

Comparison of Light and Electron Microscopic Determinations of the Number of Bacteria and Algae in Lake Water

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Determinations of the number of microorganisms in lake water samples with the bright-field light microscope were performed using conventional counting chambers. Determinations with the fluorescence microscope were carried out after staining the organisms with acridine orange and filtering them onto Nucleopore filters. For transmission electron microscopy, a water sample was concentrated by centrifugation. The pellet was solidified in agar, fixed, dehydrated, embedded in Epon, and cut into thin sections. The number and area of organism profiles per unit area of the sections were determined. The number of organisms per unit volume of the pellet was then calculated using stereological formulae. The corresponding number in the lake water was obtained from the ratio of volume of solidified pellet/volume of water sample. Control experiments with pure cultures of bacteria and algae showed good agreement between light and electron microscopic counts. This was also true for most lake water samples, but the electron microscopic preparations from some samples contained small vibrio-like bodies and ill-defined structures that made a precise comparison more difficult. Bacteria and small blue-green and green algae could not always be differentiated with the light microscope, but this was easily done by electron microscopy. Our results show that transmission electron microscopy can be used for checking light microscopic counts of microorganisms in lake water.

Well-established methods exist for the determination of the number of algae in limnic environments (22, 26). On the other hand, corresponding methods for bacteria have been more or less missing, mainly because of the small size of these organisms. Plate counts have been carried out extensively, but only a minor part of bacteria taken directly from nature is recovered by this method. For total counts of limnic bacteria, erythrosin has been used (7, 11, 13, 16, 19), but this stain seems to have been superseded by fluorescent dyes, especially acridine orange (3, 6, 9, 27).

The morphology of limnic bacteria in their natural habitat has been studied with the electron microscope (5, 8, 10, 12). However, to our knowledge only two very recent reports have described electron microscopic counting procedures for bacteria. In both studies, whole organisms, retained on filters, were observed either with the scanning (4) or with the transmission electron microscope (23). In the latter case, replicas of the membranes with adhering bacteria were prepared. The aim of the present investigation was in the first place to compare light microscopic counts of bacteria and algae in lake water with figures obtained from morphometric

measurements carried out on micrographs of sections of the same organisms and thus to check the reliability of both methods. These studies were supplemented by structural studies of the sectioned microorganisms. Some preliminary results have been published elsewhere (25).

MATERIALS AND METHODS

Bacterial and algal strains and growth conditions. Pure cultures of some bacteria and algae were used to test the counting procedures developed. *Proteus mirabilis* D 52 (21) was grown overnight in beef extract broth (Difco Laboratories, Inc., Detroit, Mich.) at 37°C. *Pseudomonas fluorescens*, a strain isolated from Lake Bysjön, was cultured overnight at 20°C in 0.1% peptone (Difco). *Pandorina morum*, strain 60/La Churda, Czechoslovakia (obtained from the Culture Collection of Algae and Protozoa, Cambridge, England), was grown at 20°C in a glycine-salts medium (15) for 30 days.

Lake water samples. All but one sample were taken from a small eutrophic lake, Lake Bysjön (area, 0.14 km²; maximum depth, 8 m) located 45 km east of the city of Malmö, Sweden. The remaining sample was taken from a pond (area, 4,800 m²; maximum depth, 3 m) at Lomma, 5 km north of the same city. Stratification did not occur in the pond. The samples were immediately fixed with Lugol solution (20) for bright-field microscopy, with glutaraldehyde to 5% for flu-

orescent microscopy, or with formaldehyde to 4% for electron microscopy.

Light microscopy. A Bürker counting chamber having a depth of 0.1 mm was used to count bacteria and algae in pure cultures under the bright-field microscope. To count algae in water samples, the samples (0.3 to 1 ml) were allowed to sediment in 2-ml chambers according to the Utermöhl method (22). The algae were counted in an inverted bright-field microscope at $\times 100$ or $\times 400$ magnification.

