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Development and Field Application of a Quantitative Method for Examining Natural Assemblages of Protists with Oligonucleotide Probes†

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A fluorescent in situ hybridization method that uses rRNA-targeted oligonucleotide probes for counting protists in cultures and environmental water samples is described. Filtration, hybridization, and enumeration of fixed cells with biotinylated eukaryote-specific probes and fluorescein isothiocyanate-conjugated avidin were performed directly on 0.4-μm-pore-size polycarbonate filters of Transwell cell culture inserts (Costar Corp., Cambridge, Mass.). Counts of various species of cultured protists by this probe hybridization method were not significantly different from counts obtained by the 4′,6-diamidino-2-phenylindole (DAPI) and acridine orange (AO) staining methods. However, counts of total nanoplankton (TNAN) based on probe hybridizations in several field samples and in samples collected from a mesocosm experiment were frequently higher than TNAN counts obtained by staining with DAPI or AO. On the basis of these results, 25 to 70% of the TNAN determined with probes were not detectable by DAPI or AO staining. The underestimation of TNAN abundances in samples stained with DAPI or AO was attributed to the existence of small nanoplanktonic cells which could be detected with probes but not DAPI or AO and the difficulty associated with distinguishing DAPI- or AO-stained protists attached to or embedded in aggregates. We conclude from samples examined in this study that enumeration of TNAN with oligonucleotide probes provides estimates of natural TNAN abundances that are at least as high as (and in some cases higher than) counts obtained with commonly employed fluorochrome stains. The quantitative in situ hybridization method we have described here enables the direct enumeration of free-living protists in water samples with oligonucleotide probes. When combined with species-specific probes, this method will enable quantitative studies of the abundance and distribution of specific protistan taxa.

Protists in the nanoplankton size class (2 to 20 μm) are integral components of marine and freshwater ecosystems. They numerically dominate the eukaryotic plankton and are responsible for important trophic processes in planktonic food webs (see references 4 and 5, references therein, and reference 7). The photosynthetic nanoplankton, which consists of a variety of pigmented flagellates, chlorophytes, and some small diatoms, often dominates total primary production in oceanic environments. The heterotrophic nanoplankton consists of colorless flagellates, amoeboid forms, and smaller ciliates. These heterotrophs are the primary consumers of bacteria, cyanobacteria, and small algae (9, 19, 21, 24, 26, 30) and have also been implicated as major nutrient remineralizers in aquatic environments (6, 8).

Although the significance of nanoplanktonic protists in aquatic food webs has been well established, relatively little information on the population structure and diversity of the nanoplankton in the water column is available. Ecological studies demonstrating the role of nanoplanktonic protists in microbial food webs tend to ignore the diversity of this group and imply that all species (aside from the distinction between photosynthetic and heterotrophic species) have similar ecological functions. The spatial and temporal distribution of taxa within this group are also poorly known. These shortcomings in our view and understanding of protistan ecology result largely from our limited ability with existing techniques to identify and enumerate individual species of naturally occurring protists.

Electron microscopy and epifluorescence microscopy are the current methods used for examining protists in natural samples. Both methods possess inherent advantages and disadvantages. Electron microscopy provides details of ultrastructure for identification of protists that are not apparent with light microscopy. This method, however, is impractical for analyzing large numbers of samples because it is time-consuming and expensive. Electron microscopy also does not permit accurate estimates of cell abundance. In contrast, epifluorescence microscopy is relatively rapid and routinely used to quantify nanoplankton abundances. A variety of fluorochrome dyes such as 4′,6-diamidino-2-phenylindole (DAPI), acridine orange (AO), primulin, fluorescein isothiocyanate (FITC), and proflavin are used to stain prokaryotic and eukaryotic cells in water samples for visualization by epifluorescence microscopy (25). While this method is versatile for counting nanoplanktonic cells, ultrastructural features that are vital for species identification are generally not apparent at the magnifications employed.

Another approach that has been applied recently for detecting and identifying microbial cells employs oligonucleotide probes complementary to specific sequences on the rRNA (29). Oligonucleotide probes that can discriminate between microorganisms at the kingdom to the subspecies level have been designed (2, 3, 11–13). When these probes are labeled with fluorescent dyes (either directly or indirectly), they can be used for the detection of target organisms by epifluorescence microscopy, confocal laser scanning microscopy, or flow cytometry (1, 10, 20, 27).

Hybridization methods applying oligonucleotide probes have

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been largely qualitative to date. Probe hybridizations are performed with preserved cells either attached to glass slides coated with gelatin or in solution (1, 13, 18). Cells processed in solution must undergo several centrifugation and resuspension steps, which may cause cell loss. Similarly, repeated washes of gelatin-coated slides typically remove some portion of the attached cells. Because of potential cell losses at these steps, hybridization procedures with oligonucleotide probes, whether for culture samples or field samples, have not been quantitative, although some attempts at relative quantitation have been made (15, 17, 22).

This article describes a new quantitative method for in situ hybridization and detection of marine and freshwater protists that employs eukaryote-specific rRNA targeted probes. Hybridization, detection, and enumeration of protists with biotinylated probes and FITC-avidin were performed with samples concentrated onto polycarbonate filters of Transwell cell culture inserts (Costar Corp., Cambridge, Mass.). Nanoplankton cell counts of cultures and field samples obtained by this probe hybridization technique were compared with cell counts obtained by the DAPI and AO methods. Probe counts of the nanoplankton by our quantitative in situ hybridization method were comparable to, and in many cases higher than, DAPI or AO counts. We conclude that the detection of total nanoplankton (TNAN) with oligonucleotide probes may provide a more representative estimate of natural TNAN abundances than has been previously possible. More importantly, we present a quantitative method for applying oligonucleotide probes as a tool for examining and enumerating natural nanoplankton assemblages.

