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Counting heterotrophic nanoplanktonic protists in cultures and aquatic communities by flow cytometry

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ABSTRACT: The food vacuole stain LysoTracker Green® was used to enumerate heterotrophic protists on a standard model flow cytometer. Appropriate stain concentration and staining time were determined using cultures of protists. Stained heterotrophic protists consistently formed distinct populations within cytograms of green fluorescence versus forward scatter. Cytometric counts of cultured species were compared to direct counts using light microscopy at cell abundances ranging from 10^3 to 10^6 cells ml^{-1}. A regression of these data was highly significant and yielded a slope of 0.95. Stained populations were accurately counted during lag, exponential and early stationary growth phases. Growth rates calculated from cytometric counts were not statistically different from those based on microscopy. The method was applied to 26 natural plankton samples, and general region definitions on the cytograms were established that identified heterotrophic protistan assemblages. A regression of cytometric counts versus direct counts yielded a slope of 1.16. LysoTracker Green® can only be used with live samples because preservation destroys membrane potential, resulting in loss of fluorescence. However, the flow cytometric method employing LysoTracker Green® is highly applicable for monitoring the growth of many heterotrophic protists in cultures and has the potential to be extremely useful for field samples, providing comparable counts to microscopical methods while allowing much faster sample processing.

KEY WORDS: Heterotrophic protists · Flow cytometry · Nanoplankton · Fluorescent staining

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INTRODUCTION

Nanoplanktonic (2 to 20 µm) heterotrophic protists play integral roles within aquatic environments. These organisms comprise a significant portion of the total living biomass of planktonic ecosystems (Stoecker et al. 1994, Caron et al. 1995) and serve as an important source of mortality for bacteria, microalgae and other heterotrophic protists (Sanders et al. 1992, Sherr & Sherr 1994). As consumers, heterotrophic nanoplankton function in the transfer of carbon and energy to higher trophic levels and as important recyclers of organic matter and macronutrients (Azam et al. 1983, Caron & Goldman 1990). Therefore, the accurate enumeration of heterotrophic nanoplankton is essential for understanding trophic dynamics and energy flow in aquatic ecosystems.

Traditionally, heterotrophic nanoplanktonic protists have been quantified using microscopical techniques. Typically, assemblages in natural water samples are preserved and stained with nucleic acid or protein stains, filtered and mounted onto slides, and counted using epifluorescence microscopy (Sherr et al. 1993). Protists are first identified based on the strong fluorescent signal of the stain, then heterotrophs and phototrophs within the sample are distinguished and quantified based on the absence or presence of autofluorescence due to photosynthetic pigments (Sherr et al. 1993). This process of counting and classifying individual cells by epifluorescence microscopy is slow, tedious, and subject to a number of potential sources of error. These sources of error can include fixation artifacts, over- or understaining, incorrectly categorizing heterotroph cells with autofluorescent prey as photo-

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trophs, and human error. Nanoplanktonic protists have occasionally been counted live (Dale & Burkill 1982), but it is difficult to differentiate small phototrophs from heterotrophs using this method. To address these issues, we sought to develop a faster and more precise method of quantification, based on flow cytometry.

Flow cytometry has been employed in aquatic microbiology to quantify autotrophic prokaryotes, small autotrophic eukaryotes, heterotrophic bacteria and viruses (Olson et al. 1983, Yentsch et al. 1983, del Giorgio et al. 1996, Marie et al. 1997, 1999). Prokaryotic and eukaryotic phototrophs can be detected and quantified based on the autofluorescence of their photosynthetic pigments. Also, heterotrophic bacteria and viruses can be detected and quantified using a variety of nucleic acid stains to distinguish them from detrital particles. The speed and accuracy with which these populations can be counted by this method has made the flow cytometer a valuable tool for ecological studies in aquatic sciences (Olson et al. 1991, Porter 1999, Campbell 2001).

Unfortunately, an effective, accurate technique for the enumeration of heterotrophic unicellular eukaryotes in natural water samples using flow cytometry has not yet emerged. Most heterotrophic eukaryotes have little or no autofluorescence, except some heterotrophic dinoflagellates which fluoresce apple-green with blue light excitation (Carpenter et al. 1991). Therefore, detection of most heterotrophic nanoplanktonic protists is not possible by cytometry without staining. Common fluorescent compounds used to stain prokaryotes and eukaryotes do not differentiate these populations on a flow cytometer. For example, one technique published recently by Rifa et al. (2002) used the nucleic acid stain SYTO-13® to quantify heterotrophic eukaryotes in cultures and in field samples. However, both the prokaryotic and eukaryotic assemblages were stained, and eukaryotes were indistinguishable from prokaryotes on cytograms when the abundance of bacteria greatly exceeded the abundance of protists. This drawback makes SYTO-13® problematic for use in growth experiments, because starting bacterial concentrations are often several orders of magnitude greater than the protistan populations. This complication may also preclude the use of SYTO-13® for many natural samples, where the ratio of bacteria to heterotrophic protists may exceed 1000 (Sanders et al. 1992).