For counting bacteria in a lake water sample with the fluorescence microscope, the sample was kept at about 5°C before staining, which was performed not more than a week after sampling. For staining, the sample (5 to 20 ml) was mixed with an equal volume of an 0.01% acridine orange solution. After 30 s, the mixture was filtered through a Nuclepore filter (pore size 0.22 μm , diameter 47 mm). The filter was rinsed twice with 10 ml of 5×10^{-4} M potassium phosphate buffer (pH 7.2) and then allowed to dry. The acridine orange solution and the buffer were filtered through a membrane filter (pore size, 0.22 μm ; Millipore Corp., Bedford, Mass.) before use. The bacteria were counted at $\times 1,250$ magnification using a Zeiss Standard RA microscope equipped with a microscope illuminator 100 for epifluorescence, a 50 W HBO super pressure mercury lamp, and a 510 reflector. The excitation filters KP 490 and 500 were used in combination together with a LP 528 barrier filter.

Electron microscopy. For electron microscopy, 5 to 10 liters of lake water was fixed with formaldehyde to 4% and centrifuged batchwise at 0°C and $17,500 \times g$ (average value) for 90 min. The pellet was washed three times by centrifugation at $48,000 \times g$ with 0.2 M Michaelis buffer (pH 6.1). The final sediment was mixed at 50°C with about five times its volume of melted 3% agar. The mixture was centrifuged at 50°C and $48,000 \times g$, and the clear supernatant was sucked off. The pellet was thoroughly stirred with a glass rod and weighed after solidification. Its density was considered close to unity. Thus, a concentration factor expressing the ratio between the volume of the original water sample and the pellet could be calculated. Before fixation, the pellet was cut into cubes. The cubes were treated with osmium tetroxide and uranyl acetate as described by Ryter and Kellenberger (17), dehydrated with alcohol, and embedded in Epon. Silver-colored sections were cut from at least four cubes by using a diamond knife and an LKB Ultratome III microtome. Diatoms were not seriously damaged when sectioned. The sections were covered by evaporation with a thin layer of carbon. Thus, shrinkage of the sections under the electron beam was avoided. To avoid observations at multiple slices of the same organisms, only one section in a ribbon of several was studied. The micrographs were taken with a Philips EM 300 electron microscope working at an accelerating voltage of 60 kV and equipped with a 30- μm objective aperture. The specimen was surrounded by a liquid nitrogen anticontamination device. The magnification of the microscope was calibrated with a carbon replica of a crossed-lines grating having a spacing in both directions of 0.463 μm . The organisms were photographed at an instrumental magnification of $\times 1,000$ to $\times 12,000$. The micrographs were enlarged 6 to 10 times when printed.

Stereology. The number of microorganisms in a concentrated sample (pellet obtained by centrifugation) was calculated from the formula (24) $N_V = (K/\beta) \times (N_A^{3/2}/V_V^{1/2})$, where N_V is the number of organisms per unit volume. K is a factor depending on the size distribution of the organisms studied. It can be put equal to unity when the highest degree of accuracy is not essential. β is related to the ratio between the length and width of the organisms. Average values of this ratio were determined from measurements made on the organisms with the fluorescence microscope. When this ratio equals 2, β is ~ 1.8 ; for spheres, β equals 1.4. N_A is the number of sectioned organisms per unit area. V_V , the volume density of the organisms, is equal to A_A , the area of the sectioned organisms per unit of containing area, i.e., the area density. The latter quantity was determined by the cut-and-weight method, i.e., all profiles were traced on paper, a known area of this paper was weighed, and the profiles were cut out and weighed.

The formula given above can be rewritten thus: $N_V = (M/c) \times [(bd^2/a)^{1/2}]$. M is the magnification of the micrograph, c the area of the section scanned for organisms, d the number of organisms counted, b the weight of the profiles (organisms) cut out, and a the weight of a unit area of the paper used for tracing the sectioned organisms. The number of organisms in a unit volume of the original lake water sample was obtained by dividing N_V by the pertinent dilution factor, i.e., the ratio between the volume of the sample and the volume (= weight) of the sediment obtained by centrifugation of the same sample. Between 60 and 1,000 photographed organisms were studied to obtain the number of each organismal type.

RESULTS

The results of light and electron microscopic determinations of the number of cells in pure cultures of algae and bacteria are shown in Table 1. The light microscopy was carried out using either bright-field illumination or fluorescence technique. As can be seen, the three counting methods gave results agreeing within the error limits.