MATERIALS AND METHODS

Cell cultures. Three heterotrophic flagellates, *Paraphysomonas imperforata* (chrysophyte; clone A obtained from J. Eccleston, Lancaster University, Lancaster, United Kingdom), *Paraphysomonas bandaiensis* (chrysophyte; clone WH1 obtained from J. Waterbury, Woods Hole Oceanographic Institution, Woods Hole, Mass.), and *Cafeteria* sp. (bicosoecid; clone Cflag from our culture collection [isolated from seawater aquarium]), and a ciliate, *Uronema* sp. (clone BBcil from our culture collection; isolated from Buzzards Bay, Woods Hole, Mass.), were grown in liquid cultures of the marine bacterium *Halomonas halodurans* (ATCC 29686). The phytoplankton cultures used were *Minutocellus polymorphus* (diatom from our culture collection; isolated from Great South Bay, Long Island, N.Y.), *Chrysochromulina ericina* (prymnesiophyte; clone NEPCC109A obtained from the Center for Culture of Marine Phytoplankton, Bigelow Laboratory of Ocean Sciences, Bigelow, Maine), *Ochromonas* sp. (chrysophyte; clone VT1 from the Center for Culture of Marine Phytoplankton), and *Alexandrium minutum* (dinoflagellate; clone 1 obtained from D. Anderson, Woods Hole Oceanographic Institution). These phytoplankton were grown on f/2 medium in natural seawater (14). Cultures in the late exponential phase of growth were preserved with formaldehyde to a final concentration of 3.7% and stored at 4°C. Samples were processed between 2 h and 3 days after fixation.

Sampling locations of field survey. Water samples were collected from three sites around the southwestern part of Cape Cod, Mass., and from one site in the Sargasso Sea in October 1994. The sampling locations were Deep Pond (Hatchville), Great Harbor (Woods Hole), and Sippiwissett Marsh (West Falmouth) in Massachusetts and the site of the Bermuda Atlantic Time-Series station in the Sargasso Sea. At each site, samples were collected at a depth of approximately 0.1 to 0.2 m and preserved with formaldehyde to a final concentration of 3.7%. Preserved samples were stored in the dark at 4°C and processed within 3 days.

Field experiment. Samples were collected from a mesocosm experiment conducted as part of a Land Margin Ecosystem Research project by the Marine Biological Laboratory Ecosystems Center, Woods Hole, Mass. This experiment was designed to examine the interaction of dissolved organic carbon (DOC) and dissolved inorganic nutrients (DIN) with the dynamics of coastal pelagic food webs. The experiment was run for 3 weeks in September 1994 off the Northeast Marine Fisheries Services jetty in Woods Hole, Mass. Each treatment consisted of $\approx$7.5 m^3 of seawater contained in polypropylene bags incubated in situ. The experimental treatments were the following: (i) no addition of DOC and DIN (control); (ii) batch addition of DOC in the form of leaf litter leachate at a final concentration of $\approx$350 μM C; (iii) daily additions of DIN which consisted of a final concentration of 5 μM KNO_3, 0.5 μM KH_2PO_4, and 7 μM $NaSiO_3 \cdot 9H_2O$; (iv) addition of DOC and DIN as in treatments (ii) and (iii) above. One mesocosm was established for each treatment. Samples from each treatment were collected and preserved with formaldehyde (final concentration, 3.7%) for nanoplankton counts at the start of the experiment and on days 2, 3, 4, 6, 8, and 10.

Oligonucleotide synthesis and labeling. The following probes were used for detection and enumeration of nanoplankton cells (the numbers correspond to *Escherichia coli* 16S rRNA sequence positions): Euk 1209 (5′-GGG CAT CAC AGA CCT G-3′) (13), Euk 502 (5′-ACC AGA CTT GCC CTC C-3′) (1), and Euk 309 (5′-TCA GGC TCC CTC TCC GG-3′) (28). These probes are complementary to regions on the small-subunit (SSU) rRNA that are conserved for all eukaryotes.

The oligonucleotides were synthesized with an amino group at the 5′ terminus (Euk 502 and Euk 309 by Protein and Nucleic Acid Center, Woods Hole, Mass.; Euk 1209 by Operon Technologies, Inc., Alameda, Calif.). The amino groups of the oligonucleotides were coupled with biotin (Molecular Probes, Inc., Eugene, Oreg.), purified through Sephadex G-25 columns, and finally purified by polyacrylamide gel electrophoresis as described by Lim et al. (18).

Sample processing for enumeration of protists by oligonucleotide probes. The in situ hybridization procedure for counting protists is summarized diagrammatically in Fig. 1. The basis of this method is described by Lim et al. (18) for hybridization of protists with biotinylated probes and FITC-avidin on glass slides. The present technique entails adapting that method for performing in situ hybridizations with Transwell cell culture inserts (Costar). The feasibility of enumerating protists in culture samples and in field samples by this method was examined. A combination of two eukaryote-specific probes was used in hybridizations of cultured protists, and three eukaryote-specific probes were used for natural assemblages of protists.

