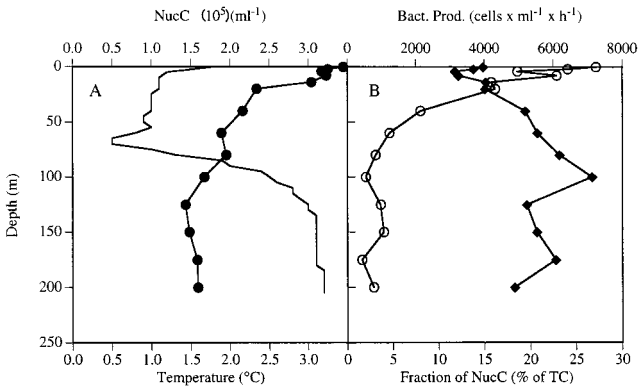

FIG. 2. Depth profile from Baltic Sea station US5b. (A) ●, NUCC bacteria; solid line, temperature. (B) ○, bacterial production; ◆, fraction of NUCC bacteria, compared with total counts.

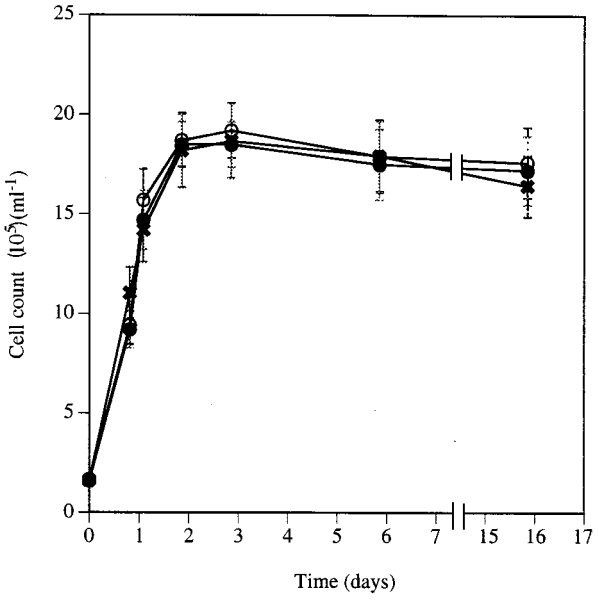

FIG. 3. Growth curve of a marine bacterial isolate in predator-free seawater culture. ○, total count; ●, NUCC bacterium count; ✕, CFU. Bars represent standard deviations.

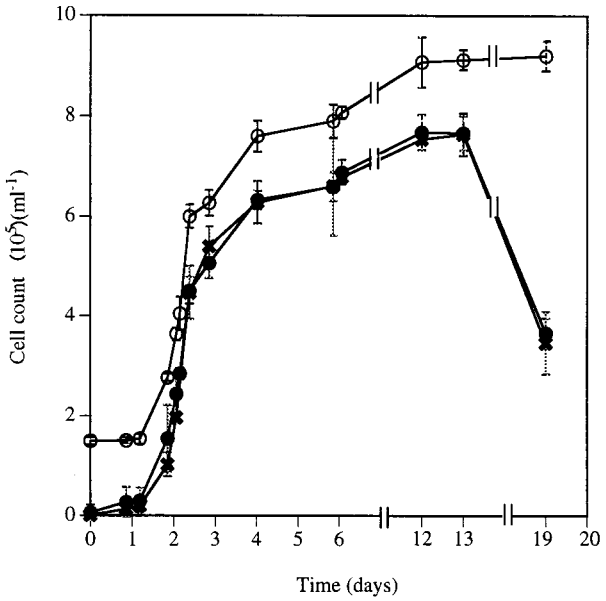

FIG. 4. Growth curve of marine bacteria in predator-free seawater culture with a natural inoculum, showing surplus of ghosts. ○, total count; ●, NUCC bacterium count; ×, CFU. Bars represent standard deviations.

at least three times before an increase in total count is seen (Fig. 5).