Most of the bacteria in lake water samples appeared red to orange, and a few green, under the fluorescence microscope. This agrees with the results of Zimmermann and Meyer-Reil (27) and of Hobbie et al. (9). The bacteria exhibited a distinct morphology and were thus rather easily distinguished from detritus particles. Even bacteria attached to such particles could be counted. It was somewhat more difficult to distinguish orange fluorescing bacteria from small blue-green algae, especially in samples from the upper part of hypolimnion, where the bacteria often were larger than in the epilimnion. Here, sizes of bacteria and blue-green algae often overlapped. Some samples were stained with erythrosin, but the counts obtained were about five times lower than those obtained using acridine orange.

TABLE 1. Comparison between different methods for counting cells in pure cultures of bacteria and algae^a

Sample (liquid culture)	Light microscopy		Electron microscopy
	Bright field	Fluorescence	
<i>Proteus mirabilis</i>	2,780 ± 200 ^b	ND ^c	3,070 ± 270
<i>Pseudomonas fluorescens</i>	290 ± 14	320 ± 38	ND
<i>Pandorina morum</i>	1.03 ± 0.07	ND	0.99 ± 0.05

^a Figures indicate number of organisms per milliliter × 10⁶.

^b Standard error of the mean.

^c Not determined.

In most cases it was possible to distinguish between bacteria and small algae, as well as between bacteria and detritus, in the electron micrographs of the sectioned sediments obtained from lake water samples. However, some ill-defined structures were encountered. Probably part of them were tangentially cut organisms. In addition, a considerable number of vibrio-like bodies having a width of only 0.1 to 0.2 μm were seen. They could not with certainty be designated as bacteria on the basis of their structure. Because of their small dimensions they were probably not seen in the light microscope, even if retained on the filters. Bacteriophages and bacteriophage-infected cells were also seen. Thus, part of the dubious structures observed could represent cell components set free by phage attacks. Most of the well-defined bacteria had envelopes consisting of two "unit membranes" and in some cases a thin, electron-dense stratum between them. The envelope was thus similar to that characteristic of gram-negative bacteria. Bacteria with thinner envelopes were also seen, but no forms similar to typical gram-positive bacteria. Many cells had a width of <0.5 μm. The categories mentioned are illustrated in Fig. 1 and 2.

The results of determinations of the number of bacteria in five water samples are given in Table 2. The figures pertaining to the hypolimnion of Lake Bysjön and to the Lomma pond showed the clearest agreement between the light and electron microscopic counts, because the number of ill-defined structures and vibrio-like bodies was lower in these samples than in the epilimnetic ones. It should also be mentioned that the greatest number of bacteriophages was seen in the epilimnetic samples.

Table 3 shows the number of algae in three water samples. The difference between the light and electron microscopic counts of the sum of the algae in one sample (Lake Bysjön 1977) is statistically significant (0.05 > P > 0.01), even if not very pronounced. The counts of the remain-

ing samples agree within the error limits (P > 0.05). It should be emphasized that a distinction between the green and blue-green algae in the Lomma pond could not be made with the light microscope. However, their electron microscopic structure was quite different (Fig. 3). Thus the two kinds of algae could be counted separately.

DISCUSSION

From the experiments carried out elsewhere (10) and by ourselves, we conclude that the method employing acridine orange or some other acridine-based fluorescent stain is the best one now available for light microscopic counts of bacteria in natural waters.

In samples from the hypolimnion prepared for light or fluorescence microscopy, minor numbers of clumped organisms or microcolonies were observed. The individual cells of these aggregates were counted. Dispersion of the aggregates by a short ultrasonic treatment did not increase the counts significantly.

Several sources of error were encountered in the electron microscopic determinations. To sediment the microorganisms in the water samples, a centrifugal field as strong as possible, considering the volumes to be handled, was used in combination with the longest practicable centrifugation time. Control experiments indicated that practically all the cells in diluted pure cultures of *P. mirabilis* and *P. fluorescens* sedimented under these conditions. This was also true for the unicellular algae in the water samples. However, investigations with the fluorescence microscope of the lake water samples revealed that a considerable number of fluorescent bodies, especially coccoid ones, remained in the supernatant fluid after the centrifugation procedure associated with the electron microscopic counts. This may be due to the small size of the limnic organisms or to gas vacuoles present in these organisms. The numbers given in Table 2 and pertaining to electron microscopy are corrected for the incomplete sedimentation of the samples during centrifugation.