Preserved samples (2 to 25 ml) were drawn down onto polycarbonate filters (25-mm diameter, 0.4-μm pore size) of Transwells at a vacuum no greater than 200 mm Hg (ca. 26.7 kPa). A 3.0-μm-pore-size 25-mm Millipore filter was placed between the Transwell and the fritted-glass filtration base (Millipore) to promote even dispersion of cells on the filters of the Transwell. Transwells containing the concentrated samples were then transferred to six-well tissue culture dishes, and 400 μl of 50% ethanol was added to each Transwell, incubated for 2 min, and removed by gentle vacuum. This process was sequentially repeated with 75 and 100% ethanol to dehydrate the samples. The Transwells were similarly placed in tissue culture trays during subsequent incubations, and solutions were drawn away by vacuum filtration following each incubation throughout the hybridization procedure. After dehydration, the Transwells were air dried in the tissue culture tray, and 200 μl of hybridization buffer (10× Denhardt's solution, 0.1 mg of polyadenylic acid ml^{-1}, 5× SET buffer [750 mM NaCl, 100 mM Tris-HCl {pH 7.8}, 5 mM EDTA], 0.1% sodium dodecyl sulfate) was added to each Transwell. Each tray of Transwells was then placed in a resealable bag that contained a piece of buffer-saturated tissue paper at the bottom of the bag and incubated at 40°C for 45 min. Following this prehybridization step, 1 μl of each probe (stock concentration, 0.5 $\mu g\ \mu l^{-1}$) was added to each Transwell to obtain a final probe concentration of 2.5 ng μl^{-1} and allowed to hybridize at 40°C overnight. The trays were shaken occasionally during hybridization to ensure that the surface of the filters remained wet. At the completion of probe hybridization, each filter was washed by adding 2 ml of 0.2× SET buffer (30 mM NaCl, 4 mM Tris-HCl [pH 7.8], 0.2 mM EDTA) prewarmed to 45°C and incubating for 10 min. The filters were then rinsed once with 0.2× SET buffer and allowed to air dry. To detect the probed cells, 200 μl of FITC-avidin solution (20 $\mu g\ ml^{-1}$ in 100 mM $NaHCO_3$-buffered saline [pH 8.2]; Vector Laboratories, Inc., Burlingame, Calif.) was added to each Transwell and incubated in the dark for 10 min at 4°C. Samples were subsequently washed three times with cold $NaHCO_3$-buffered saline (100 mM) and allowed to air dry in the dark.

To examine the samples, the filters were cut out of the Transwells with a dissecting knife and placed as flat as possible on glass slides coated with a thin film of immersion oil (Citifluor, Ltd., London, England). One drop of Citifluor, followed by a coverslip, was then placed on the center of each filter. The filters were flattened by inverting the slide onto a flat surface and pressing gently. Cells were observed and counted with a Zeiss standard microscope equipped for epifluorescence microscopy. The area of the fritted-glass filtration base was used as the area covered by the samples for calculating cell density. Filter sets used for fluorescence observations were as follows: for DAPI, a G365 exciter filter, an FT420 chromatic beam splitter, and an LP418 barrier filter; for AO and FITC, a BP450-490 exciter filter, an FT510 chromatic beam splitter, and an LP520 barrier filter. Epifluorescence micrographs of the samples were taken with an Olympus OM-4T camera with Ektachrome 200 ASA color film.

Duplicate hybridizations were performed with each culture examined, and triplicate hybridizations were conducted with each of the samples collected from different locations in the field survey. Only one hybridization was carried out for each sample collected during the mesocosm experiment because of the large number of samples that had to be processed in a short period. To enumerate the nanoplankton cells, approximately 15 to 40 fields were observed. The total number of cells counted ranged from approximately 35 (Sargasso Sea samples) to 1,000 (culture and enriched mesocosm samples).

Sample processing for enumeration of protists by DAPI and AO staining. Probe counts of protists were compared with counts obtained by the conventional DAPI and AO staining methods to examine the accuracy of cell counting by the quantitative in situ hybridization method. The procedures for fluorochrome staining were as described by Sherr et al. (25). Briefly, preserved water samples were vacuum filtered onto 0.8-μm-pore-size blackened polycarbonate

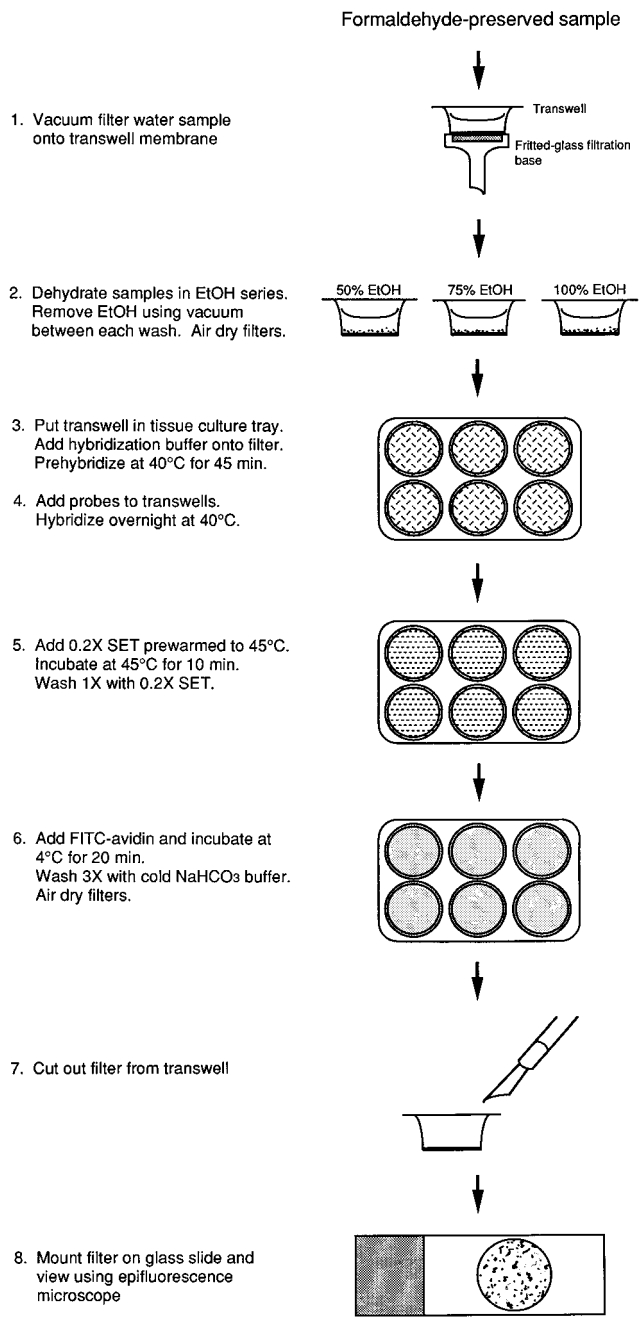

FIG. 1. Flow diagram of the quantitative in situ hybridization method with biotinylated probes and FITC-avidin. EtOH, ethyl alcohol.

filters (Nuclepore) and stained with either DAPI or AO for 10 and 3 min, respectively. The final concentration of DAPI used was 5 μg ml^{-1}. In addition, all of the field samples were stained with DAPI at a final concentration of 50 μg ml^{-1} and counted to ensure that the 5-μg ml^{-1} concentration was sufficient to thoroughly stain the nanoplanktonic cells. The final concentration of AO used was 0.01%.