A new cytometric method was developed that employs a fluorogenic compound that selectively stains eukaryotic cells that possess acidic vacuoles, in order to avoid problems distinguishing prokaryotes from eukaryotes by cytometry. The method employs a pH-specific compound, LysoTracker Green® (Molecular Probes) to allow the discrimination of eukaryotes from prokaryotes by flow cytometry. The stain concentrates within acidic organelles of eukaryotes such as intracellular food vacuoles, lysosomes and chloroplasts. Prokaryotes do not possess acidic organelles and thus display low fluorescence. Phototrophic eukaryotes are distinguished from heterotrophic eukaryotes by the autofluorescence of photosynthetic pigments. This method was developed using cultures of heterotrophic protists and a standard benchtop flow cytometer in order to demonstrate the efficacy of LysoTracker Green® for enumerating these species. The stain was then applied to natural samples of plankton.

MATERIALS AND METHODS

Cultures. The LysoTracker Green® compound was tested on 1 strain of cyanobacteria and 11 species of protists. The phototrophic dinoflagellate *Heterocapsa triquetra* (CCMP 448), the prasinophyte *Micromonas pusilla* (CCMP 494) and the cyanobacterium *Synechococcus* sp. (CCMP 837), were obtained from the Provasoli–Guillard National Center for the Culture of Marine Phytoplankton, Boothbay Harbor, Maine. *Paraphysomonas vestita* was obtained from R. W. Sanders, Temple University, Philadelphia. *P. butcheri* and *Bodo caudatus* were obtained from D. Bratvold, College of Charleston, and *P. bandaiensis* from J. Waterbury, Woods Hole Oceanographic Institution. *P. imperforata*, *Cafeteria roenbergensis*, *Pteridomonas* sp., an unidentified chrysophyte and a scuticociliate (*Uronema* sp.) were isolated from a variety of aquatic environments and cultured in the laboratory of D.A.C. All protists other than *Paraphysomonas vestita* were marine. All heterotrophic protists were cultured on bacteria endogenous to the cultures.

Natural samples. We sampled 8 coastal locations from Connecticut to Maine over the course of 3 d: 150 ml samples were collected in 75 cm^2 tissue-culture flasks from a single location within Milford, Connecticut; Noank, Connecticut; East Greenwich, Rhode Island; Cohasset, Massachusetts; Portsmouth, New Hampshire; Portland, Maine; and 2 locations within Boothbay Harbor, Maine. We also sampled 18 sites along the California coast, between Carlsbad and Malibu: 1 l samples were collected in polycarbonate flasks from Agua Hedionda and Carlsbad State Beach, Carlsbad; Buena Vista Lagoon, Oceanside; Dana Cove Park, Dana Point; Newport Beach; Newport Beach Harbor; Huntington City Beach; Huntington Beach Harbor; Seal Beach; Bluff Park, Long Beach; Los Angeles Harbor; Cabrillo Beach, Los Angeles; Redondo County Beach, Redondo; Dockweiler State Beach, El Segundo; Santa Monica State Beach, Santa Monica; and Leo Carrillo Beach and Topanga County Beach, Malibu. Samples

were kept on ice in the dark, prior to analysis, and then allowed to come to room temperature before being analyzed on the flow cytometer and preserved for epifluorescence microscopy.

Microscopical counts. Transmitted light microscopy was used to count protists in culture. We removed 5 ml samples from experimental flasks and preserved them in 0.1 ml acid Lugol's solution (2% final concentration). Subsamples were removed, and 0.1 ml aliquots were counted in a Palmer–Maloney counting chamber. At least 200 heterotrophic protists were counted per slide.

Heterotrophic protists in natural samples were counted using epifluorescence microscopy (Sherr et al. 1993). A subsample was preserved for microscopical analysis at the same time as the live sample was analyzed on the flow cytometer. Samples were preserved with 0.3% glutaraldehyde and stored in the dark overnight at 5°C. Samples of 15 to 45 ml were placed in a glass filtering tower, stained for 5 min with 5 to 25 µg ml^{-1} DAPI (final stain concentration) and filtered onto 25 mm, 0.8 µm, black polycarbonate filters. The filters were immediately placed on slides, mounted with a drop of immersion oil, covered with a coverslip, sealed with wax, and stored in the dark at –20°C until they were counted. Duplicate slides were prepared for each sample. Slides were counted by comparing 2 digital images for each field of view examined; one obtained using blue light excitation and the other using UV light excitation. Protists were distinguished based on the signal of DAPI with UV light excitation, then classified as either phototrophic or heterotrophic based on the presence or absence of photosynthetic pigments using blue light excitation. Multiple fields of view were examined until at least 100 heterotrophic protists were counted per slide.

