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# Enumeration and Biomass Estimation of Planktonic Bacteria and Viruses by Transmission Electron Microscopy

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**Bacteria and virus particles were harvested from water samples by ultracentrifugation directly onto Formvar-coated electron microscopy grids and counted in a transmission electron microscope. With this technique, we have counted and sized bacteria and viruses in marine water samples and during laboratory incubations. By X-ray microanalysis, we could determine the elemental composition and dry-matter content of individual bacteria. The dry weight/volume ratio for the bacteria was 600 fg of dry weight $\mu m^{-3}$. The potassium content of the bacteria was normal compared with previous estimates from other bacterial assemblages; thus, this harvesting procedure did not disrupt the bacterial cells. Virus particles were, by an order of magnitude, more abundant than bacteria in marine coastal waters. During the first 5 to 7 days of incubation, the total number of viruses increased exponentially at a rate of 0.4 $day^{-1}$ and thereafter declined. The high proliferation rate suggests that viral parasitism may effect mortality of bacteria in aquatic environments.**

Accurate estimation of bacterial biomass has been important for the study of microbial ecology. Currently, bacterial total counts are routinely obtained with epifluorescence microscopy of bacteria collected on filters and stained with fluorescent dyes (16, 23, 24, 32). Counting in the scanning electron microscope of bacteria on membrane filters has also been reported (18, 29), but the use of transmission electron microscopy (TEM) for biomass estimation has not become common, partly because it has been difficult to produce quantitative preparations. The resolution of the electron microscope makes it superior to light microscopy for estimation of bacterial size distribution. We have developed a simple and rapid method for counting bacteria in the TEM, and we have used the same preparations to count virus particles (3).

In current theories in aquatic microbial ecology, it is often assumed that bacterial production is balanced by grazing (2, 9, 31). Parasitism by bacteriophages has been neglected as a source of bacterial mortality because the concentrations of viruses and bacteria are thought to be too low for this process to be important (30). Concentrations of bacteriophages in natural aquatic environments as determined by counting of PFU on various host bacteria usually are in the order of 1 to 10 PFU $ml^{-1}$ (8, 21), but in the Kiel Bight up to $3.65 \times 10^4$ PFU $ml^{-1}$ has been reported (1). Torrella and Morita (28) used electron microscopy and estimated the total number of phage particles to be $>10^4$ $ml^{-1}$ in seawater from Yaquina Bay, Oregon. In a recent paper, Bergh et al. (3) found abundances of $1 \times 10^5$ to $2.5 \times 10^8$ viruses $ml^{-1}$ in various aquatic samples. These studies suggest that the concentration of phages in marine waters may be much higher than indicated by the concentrations obtained by culturing methods.

We describe and discuss a method for counting and biomass estimation of bacteria and viruses, and we demonstrate its usefulness for studying the development of viruses and bacteria in incubations of seawater.

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## MATERIALS AND METHODS

Seawater samples were collected at 5-m depth in Raunefjorden, W. Norway (60°16.2′ N, 5°12.5′ E). The incubations were done in loosely capped 10-liter polypropylene bottles: at 21°C in the light for 1 week (sampling date, 12 July 1988), and at in situ temperature (15°C) in the dark for 19 days (sampling date, 10 August 1988).

Harvesting of bacteria and viruses was performed by ultracentrifugation of the particles directly onto grids. The centrifuge tubes were modified by molding a flat supporting bottom of two-component epoxy glue. After the glue was added, the tubes were centrifuged at 80,000 × *g* for 30 min to shape the glue and remove air bubbles, and tubes were left in an upright position at 60°C overnight for hardening.

Water samples were filled in the centrifuge tubes, and electron microscopy grids (nylon or 400-mesh Cu grids; Agar Scientific) supported with carbon-coated Formvar film were submersed below the surface and dropped with the Formvar film upwards. The samples were centrifuged in a Beckmann L8-70M ultracentrifuge, using a swing-out rotor (SW27.1) run at 22,000 rpm (80,000 × *g*) for 90 min at 12°C. After centrifugation, the supernatant was withdrawn with a pipette and the grids were air dried.

For counting and sizing of bacteria, unstained grids were examined in a JEOL 100CX TEM operated at 80 kV and at a magnification of ×20,000. The grid squares were used to keep track of the area counted. Length and width of bacteria were measured, and notes on morphology were recorded for each individual cell. More than 150 cells were measured from each preparation. Cell volumes were calculated as $\pi/4 \cdot W^2 \cdot (L - W/3)$, where $W$ is width and $L$ is length of the cell. Quantitative energy-dispersive X-ray analysis of individual bacterial cells was performed by the method of Heldal et al. (14). For comparison, bacteria were counted with epifluorescence microscopy after staining bacteria collected on 0.2-μm-pore size Nuclepore filters with 4′,6′-diamidino-2-phenylindole (DAPI) (24). Bacteria were collected by filtering or by placing the filter on the bottom of the centrifuge tube during ultracentrifugation under conditions described above.