Enumeration of TNAN by each of the three labeling techniques (probes, AO staining, and DAPI staining) was performed for slides prepared from subsamples of the same preserved sample. Several samples were also dual labeled with probes and DAPI for taking photographs. DAPI and AO counts were performed for all of the samples collected and processed for cell counts by probe hybridization with one exception. In the mesocosm experiment, AO counts were performed only on the final samples of the experiment (day 10). The number of slides prepared and fields examined for each sample were otherwise the same as those described for the hybridization method.

Because filters with two different pore sizes were used for enumerating protists (0.4 μm for the in situ hybridization method versus 0.8 μm for the DAPI and AO methods), counts of TNAN with 0.4- and 0.8-μm filters were compared to ensure that both types of filters trap the same number of cells. A seawater sample collected from Eel Pond, Woods Hole, Mass., was stained with DAPI, and a culture of a *Nannochloris*-like alga (clone BT3; isolated from Great South Bay, Long Island, N.Y.), 1 to 3 μm in diameter, was stained with 5-(4,6-dichlorotriazin-2-YL)-aminofluorescein (DTAF) and enumerated.

RESULTS

Quantitative in situ hybridization of protists with probes. The key to the quantitative in situ hybridization technique was the use of Costar's Transwell cell culture inserts. The Transwell is essentially a small chamber with a porous polycarbonate membrane filter at the base. This design allowed the insert to serve as a filtration cup in which samples could be concentrated and processed without cell loss due to rinsing. The Transwells were used directly without pretreatment of the filters to reduce background fluorescence, a routine procedure in epifluorescence microscopy (16). However, it was crucial to include a prehybridization step with hybridization buffer which contained 10× Denhardt's reagent and 0.1 mg of polyadenylic acid ml^{-1} (23). These blocking agents suppressed nonspecific binding of the probes to the filter and thus greatly reduced background fluorescence. The following points were also found to be important: (i) a volume of 200 μl adequately covered the surface of the filters with hybridization buffer or FITC-avidin solution yet minimized the amount of each probe added so that the procedure remained economical and (ii) the hybridization time could be reduced to 3 h without noticeable loss in the fluorescence intensity of the probed cells.

Samples were typically processed and enumerated from 1 to 3 days after preservation in formaldehyde. However, the fluorescence of individual cells and the nanoplankton counts of field samples preserved for up to 10 days were not different from the fluorescence intensity and the counts obtained when they were probed 1 day after preservation (data not shown). The signal-to-noise ratios of the probed samples were very high, and protists could be clearly visualized against a dark background by epifluorescence microscopy (Fig. 2a to d, f, and j). A variety of protists, including small flagellates, dinoflagellates, ciliates, amoebae, and diatoms, could be distinguished in the field samples. Bacteria and cyanobacteria were not labeled by the probes, and nonspecific staining of detrital material was extremely low. This combination of factors made enumerating probed nanoplankton cells easy and rapid. It was not possible to obtain reliable estimates of the percentage of nanoplankton cells that were photosynthetic in natural water samples (i.e., separate them from the heterotrophic nanoplankton) because the ethanol washes extracted the chlorophyll from some of the small photosynthetic cells. Therefore, only TNAN counts were obtained in this study. In the future, this problem might be circumvented by counting autofluorescent cells in samples processed without ethanol dehydration.

Comparison of cell counts by hybridization probes, DAPI, and AO staining. (i) Cell cultures. Eight cultures of protists representing a range of sizes and taxonomic groups were selected for enumeration by in situ hybridization and DAPI and AO staining. All of the cultures tested were easily counted, but the fluorescence signal of probed cells varied among the species tested owing largely to differences in cell size and perhaps physiological state (which may affect ribosome numbers). Cell densities obtained by the three methods (expressed as the percentage of cells detected by eukaryote-specific probes relative to DAPI and AO staining) were not significantly different (two-way analysis of variance, $P = 0.14$) at the α equals 0.01