The high centrifugal field applied and the treatment at 50°C might damage sensitive organisms. However, the general appearance of the algae and bacteria did not suggest damage in the form of shrunken or deformed cells, coagulated cell contents, or similar abnormalities.

It could be assumed that the volume of the agar blocks containing the sedimented water organisms would change during the dehydration and embedding procedures. Control experiments with agar disks showed, however, that no significant changes occurred. On the other hand, it was found that the surface of the sectioned material was compressed on an average by 10%

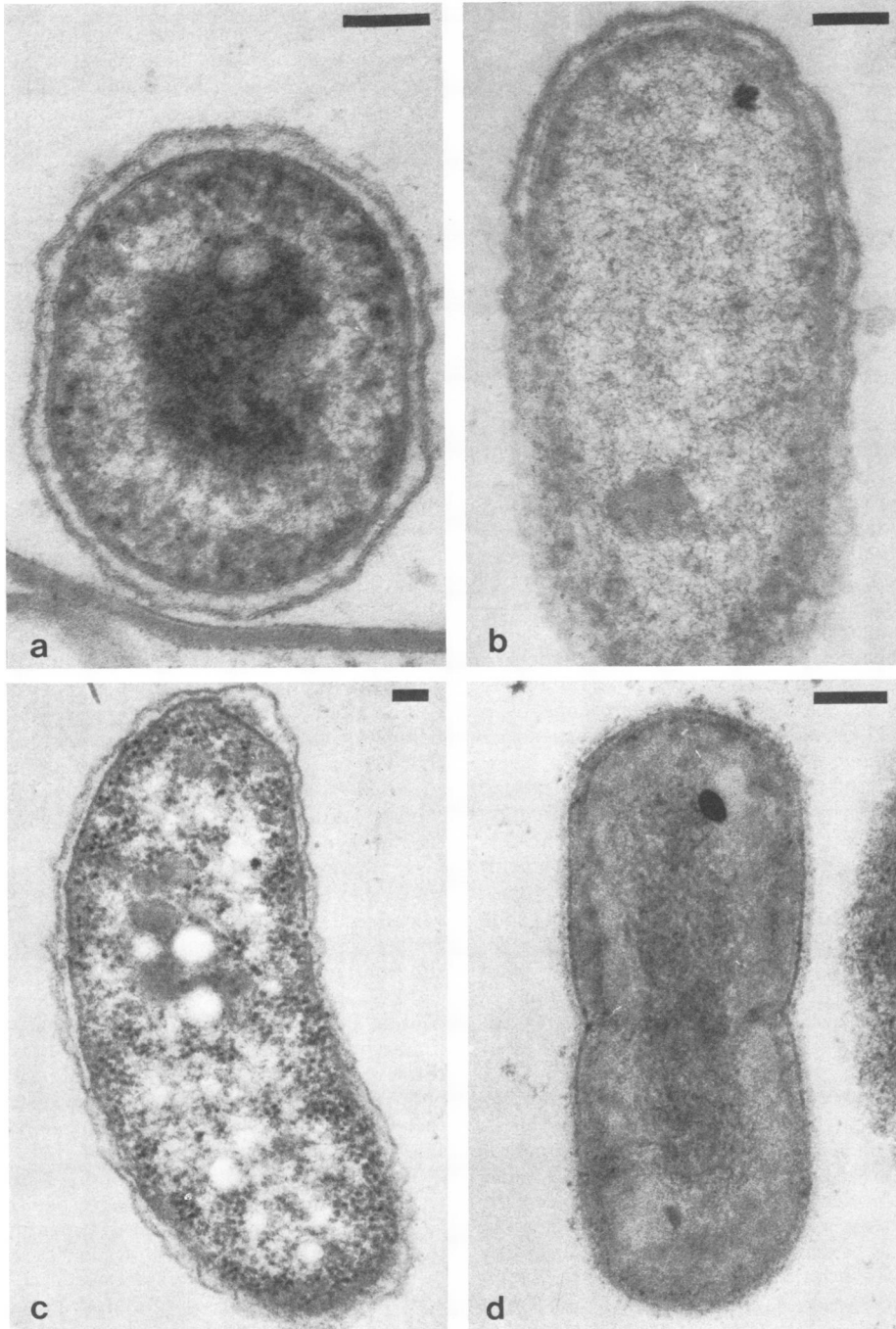


FIG. 1. Bacteria found in water samples from Lake Bysjön (a-c) and from a pond at Lomma (d). (a) Bacterium with a dark central body of unknown nature and an envelope consisting of two unit membranes. (b) Bacterium with an envelope consisting of two unit membranes and a thin, central, electron-dense layer. (c) Slightly curved bacterium. (d) Bacterium with a rather compact envelope and cytoplasm. All bars represent 0.1 μm .