Since NUCC cells are denser than nucleoid-devoid cells, we used centrifugation to separate these two fractions (Fig. 6). The NUCC fraction in the original sample was 28%. After 10 min of centrifugation, two replicate tubes were withdrawn and cells were clearly distributed between separate layers. Twenty-eight percent of cells were found in the bottom layer or sedimented material, and 89% of these cells were NUCC cells. Additional centrifugation yielded a slightly higher level of sedimentation and a corresponding figure of 29% of cells collected, of which 91% were NUCC cells.

The accuracy of NUCC bacterium counts was further tested by comparing NUCC bacterium counts with the numbers of

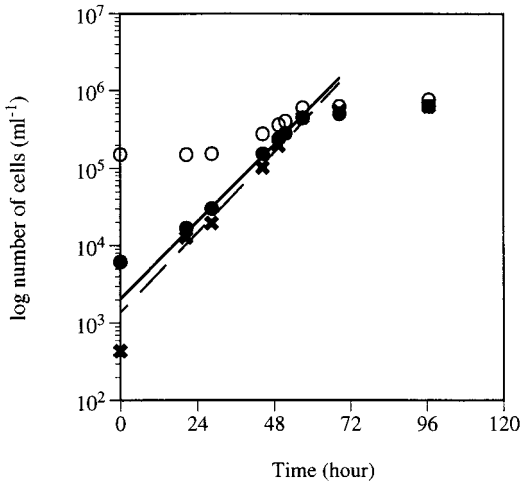

FIG. 5. Logarithmic plot of bacterial growth in seawater culture (as in Fig. 4). ○, total count; ●, NUCC bacterium count; ×, CFU; solid line, exponential curve fit to NUCC bacterium count; dashed line, exponential curve fit to CFU.

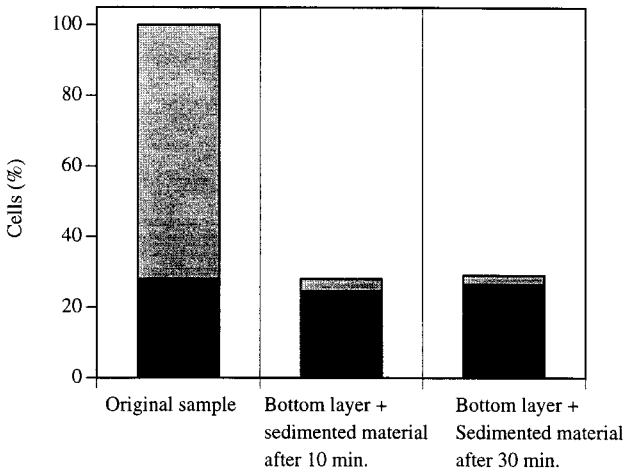

FIG. 6. Distribution of NUCC (black bars) and nucleoid-devoid (gray bars) cells after centrifugation. Bar 1, original distribution in untreated water (original sample); bar 2, distribution in the bottom layer and sedimented material after 10 min of centrifugation; bar 3, distribution in the bottom layer and sedimented material after 30 min of centrifugation.

16S rRNA genes in seawater samples. Bacterial DNAs that had been isolated from samples for a depth profile and collected on filters were hybridized to a universal deoxyoligonucleotide 16S rRNA probe (*E. coli* positions 519 to 536) (22). A positive correlation was found between NUCC bacterium count and the hybridization signal toward bacterial DNA ($r = 0.903$, $P < 0.04$, $n = 5$). No significant correlation was found between total count and hybridization signal.

DISCUSSION

In this paper, we have presented data that suggest that only a fraction of the particles that are enumerated as bacteria by traditional epifluorescence techniques are truly NUCC cells. These data rely on a new staining protocol, and the accuracy of this protocol is crucial for interpretation of the results. In cultures, we found matched increases in the number of cells as determined by total counts of bacteria, NUCC bacterium counts, and CFU. This result shows that there is no loss of NUCC bacteria due to the treatment of samples and that the protocol serves as a quantitative measurement. Nucleoids were also observed when bacteria had been deprived of nutrients for 2 weeks, indicating that starved cells are also susceptible to this staining protocol. By centrifuging natural samples, we observed that NUCC bacteria sank faster than did presumably empty cells or cell residues. This is consistent with the higher densities of cells that contain DNA, compared with those of non-DNA-containing cells. Finally, the correlation with the 16S RNA probe suggests that the results of the microscopic counting procedure match the results of an independent molecular assay. Taken together, we believe that this protocol is an accurate method for estimating NUCC bacteria in natural seawater samples.