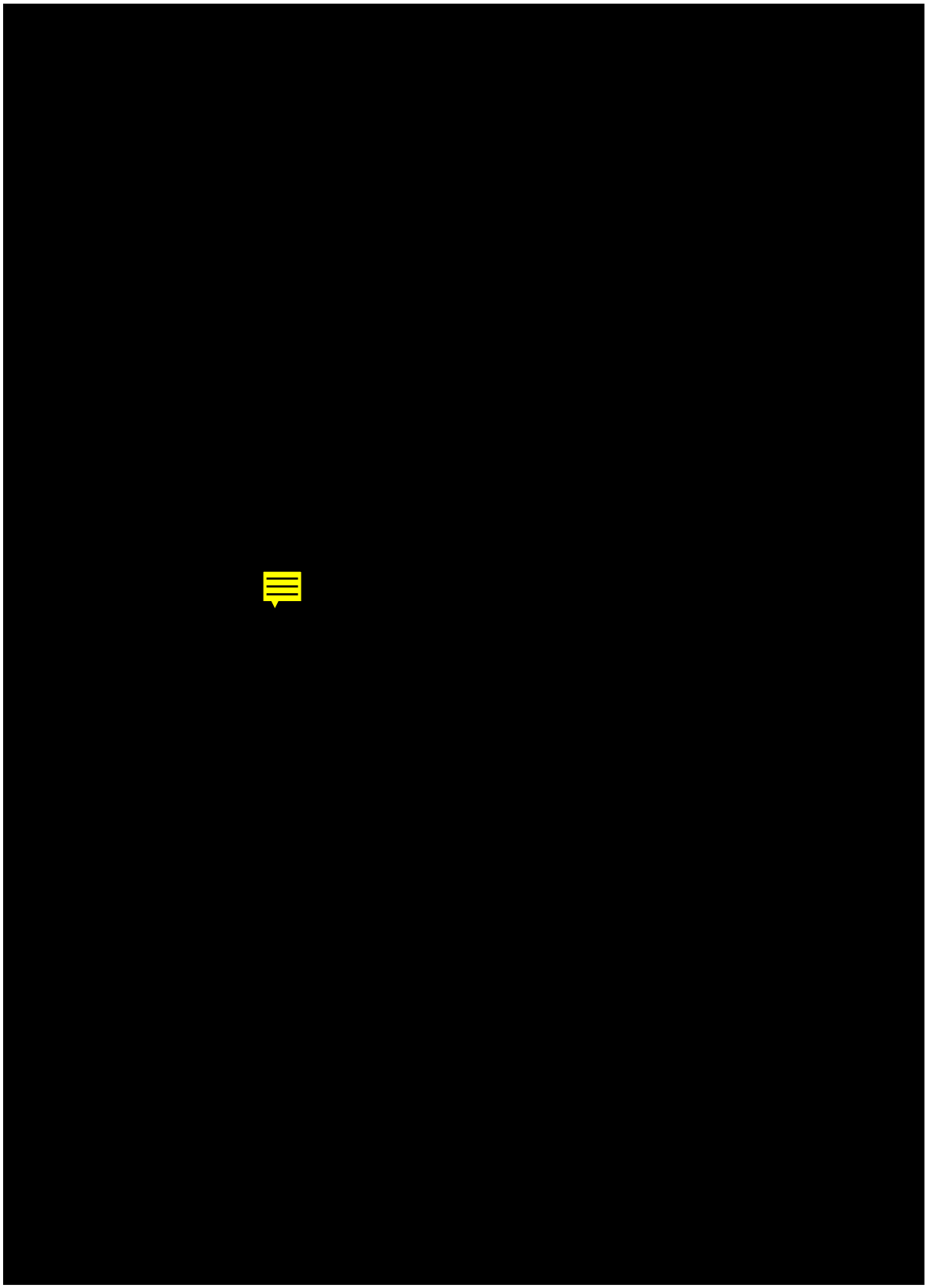

FIG. 2. Epifluorescence micrographs of naturally occurring protists in the DOC plus DIN treatment of the mesocosm experiment at day 10. (a to d, f, and j) A variety of protists hybridized with eukaryote-specific biotinylated oligonucleotide probes and FITC-avidin showing FITC fluorescence, including a diatom (a, arrow), a dinoflagellate (b), and an amoeba (d); (e and f) probed protists, dual-stained with DAPI and FITC-avidin, irradiated with either UV light (e) to preferentially excite DAPI or blue light (f) to preferentially excite FITC (note the 2-μm cell at the bottom of the micrograph [f, arrow] that is easily distinguished by its FITC fluorescence but not by its DAPI fluorescence [e, arrow]); (g and h) AO-stained preparations of free-living protists (g) and an aggregate with associated protists (h); (i and j) DAPI (i) and the corresponding FITC (j) fluorescence of aggregate-associated protists in a dual-stained preparation. It is important to note that protists associated with aggregates are clearly distinguished by oligonucleotide probes. Scale bars, 5 μm.

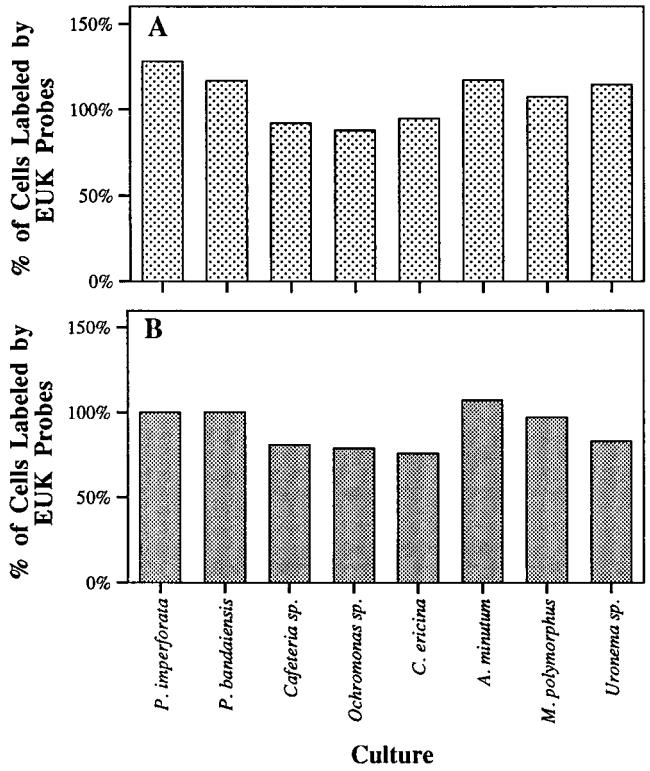

FIG. 3. Percentage of cultured heterotrophic flagellates (*Paraphysomonas imperforata*, *Paraphysomonas bandaiensis*, and *Cafeteria* sp.), algae (*Ochromonas* sp., *Chrysochromulina ericina*, *Alexandrium minutum*, and *Minutocellus polymorphus*), and a ciliate (*Uronema* sp.) labeled by in situ hybridization with eukaryote-specific probes. All cell abundances obtained by eukaryote-specific probes were normalized to cell counts obtained by DAPI (A) and AO (B) staining.

significance level (Fig. 3). The use of eukaryote-specific probes and the filter hybridization method thus gave counts of cultured protists that were comparable to those of DAPI and AO staining.

(ii) Field samples. The ability of the probe hybridization method to provide accurate counts of mixed assemblages of protists relative to DAPI and AO counts was examined with water samples collected from a variety of environments. The nanoplankton assemblages in the oligotrophic freshwater (Deep Pond) and seawater (Sargasso Sea) samples as well as the Woods Hole coastal water sample were mainly dominated by small protists approximately 2 μm in diameter. Nanoplanktonic protists in the Sippiwissett Marsh samples, on the other hand, were predominantly photosynthetic dinoflagellates approximately 15 to 18 μm in size.