For counting of viruses, the grids were stained with uranyl

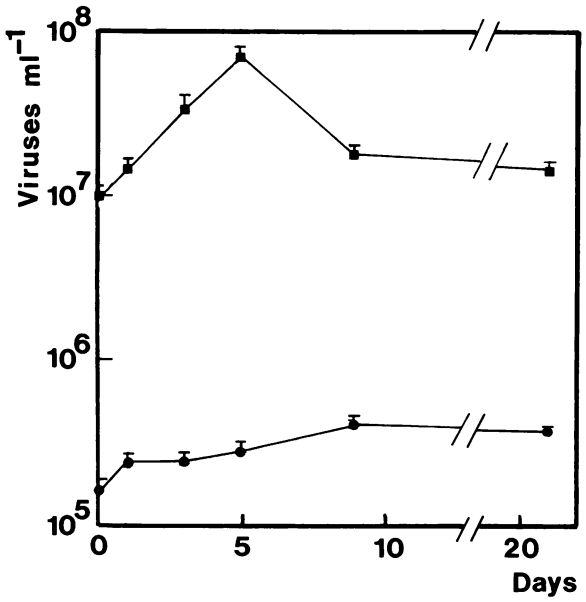


FIG. 1. Time course of total counts of bacteria and viruses in seawater sample incubated in the dark at in situ temperature. Symbols: ●, bacteria; ■, viruses. Vertical bars indicate standard errors.

acetate and examined at a magnification of ×100,000. View fields were randomly selected and counted until the total counts exceeded 200. The size of the view field was used to keep track of the area counted.

## RESULTS

**Bacteria.** Bacterial cells were easily distinguished from other particles on the basis of shape and electron density. During the spring of 1989, nine samples were collected from the Raunefjorden location, and total counts with the DAPI method and the TEM were compared. Total counts of bacteria in the TEM were not significantly different from total counts of DAPI-stained bacteria in the epifluorescence microscope (Student's $t$ test; $P < 0.05$).

Total counts of bacteria increased during the incubation, but much less than total counts of viruses (Fig. 1). Average cell volume of the different morphological groups of bacteria increased during the first 24 h of incubation, declined thereafter to the initial level, and then increased at the end of the experiment (Table 1). The relative distribution of the different morphological groups changed gradually. The percentage of curved rods declined, whereas rod-shaped bacteria became more dominant (Table 1). During incubation, a marked increase in flagellated bacteria was observed. In preparations from natural communities, flagellated cells are present in low numbers, typically <1% of the total number (M. Heldal, unpublished data).

Figure 2a shows two bacteria with markedly different staining properties, and the bacterium with low electron density seems to be lysed and surrounded by extruded phage particles. Figure 2b shows a cell with normal electron density that has several viruses attached to it. Such associations were not seen in the fresh sample, but became common during incubation of the sample. X-ray microanalysis of single cells (July sample) is shown in Table 2. The dry weight/volume ratio was approximately 600 fg μm$^{-3}$.

TABLE 1. Mean cell volume and distribution of different morphological types of bacteria during incubation[a]

| Day | Rods | | Cocci | | Curved rods | | Spirillae | | All (vol, μm$^3$) |
|---|---|---|---|---|---|---|---|---|---|
| | Vol (μm$^3$) | %[b] | Vol (μm$^3$) | % | Vol (μm$^3$) | % | Vol (μm$^3$) | % | |
| 0 | 0.082 | 53 | 0.049 | 9 | 0.031 | 36 | 0.029 | 2 | 0.060 |
| 1 | 0.137 | 67 | 0.181 | 13 | 0.054 | 17 | 0.042 | 2 | 0.127 |
| 3 | 0.084 | 69 | 0.025 | 12 | 0.031 | 19 | 1.235 | 0 | 0.067 |
| 5 | 0.075 | 68 | 0.083 | 11 | 0.031 | 20 | 0.025 | 1 | 0.066 |
| 9 | 0.075 | 78 | 0.089 | 14 | 0.044 | 8 | 0.038 | 0.03 | 0.074 |
| 21 | 0.12 | 81 | 0.110 | 17 | 0.122 | 2 | | 0 | 0.110 |

[a] Some 150 to 200 cells were measured at each sampling.
[b] Percentage of total number.