The different growth patterns of bacterial isolates and seawater cultures are an important observation. Isolated bacteria had been pregrown, and when tested, the inoculum had 100% NUCC bacteria. The growth of this isolate was thereby exponential from the start. In seawater cultures, the natural sample used as an inoculum (0.6-μm filtrate) contained only 4% NUCC bacteria, compared with the traditional total count, giving rise to a pronounced lag phase and a surplus in total cell

TABLE 2. Growth rates of marine bacteria calculated from estimates of community production and various methods of cell counts

Sea, sampling site, and depth (m)	Temp (°C) (salinity [‰])	Bacterial production[a]	Growth rate[b]		
			Total count	NUCC	MPN
Baltic Sea, NB1, 0–30	7–8 (4)	1,200–2,100	0.0003–0.0005	0.005–0.008	0.14–0.33
Baltic Sea, SR5, 0–100	0.8–2.5 (5–7)	400–2,200	0.0004–0.0014	0.002–0.02	0.008–0.03
Baltic Sea, US5b, 0–200	0.9–3.2 (5–7)	400–7,200	0.0006–0.002	0.003–0.015	0.008–0.03
North Sea, Skagerrak, 0–25	11–20 (31)	800–6,200	0.0006–0.003	0.06–0.3	0.1–0.5
North Sea, Skagerrak, 25–340	5–11 (35)	50–200	0.0001–0.0005	0.006–0.07	0.06–0.10
Mediterranean Sea, Point B[c]	19 (35)	6,000	0.012	0.06	0.08
Other[d]	4		0.02–0.05	0.02–0.05	
	15		0.07–0.3	0.07–0.3	

[a] Measured by tritiated thymidine incorporation and expressed in cells ml^{-1} h^{-1}.
[b] Calculated as (ln 2/number of cells ml^{-1})/bacterial production (cells ml^{-1} h^{-1}) with total count, NUCC, and MPN bacterial abundance measurements, as indicated.
[c] Villefranche-sur-Mer, France.
[d] Cultured marine bacteria. At 4°C, there were 3 samples; at 15°C, there were 11 samples.

count. This surplus remained constant throughout the growth cycle, showing that ghosts are unable to return to active growth. Mixed marine bacterial communities that grow in seawater cultures always show a lag phase in the traditional total cell count. An initial lag phase is seen even when the inoculum (gravity filtrate, <0.6-μm-pore-size-filtered predator-free bacteria) is collected from seawater and added to cultures within 30 min. This paradox becomes even more complicated with the observation that if a culture in log phase is once again filtered and diluted with particle-free seawater, growth with no lag phase is observed (7). Our observation that initially a few live cells must double several times before the traditional total count shows increased numbers explains the paradox of the S-shaped growth curve.

At initial sampling of mixed culture, CFU were 0.3% of the total count and 7% of the NUCC bacterium count, numbers that fall within the range of normal plating efficiencies of natural samples. However, the difference between NUCC bacterium and CFU determinations was only 5.8×10^3 cells ml^{-1} in absolute numbers. From the data presented here, it is not possible to determine whether this difference remained throughout the growth cycle since the number falls within the standard deviations of these enumeration techniques. From the exponential curve fits of these two data sets, CFU and NUCC bacterium determinations predict an initial stock of 1.3×10^3 to 2×10^3 living cells ml^{-1} in culture (Fig. 5). Since the inoculum was diluted 20-fold, the number of living bacteria in the original sample would have been 2.6×10^4 to 4×10^4 cells ml^{-1}.

NUCC bacterium counts represent maximum estimates of the number of active bacteria since the presence of a nucleoid does not ensure that the bacterium is growing. A fraction of these cells may still be nonviable, such as cells in late viral infection (21), or nongrowing cells. This may explain the differences among NUCC bacterium, MPN, and CFU determinations for natural samples and the initial difference between NUCC bacterium and CFU determinations for seawater culture. Nonviable bacterium are incapable of division and therefore are not recorded as viable by the MPN and CFU techniques. They do, however, contain visible nucleoids and maintain metabolic activities until they are lysed. In the future, more work has to be done to elucidate the relationships among total count, NUCC bacterium, MPN, and CFU determinations.