TNAN counts obtained from samples stained with 5 μg of DAPI ml^{-1} were similar to counts from samples stained with 50 μg of DAPI ml^{-1} (data not shown). Estimates of TNAN by use of 0.4- and 0.8-μm filters also were not different. In the Eel Pond water sample, TNAN abundances estimated by use of 0.4- and 0.8-μm filters were $(1.34 \pm 0.20) \times 10^3$ ml^{-1} and $(1.36 \pm 0.27) \times 10^3$ ml^{-1}, respectively, and in the algal culture, they were $(9.03 \pm 0.16) \times 10^4$ ml^{-1} and $(9.31 \pm 0.20) \times 10^4$ ml^{-1}, respectively. The density of TNAN in all the field samples enumerated by in situ hybridization was similar to or higher than TNAN densities obtained with DAPI or AO staining (Fig. 4). The greatest difference observed was for the Woods Hole water sample. Probe counts of TNAN for this water sample were approximately two times greater than TNAN densities obtained with DAPI and AO staining.

(iii) Mesocosm experiment. The additions of DOC and DIN in the mesocosm treatments stimulated the growth of the bacterial and algal assemblages and, subsequently, other organisms at higher trophic levels as they responded to the elevated production. The abundance of heterotrophic nanoplankton (consisting largely of bacterivorous flagellates) increased probably in response to the elevated bacterial densities, while the photosynthetic nanoplankton bloomed presumably as a result of the inorganic nutrient additions. This experiment was ideal for testing the in situ hybridization method under a field setting because the various DOC and DIN additions resulted in a range of TNAN abundances during the 10-day sampling period.

TNAN concentrations of the three enriched treatments (DOC, DIN, and DOC plus DIN) increased dramatically relative to that of the control treatment during the first 10 days of the experiment (Fig. 5). TNAN abundance in the control treatment was two-fold higher than its initial value at the end of the experiment. In contrast, TNAN abundances in the DOC, DIN, and DOC plus DIN treatments increased by approximately 10-fold during the 10-day period.

TNAN counts obtained by probe hybridization were consistently higher than DAPI counts of TNAN for all four treatments over the 10-day period (Fig. 5). Probe counts at the beginning of the experiment were nearly twofold greater than DAPI counts (average of time zero samples from all four treatments) and ranged from approximately 1.3 to 3.3 times the DAPI counts over the duration of the experiment. By day 10, probe counts of TNAN remained approximately twofold greater than the corresponding DAPI counts in the control, DOC, and DIN treatments but were more than threefold greater than the DAPI counts in the DOC plus DIN treatment. These results indicated that the probes consistently detected a portion of the nanoplankton assemblage that was not detected

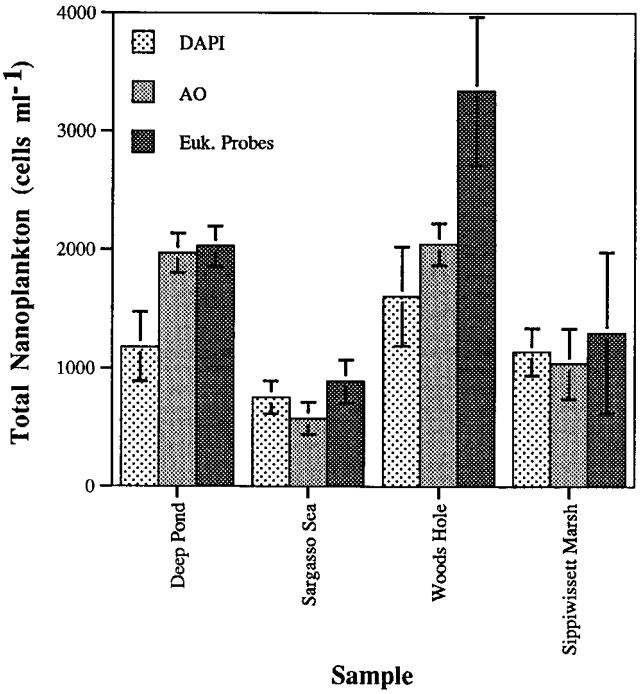

FIG. 4. TNAN densities in a freshwater sample (Deep Pond) and three seawater samples determined by DAPI staining, AO staining, and eukaryote-specific (Euk.) probes. Error bars represent 1 standard deviation of the mean of triplicate counts.