**Viruses.** Viruses were recognized on the basis of sizes and shapes. The total number of viruses in the seawater sample collected on 12 July 1988 was $1.8 \times 10^7$ ml$^{-1}$. After 1 week of incubation, the total number of viruses had increased to $1.5 \times 10^8$ ml$^{-1}$. We repeated the incubation experiment with the sample collected 10 August 1988 and followed the development of both bacteria and viruses in more detail. We tentatively divided the viruses in this sample into three size groups, 30 to 60, 60 to 80, and >80 nm in head diameter. The initial numbers of viruses in each size group were 62, 39, and 8% of the total count, respectively. The smallest size group showed a hexagonal outline but viruses were mainly without

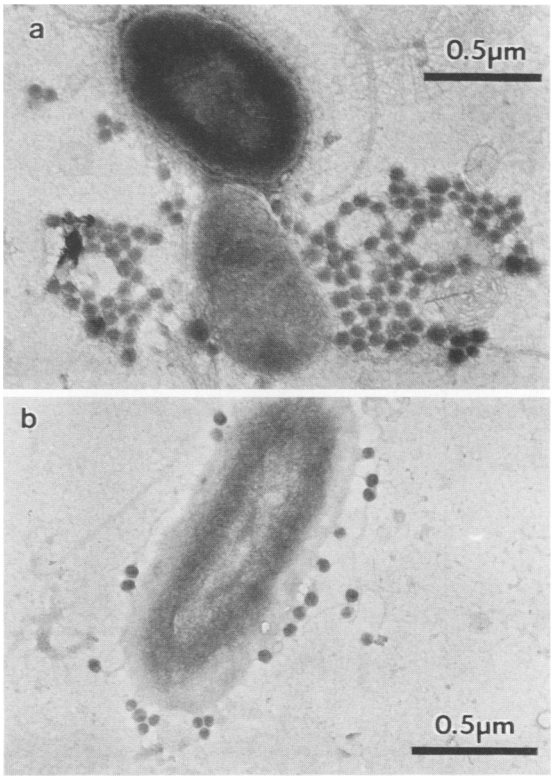


FIG. 2. Phages with head diameter of approximately 50 nm associated with bacteria after 48 h of incubation of a seawater sample. (a) The bacterium with high electron density looks normal and healthy; the cell with low electron density appears to be lysed and is surrounded by 110 to 120 phage particles. (b) Bacterium probably undergoing infection.

TABLE 2. Elemental composition and dry weight of bacteria in the sample collected on 12 July 1988

| Measurement | Vol ($\mu m^3$) | Dry wt (pg) | P (fg) | S (fg) | K (fg) |
|---|---|---|---|---|---|
| Mean ($n$ = 13) | 0.70 | 0.43 | 8.86 | 3.54 | 1.33 |
| SE | 0.21 | 0.12 | 3.76 | 0.94 | 0.42 |

tails (Fig. 2a and b), while ca. 50% of the viruses in the two other size groups had tails or tail-like structures.

A time course of the proliferation of the three size classes of viruses in this incubation is shown in Fig. 3. The smallest class of viruses increased exponentially at a rate of 0.41 $day^{-1}$ for at least 5 days. After approximately 7 days, the concentration started to decrease and declined to almost the same level as at the beginning of incubation. For the larger size classes, proliferation was slower and the increase ceased earlier (Fig. 3).

## DISCUSSION

**Centrifugation.** The direct harvesting technique for examination of bacteria and viruses in aquatic samples by TEM has several advantages. It is the only technique available that permits direct counting of viruses. For bacteria, the method gives results comparable to those obtained by epifluorescence counting. The reliability of the virus count will depend on the efficiency of the centrifugation. The time required for harvesting particles during centrifugation is related to the relative pelleting efficiency of the rotor and the sedimentation coefficient of the particles. The pelleting efficiency depends on the maximum and minimum radial distances of the rotor and on the speed applied. In distilled water at 20°C, the sedimentation coefficient for isometric DNA and RNA viruses is between 75S and 120S, and for filamentous viruses it is between 39S and 45S (19). According to Stokes law for the velocity of sedimentation, these values will be approximately 25% lower in seawater at 12°C.