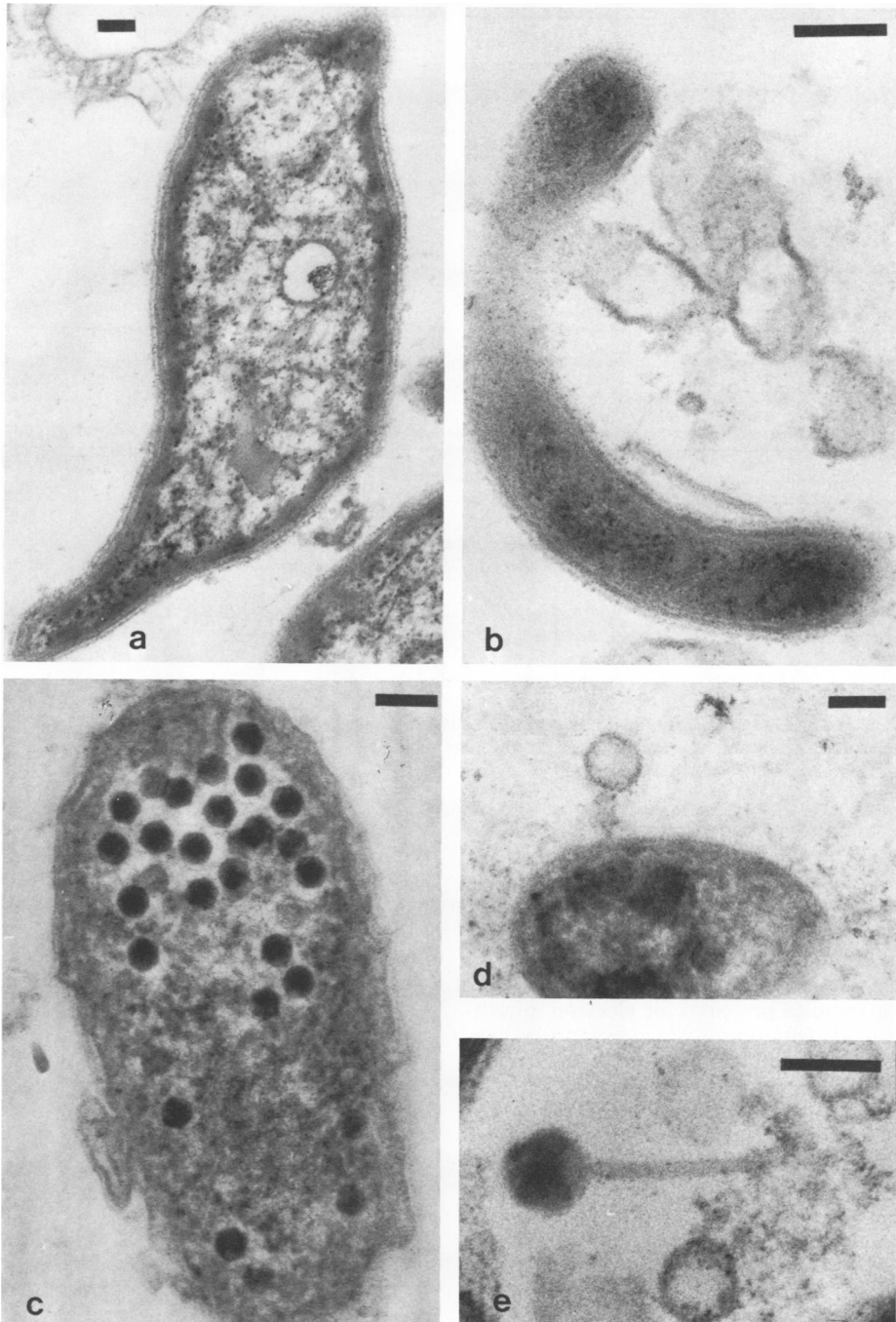


FIG. 2. Bacteria and structured bodies found in water samples from Lake Bysjön. (a) Photosynthetic bacterium, probably a *Pelodictyon* sp. (b) Small, vibrio-like body. (c) Bacteriophage-infected cell. (d) Bacterium with attached, empty bacteriophage. (e) Free bacteriophage. All bars represent 0.1 μm .

TABLE 2. Number of bacteria in water samples determined by light (fluorescence) and electron microscopy^a

Sample	Fluorescence microscopy: bacteria	Electron microscopy		
		Well-defined bacteria	Small vibrio-like bodies	Ill-defined structures, perhaps bacteria
Hypolimnion, Lake Bysjön, 28 July 1975	6.9 ± 0.6 ^b	5.1 ± 0.7	1.8 ± 0.4	0.7 ± 0.1
28 July 1976	12.6 ± 1.6	13.4 ± 1.2	0.5 ± 0.1	1.8 ± 0.5
Epilimnion, Lake Bysjön, 29 April 1976	3.2 ± 0.5	5.1 ± 1.0	8.2 ± 2.4	3.5 ± 0.7
28 April 1977	4.5 ± 0.4	3.6 ± 0.3	2.2 ± 0.2	0.7 ± 0.1
Pond, Lomma, 5 April 1977	18.5 ± 2.0	21.9 ± 2.4	<0.1	2.4 ± 0.3

^a Figures indicate number of organisms per milliliter × 10⁶.

^b Standard error of the mean.

TABLE 3. Number of algae in water samples determined by light (bright-field) and electron microscopy^a

Sample	Algal species	Light microscopy	Electron microscopy
Hypolimnion, Lake Bysjön, 28 July 1976	<i>Cryptomonas</i> sp.	7.7 ± 0.4 ^b	9.6 ± 1.0
	<i>Chlamydomonas</i> sp.	23.1 ± 1.9	25.5 ± 0.9
	<i>Cryptomonas</i> or <i>Chlamydomonas</i> sp.	0	2.2 ± 0.5
Epilimnion, Lake Bysjön, 28 April 1977	<i>Stephanodiscus hantzschii</i> (Grunow)	23.8 ± 1.1	18.6 ± 2.6
Pond, Lomma, 5 April 1977	Small blue-green algae	3.0 ± 0.4	1.0 ± 0.1
	Small green algae		3.3 ± 0.6

^a Figures indicate numbers of organisms per milliliters × 10³.

^b Standard error of the mean.

during sectioning. A further shrinkage of the sections occurred under the electron beam, if the sections were not covered by a carbon layer. The carbonizing effectively prevented this shrinkage. Finally, the magnification of the microscope had to be checked carefully. Deviations of up to 10% from the nominal magnifications were noted.