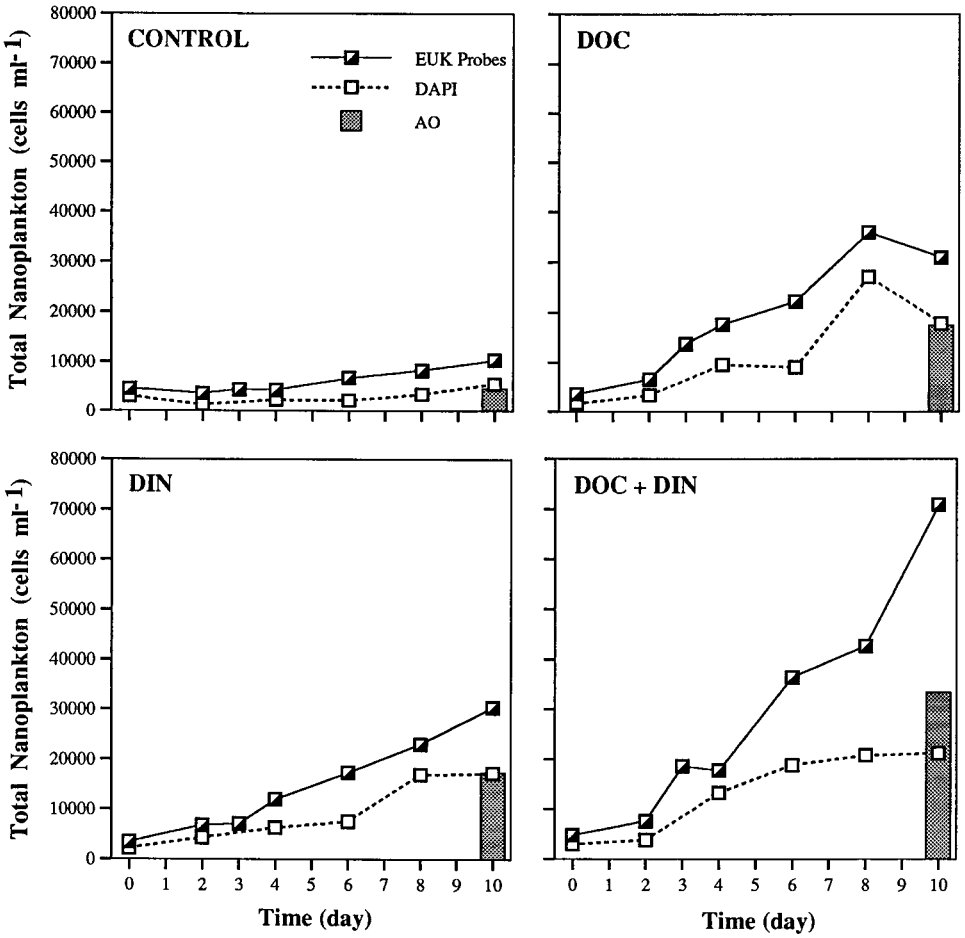

FIG. 5. Changes in TNAN densities as measured by eukaryote-specific (EUK) probes, DAPI staining, and AO staining in four treatments of a mesocosm experiment. The treatments were unamended seawater (control), DOC addition, DIN additions, and DOC plus DIN additions.

by DAPI staining for all the treatments. This difference was greatest for the DOC plus DIN enrichment.

TNAN densities determined by AO staining on day 10 were comparable to DAPI counts for the control, DOC, and DIN treatments but were approximately 1.5 times higher than DAPI counts for the DOC plus DIN treatment. Nevertheless, all TNAN abundances determined by AO staining were lower than estimates obtained by the probe hybridization method.

DISCUSSION

A major goal in the application of molecular approaches in ecological studies has been the identification and enumeration of individual species of microorganisms in their natural environment. The quantitative in situ hybridization technique described in this study is a significant step towards this goal. The method permits direct enumeration of both cultured and naturally occurring protists by in situ hybridization with oligonucleotide probes. In addition, the method has wide applicability for conducting in situ hybridization to identify and enumerate other microbial cells in aquatic samples.

Hybridization experiments performed in this study with several cultures of nanoplanktonic protists demonstrated that the method provided accurate cell counts in comparison to the conventionally employed DAPI and AO methods for enumerating protists (Fig. 3). Because cultures do not present many of the difficulties associated with field samples (diverse species assemblages resulting in a range of cell types, sizes, physiological states, and aggregations), they served as simple controls for testing the reliability of oligonucleotide probes and of the hybridization procedure for quantifying nanoplanktonic protists. We have shown that, for the species tested in this study, the quantitative in situ hybridization method gave estimates comparable to those of the fluorochrome staining techniques.

Our quantification of mixed assemblages of protists with eukaryote probes has also provided an independent means other than conventional staining methods to examine the natural abundance of nanoplanktonic protists. In a survey of water samples collected from four environments of differing trophic status, probe counts of TNAN were up to twofold greater than counts obtained by DAPI or AO staining (Fig. 4). This observation was further supported by results from the mesocosm experiment which demonstrated that TNAN abundances obtained by probe hybridization ranged from 1.3 to 3.3 times (average, 2) higher than DAPI counts of TNAN in all four enrichment regimes over a 10-day period (Fig. 5). If the probe counts of TNAN are assumed to be an accurate estimate of TNAN in these water samples, our results indicate that DAPI or AO staining failed to detect a portion of the nanoplanktonic assemblage constituting approximately 25 to 70% of the TNAN assemblage.

The differences observed in TNAN abundances among samples treated with probes, DAPI, or AO may be explained by the presence of nanoplanktonic cells that were obscured in detrital aggregates or poorly stained by DAPI or AO but readily detectable by in situ hybridization with oligonucleotide probes. We have observed that the nuclei of some cultured species of protists as well as the nuclei of some protists in natural water samples are not well stained by DAPI. A prevalence of such cells in water samples collected in this study could have contributed to an underestimation of TNAN densities in the DAPI-stained samples. Water samples collected from Deep Pond, Sargasso Sea, and Woods Hole at the time of this study also contained many small nanoplanktonic cells. Small nanoplanktonic cells, typically those about 2 μm in size, are often extremely difficult to distinguish when stained with DAPI or AO. DAPI stains primarily the nucleus; therefore, the nuclei of 2-μm protists often appear as small, bright dots that can be confused with stained bacteria (Fig. 2e, arrow). Probes, on the other hand, bind to ribosomes that are dispersed in the cytoplasm. As a result, protists are more uniformly stained and thus easier to visualize (Fig. 2f, arrow). Tiny (≈2-μm) cells were routinely observed in samples examined by the in situ hybridization method during this study.

The low estimates of TNAN obtained from DAPI counts may also be caused by bacteria and aggregate material interfering with nanoplankton counts, particularly in the DOC plus DIN treatment of the mesocosm experiment. Enrichment of the water sample in the mesocosm experiment with DOC plus DIN resulted in the greatest difference between DAPI counts and probe counts of TNAN (Fig. 5). Macro- and microscopic aggregates composed primarily of phytodetrital aggregates and filamentous bacteria were particularly extensive in the DOC plus DIN treatment as a result of elevated phytoplankton and bacterial abundances. The high concentration of bacteria and aggregate material may have been responsible for the underestimation of TNAN in DAPI-stained samples because DAPI (as well as AO) stained the bacteria and detrital material intensely and, as a result, masked the fluorescence of stained protists within aggregates. Figures 2e and g show DAPI- and AO-stained protists, respectively, that were not associated with detrital aggregates and were reasonably easy to enumerate. However, in aggregates stained by AO (Fig. 2h) or DAPI (Fig. 2i), the fluorescence of detrital material and aggregated bacteria masked the fluorescence of the protists associated with the aggregates. These attached protists, in contrast, were easily distinguished when treated with oligonucleotide probes because the probes and FITC-avidin labeled the protists but not the detrital material and bacteria (Fig. 2j, same field of view as that of Fig. 2i but showing FITC fluorescence of probed cells).