On the basis of doubling times as calculated from bacterial production rates and total cell counts, it has been suggested that marine bacteria grow quite slowly. Growth rates that range from 0.0004 to 0.01 h^{-1} in cold water and from 0.0003 to 0.09 h^{-1} in temperate water can be calculated by using the frequency of dividing cells (8) or uptake of tritiated thymidine (6). However, mixed and isolated marine bacteria that grow in seawater cultures at in situ temperatures usually have significantly higher growth rates (32) (0.02 to 0.5 h^{-1}), depending on temperature (Table 2). In depth profiles, we measured the production of the bacterial community in terms of the uptake of tritiated thymidine (25). Using the numbers of bacteria determined by various techniques (Tables 1 and 2), we calculated the growth rate of bacteria that was necessary to support the measured bacterial production. If all of the particles enumerated as bacteria by traditional total counts are actively growing, growth rates as low as 0.0002 to 0.003 h^{-1} can be reached. Calculations of growth rates that are derived from the NUCC protocol and the MPN technique yield numbers that, if they represent live bacteria, are in close agreement with the rates found for seawater cultures at ambient temperatures. In the Skagerrak surface layer, growth rate estimates based on NUCC bacterium determinations were as high as 0.3 h^{-1}, arguing that a small fraction of fast-growing bacteria supports the observed bacterial production.

The findings presented in this paper do not overthrow the common concept of carbon flow in the microbial food web. The bacterial production rates and bacterial uptake capabilities that have been reported are probably as accurate as the various methods allow. However, our findings imply that fewer bacteria are growing faster than previously estimated and that a considerable amount of carbon may be channelled between bacterial detritus and dissolved organic matter. We infer that a large fraction of total counts of bacteria consists of non-NUCC bacterial ghosts and that this is the major explanation for the small fraction of marine bacteria that is active. This implies that bacterium-shaped and -sized particles, such as cell residues, are included in traditional total counts. Virus-lysed bacteria are likely to be part of this material since virus activities in marine environments have been shown to be high, with a potential for 2 to 24% turnover in the traditional total bacterial community per h (9). An other source for this material may be egestion of cell walls during protozoan grazing (15).

Why have these findings escaped marine microbiologists? The answer can be found in the specificity of the fluorescent stain. DAPI is a highly DNA-specific fluorochrome that is used to stain DNA and to visualize chromosomal structures in cells (1, 16, 27). When we collected samples of NUCC bacteria in the high-saline North Sea (Skagerrak), staining of bacteria was much less effective than it was for samples from the brackish Baltic Sea (Gulf of Bothnia) (salinity, 5 to 7‰). It became obvious that DAPI binds poorly to DNA at high salt concen-

trations, which is common knowledge in biophysics literature (28), but to our knowledge, this fact has not been discussed in the context of marine ecology. In marine microbiology, DAPI is used to stain for total counts of bacteria by a protocol that requires formaldehyde-fixed cells (20). In formaldehyde-fixed samples, DAPI binding most likely occurs on reactive bacterial surfaces that have been created by formaldehyde. This is indicated by the fact that in high-saline samples, DAPI poorly stains live and sodium azide-killed bacteria; the stain is totally removed when it is washed with 2-propanol. In practice, DAPI staining of bacterial nucleoids was impossible at salt concentrations above 12‰, which is well below the salt concentrations in marine waters. However, when high-saline samples were fixed with sodium azide, diluted with 0.2-μm-pore-size-filtered Milli-Q water, and then stained with DAPI, nucleoids became clearly visible. Although this procedure can be anticipated to cause considerable osmotic stress, total numbers (acridine orange stain [10]) remained constant and no changes in apparent bacterial shape or volume were observed. In fact, marine isolates were diluted without fixation with no loss in traditional total counts or CFU. Thus, we argue that our staining procedure can be used for live and sodium azide-killed bacterial cells, but the internal ion contents of bacteria in high-salinity samples must be lowered before DAPI binding to DNA can occur.

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