In the samples prepared for electron microscopy, the distribution of the organisms was somewhat uneven. This unevenness, reflected in the standard errors of the counts, could not be entirely eliminated by vigorous stirring of the mixture of pelleted organisms and melted agar obtained during the preparation procedure (see Materials and Methods).

The cut-and-weigh method used to calculate the area density of the microorganisms in the sectioned material is laborious, but it was considered the most suitable one for the present study, since this density was rather low, and the area of many profiles was small. Thus, linear integration or point counting (24) would have given results of lower precision. However, these methods might be useful in combination with more extensive series of electron microscopic counts than those performed by us.

The electron microscopic counts of bacteria reported by other investigators (4, 23) were carried out on whole cells. As pointed out in a study of the morphology of soil bacteria (2), investigations on whole organisms provide more information on cell shapes than do studies of sections, whereas the reverse is true with regard to cell structures. The sectioning method is probably more laborious, but it is doubtful whether differential counts of the kind described by us between bacteria and small algae and between different types of bacteria or small algae could be performed on whole cells.

It is obvious that hardly any electron microscopic counting procedure could be used as a routine tool in ecological investigations. With respect to the morphology of well-defined bacteria, our observations largely agree with those made by other investigators on limnic and terrestrial bacteria in their natural habitat (1, 5).