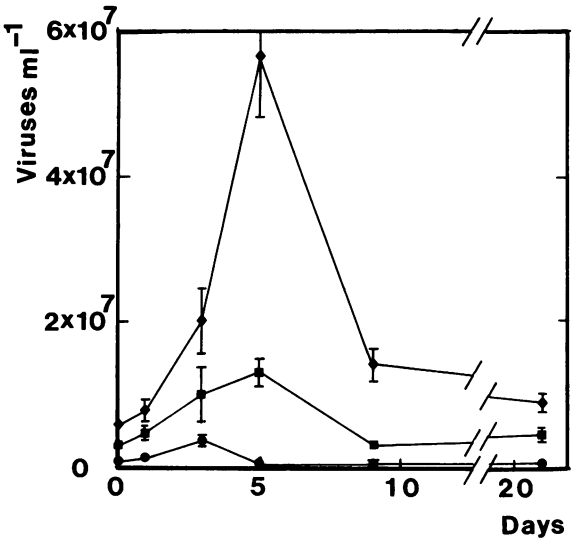


FIG. 3. Time course of virus concentration in seawater sample incubated in the dark at in situ temperature. Symbols: ●, viruses with head size of >80 nm; ■, head size of <80 and >60 nm; ◆, head size of <60 nm. Vertical bars indicate standard errors.

For the conditions we used for centrifugation, it may be calculated that particles larger than 280S were harvested with 100% efficiency while particles of 40S and 100S were harvested with efficiencies of 18 and 43%, respectively. Our virus counts may therefore be somewhat underestimated. For future work, we recommend the use of a centrifugation time and speed that at least is high enough to pellet 100S particles with 100% efficiency.

**Bacteria.** Use of the TEM allows for more accurate sizing and it gives more information about bacterial morphology than epifluorescence microscopy. It is possible to observe DAPI-stained viruses in the epifluorescence microscope (25, 26). We found no difference between total count of bacteria in the TEM and in the epifluorescence microscope, which may indicate that viruses are not counted as small bacteria during routine work. This does not exclude, however, the possibility that some of the "ultramicrobacteria" reported from epifluorescence microscopy studies may have been viruses.

The change in mean volume of bacteria observed during the incubation was partly due to population changes in which the frequency of the smallest size group (curved rods) showed a marked decrease. The mean bacterial volumes of the incubation experiment are based on random samples and therefore reflect the true population mean (Table 2). We suspected that the centrifugation might disrupt or distort the cells, and for X-ray analysis we chose large cells because such cells may be most affected. Therefore, the mean size of the bacteria in Table 1 is biased compared with the population mean.

For analytical purposes, the use of unfixed and unstained cells is important (14). Fixation and staining will lead to loss of unpredictable amounts of elements, especially potassium and chlorine, but also other elements, and low-molecular-weight organic constituents from the cells. The dry weight/volume ratio for bacteria in this study was higher than the ratios determined by the same method for freshwater and cultured bacteria (22). If carbon is assumed to constitute 50% of dry weight, a volume-to-carbon conversion factor of 300 fg of C $\mu m^{-3}$ can be calculated. This factor is approximately three times the frequently cited value of Watson et al. (29), 1.4 times higher than the conversion factor for unfixed bacteria suggested by Bratbak (6), and lower than factors suggested for fixed marine bacteria (4, 6, 20). The mean potassium content of the cells was in the range of that for *Nevskia* (15). In natural microbial communities, a fraction of 10 to 50% of the bacterial cells shows a potassium content close to or below the detection limit for the method, ca. 0.5 mM (Heldal, unpublished observations). For the analysis shown in Table 2, 25% of the cells were apparently without potassium. The phosphorus and sulfur contents at levels of 2 and 1% dry weight, respectively, are in the normal range for bacteria. Thus, we may conclude that sampling of bacterial cells at high gravitational force does not seriously disrupt the cells. For analysis of elemental composition, the samples should be harvested prior to any fixation and within 2 to 3 h after sampling. From our experience, we found that it was most convenient to first analyze and count bacteria and thereafter stain and count viruses on the same grid. Alternatively, we have used 400-mesh Cu grids for quantification of viruses and nylon grids for analysis of bacteria.

**Viruses.** Other investigators have used filtration to collect viruses for electron microscopy enumeration (28; L. Proctor, J. A. Fuhrman, and M. C. Ledbetter, EOS Transact. Am. Geophys. Union **69**:1111, 1988) or centrifugation combined with agar replica transfer techniques (7). Although we

have made no direct comparison, the reported numbers suggest that the direct harvesting method gives higher numbers than previous approaches. This is not unreasonable, because filtration procedures may lose the smallest size groups, and agar replica transfers must be expected to be less efficient than the very simple direct harvesting approach.