We have demonstrated in this study that counts of cultured protists obtained by probe hybridization were comparable to DAPI and AO counts and that counts of TNAN in field samples obtained by probes were frequently higher than DAPI or AO counts of TNAN. It is difficult to assess whether probes provide accurate determinations of the abundance of TNAN in natural, mixed assemblages, but the results of our study indicate that in situ hybridization with probes gave estimates that are at least as high as (and in some cases higher than) counts obtained by use of commonly employed fluorochrome stains.

Identification of a variety of microorganisms in diverse habitats by rRNA-based phylogenetic identification techniques has greatly enhanced our understanding of species diversity and distribution (see reference 3 and references therein). Knowledge of the species composition in a particular environment, in turn, can be used to relate community structure to various aspects of community function and biogeochemical activity. Similarly, demonstration of specific taxa in an environment can be indicative of particular physiologies within the assemblage. However, to estimate the importance of these processes in the environment, it is necessary to quantify the abundance of the microorganisms responsible for them. The quantitative in situ hybridization method that we have developed provides an effective means to enumerate the total assemblage of nanoplanktonic protists in environmental samples. Our laboratories have also recently developed oligonucleotide probes specific to several ecologically important species of heterotrophic flagellates common in aquatic environments (unpublished data). With the availability of these probes and the quantitative method described here, it should now be possible to gather data on the abundance and distribution of different taxonomic groups of protists and to measure the contribution of these species to energy flow in aquatic ecosystems.

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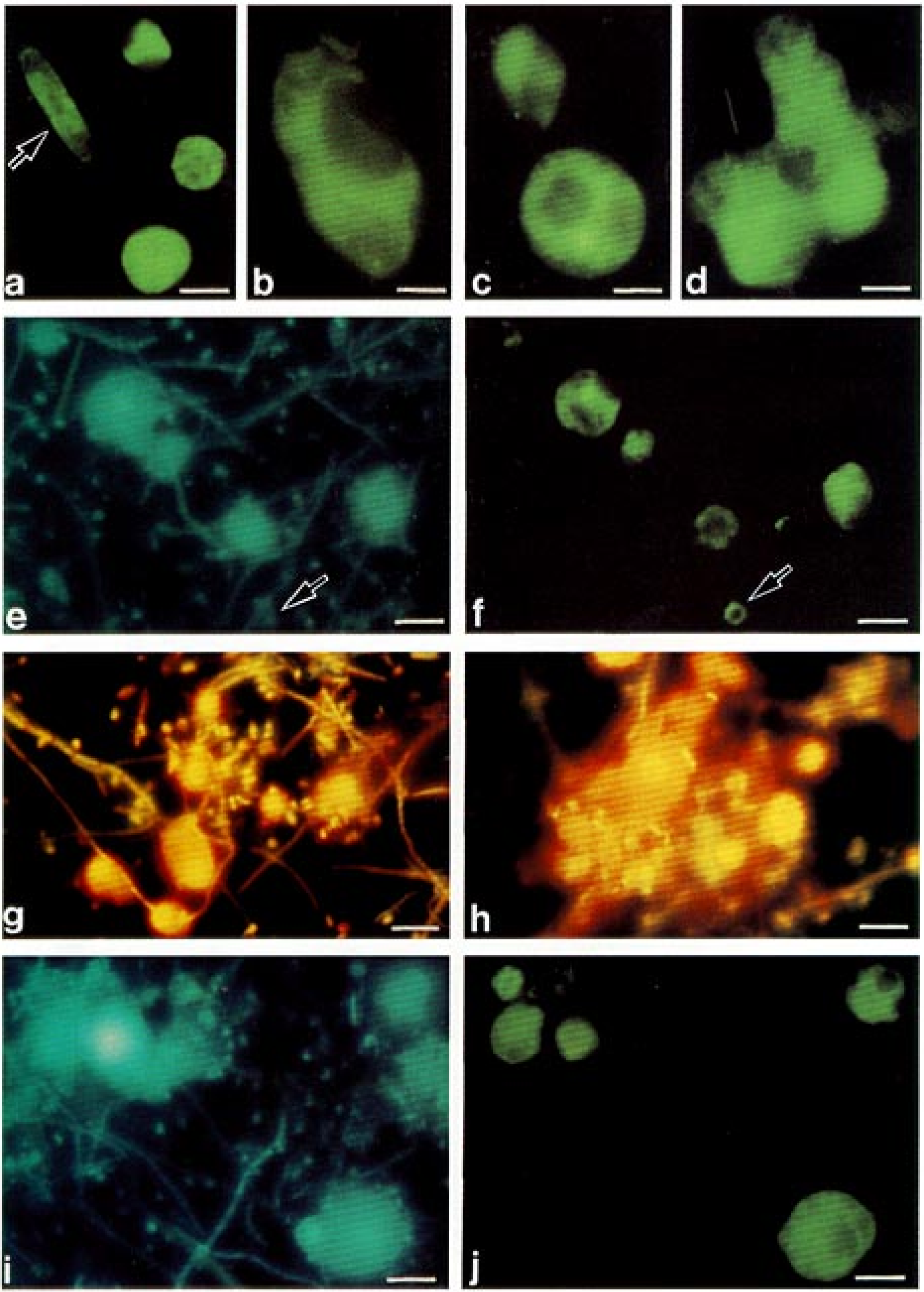
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