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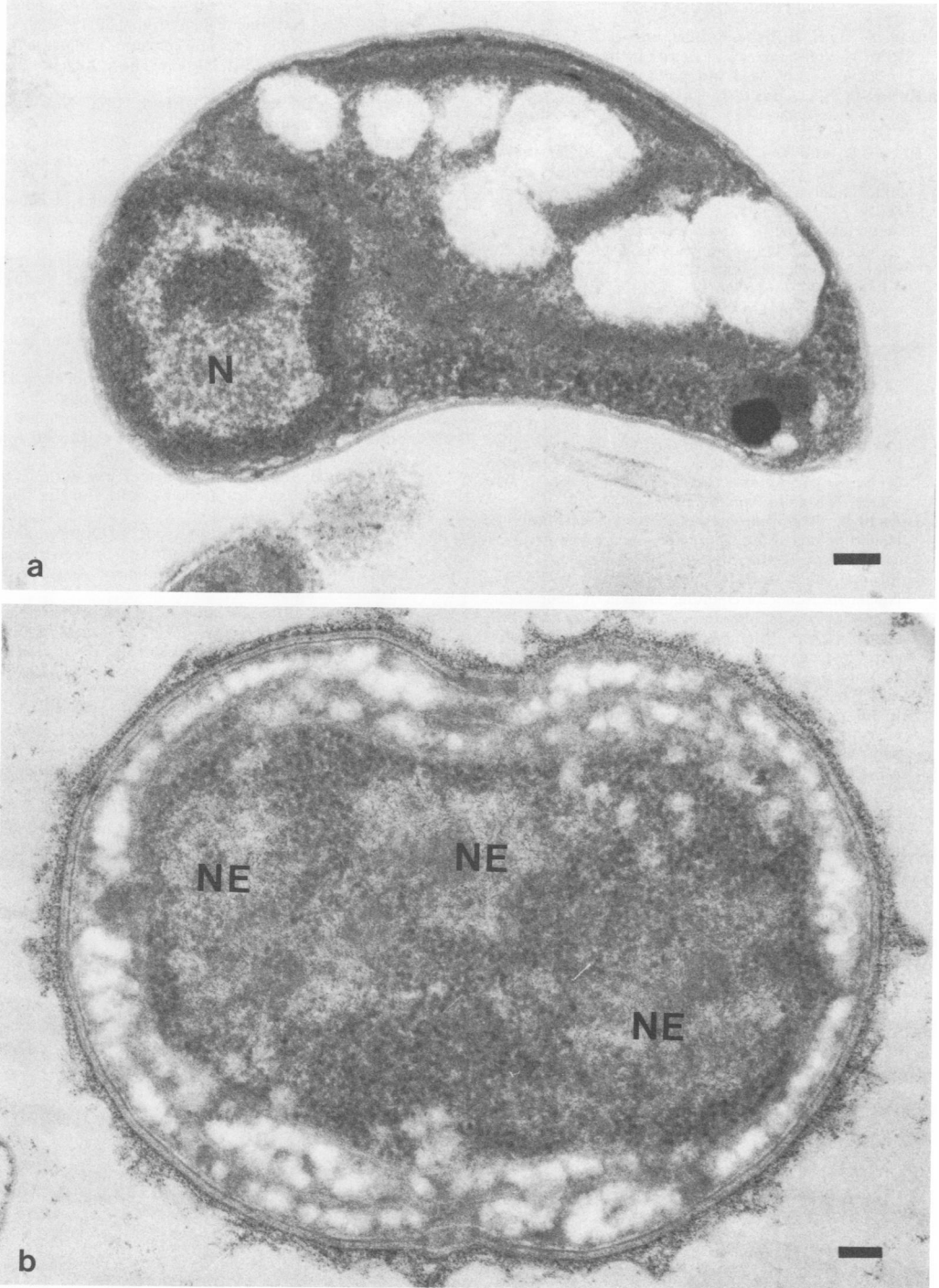


FIG. 3. Algae found in a pond at Lomma. (a) Green alga with nucleus (N). (b) Blue-green alga with nuclear equivalent (NE). The bars represent 0.1 μ m.

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