Studies of pure cultures of marine bacteriophages have shown that large variations in morphology and host specificity can be detected (11). In the present study, and in Bergh et al. (3), we found that the smallest size class of viruses dominated. Tails were rarely seen on these particles at the standard magnification used for counting, but closer examination of one sample at higher magnification showed that tails were severely underestimated in this size class. In the two larger size classes, tails or tail-like structures were common, and the viruses could be assigned to Bradley (5) groups A and B of bacteriophages. Typical viruses for eucaryotic hosts were rarely seen, but the tail-less viruses may belong to a wide range of hosts, including eucaryotes. However, we cannot rule out the possibility that tails were lost during the preparation. Because of the high concentrations and the high proliferation rates in the incubation experiment, we believe that the majority of the total counts of viruses in the samples examined were bacteriophages.

Bergh et al. (3) concluded that the high concentrations of bacteriophages in aquatic environments suggest that viruses may play important roles as agents of bacterial mortality and also mediate the genetic transfer between bacteria in nature. It is implicated that the rate at which phage infection and proliferation occur or have the capability of occurring becomes important for analysis of the role of viruses in microbial ecology.

The increase in total counts of viruses in the incubated seawater samples indicates that viruses of the kind we have observed are capable of fast proliferation. We do not know what factor triggered this proliferation, but confinement of water has been shown to influence bacteria in general (10). The observed increases in concentration of viruses may have been a result of lowered resistance of the bacteria to infection and lysis by virulent phages that has been induced by environmental changes involved in the handling of the sample. Another possible source of viruses is the release of prophages from previously infected bacteria, which also may have been induced by the handling of the samples. Bradley (5) argued that the primary role of bacteriophages in nature is to affect the evolution of associated bacterial populations. The high proliferation rates we observed suggest that viruses have the potential to act as selection factors also for aquatic bacteria. It may also be speculated that viruses may have been an important part of the changes usually denoted as bottle effects. We do not believe that the growth rate of viruses is representative of in situ production of phages, but the results indicate that an impressive proliferation potential of phages is present. We do not know which mechanisms control the phages in the undisturbed environment, but minor manipulations were enough to induce fast growth.

It may be interesting to compare the growth rates of bacteria and phages in terms of synthesis of genetic material. Bacterial production of DNA may include synthesis of bacteriophage DNA as well as synthesis of bacterial genomes. Incorporation of radiolabeled thymidine into DNA is one of the most widely used methods for measuring bacterial production in aquatic ecosystems (12). One of the implicit assumptions of this method is that the bacterial DNA synthesis is correlated with the growth of the bacterial population. In our incubation experiment, a substantial proportion of the bacterial DNA replication must have led to the production of phages. The average DNA content of marine bacteria is approximately 2.6 fg cell$^{-1}$ (13). The average DNA content of bacteriophages is about 0.08 fg of DNA virus$^{-1}$ (standard deviation of 0.06; $n = 96$ [data from reference 19]). During the first 24 h of incubation, the number of bacteria increased by $0.08 \times 10^6$ cells ml$^{-1}$ and the number of viruses increased by $4.3 \times 10^6$ viruses ml$^{-1}$. These values correspond to a calculated increase in bacterial and viral DNAs of 0.2 and 0.34 μg of DNA liter$^{-1}$, respectively. This shows that, during the first part of this incubation, viral DNA synthesis may have constituted approximately 62% of the total bacterial DNA synthesis.

It is interesting to note that the concentration of viruses decreased after the initial increase. This indicates that mechanisms for removing phage particles exist, as would be expected in a dynamic system. Viruses may adsorb to particles, either by specific adsorption to hosts that are resistant or nonspecifically to other types of particles. High activity of extracellular proteolytic enzymes has been shown in various aquatic environments (17), and enzymatic breakdown of capsid proteins may be one of the causes for the observed decline of virus total count. It has also been demonstrated that some protozoa are capable of ingesting high-molecular-weight substances (27). Protozoa may, therefore, also be able to ingest viruses and thus may be a factor in the control of viruses in natural communities. This hypothesis needs further investigation of uptake rates and digestion of particles within the viral size range of marine protozoa.

Reliable estimation of bacterial biomass has been important for progress in the study of microbial ecology, and by analogy to the history of bacterial total counts, it may be inferred that methods for counting viruses may aid in revealing the quantitative importance of these parasites in aquatic environments. From our point of view, the direct harvesting method we describe offers great advantages for studies of microbial communities. High-resolution power and analytical possibilities given important information on both structure and changes in planktonic microbial assemblages down to the level of single particles in the nanometer size range.

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