Staining protists with LysoTracker Green®. The LysoTracker Green® stain (Molecular Probes) consists of a fluorochrome attached to a weak base, which causes the compound to accumulate in acidic organelles. The stain has a peak excitation at 504 nm, but can be excited by the 488 nm argon-ion laser found in some commercially available benchtop flow cytometers. Peak emission occurs at 511 nm, so the photomultiplier tube of the FL1 channel of the FACScan and FACScalibur flow cytometers (Becton Dickinson) collects the strongest signal (515 to 545 nm, green fluorescence). There is no significant detectable emission at wavelengths detected by the FL3 or FL4 channels (>653 nm).

Optimizing flow cytometer settings and staining procedures with cultured protists. A FACScalibur flow cytometer (Becton Dickinson) equipped with a 15 mW air-cooled 488 nm argon-ion laser was used to count heterotrophic protists. The salinity of the sheath fluid (the fluid surrounding the sample stream within a flow cytometer) was matched to that of the sample to avoid distortion of the forward scatter signal (Cucci & Sieracki 2001). Organisms stained with LysoTracker Green® were recorded by the FL1 (green) fluorescence detector. Uniprotistan cultures of heterotrophic protists in bacterized cultures were distinguished by their high fluorescence and high forward scatter on a plot of green fluorescence (FL1) versus relative size (forward scatter, FSC). Polygon gates were drawn around populations of protists, and events falling within these polygons were counted using the software program CellQuest (Becton Dickinson).

The number of events on the flow cytometer was converted to cell abundance (protists ml^{-1}) using 1 of 2 methods: (1) A known concentration of green fluorescent 2.5 µm polystyrene beads (Molecular Probes) was added to samples of cultured protists and served as an internal standard for enumeration of the protistan population. The beads were diluted 1:1000 to create a working stock that was enumerated at the beginning of each experiment using a hemacytometer. Samples were acquired on the high (HI) flow-rate setting (approximately 50 µl min^{-1}) until at least 200 beads and 200 protists were recorded. Alternatively (2), the sample tube was weighed on an analytical balance before and after each analysis on the flow cytometer to determine the volume examined over the course of the analysis. The total event count was converted to cell abundance (protists ml^{-1}) after each timepoint using the processed sample volume. The first method was employed during growth experiments with cultured heterotrophs, the second method was employed during the experiments optimizing stain conditions as well as in natural water samples. Both methods yielded comparable results, but the second method required much less effort and therefore less time to prepare.

Stain concentration and appropriate staining time were determined for the LysoTracker Green® stain based on experiments with a medium-sized ciliate (*Uronema* sp., 10 to 20 µm) and a small heterotrophic flagellate (*Bodo caudatus*, 4 to 6 µm) because they represented the approximate range of sizes of heterotrophic protists in natural samples that might be expected to be encountered and effectively counted by flow cytometry. We examined 11 different incubation times and 12 different stain concentrations to determine appropriate stain conditions for both species.

A 1 mM stock of LysoTracker Green® was diluted 1:10 with 0.2 µm-filtered sterile seawater, and this working stock was added to 2 live cultures of heterotrophic protists at a final stain concentration of 75 nM. This stain concentration was chosen based on a recommendation from Molecular Probes (Molecular Probes,

Technical Bulletin #07525). Subsamples were analyzed on the flow cytometer immediately for 30 s on the HI setting, then again after 3, 5, 7, 10, 15, 20, 30, 45, 60 and 90 min. The samples were kept at room temperature between cytometric analyses and kept in the dark to prevent the stain from fading. Samples of each culture were also removed immediately prior to staining with LysoTracker Green® and preserved with acid Lugol's solution (2% final concentration) for direct determinations of cell abundances by light microscopy.

Stain concentration was examined for its efficacy for staining heterotrophic protists. A serial dilution of a 1 mM stock of LysoTracker Green® was used to prepare final stain concentrations of 4, 7, 10, 16, 25, 38, 58, 89, 137, 211, 325 and 500 nM. Cultures were stained for 10 min, based on the results from the incubation time-series, and then analyzed on the flow cytometer. Cell abundances were determined as described above.

We examined 3 species of heterotrophic protists and 1 species of heterotrophic bacterium for the effect of preservative on the fluorescence of stained cultures: *Uronema* sp., *Pteridomonas* sp. (~5 µm), *Paraphysomonas bandaiensis* (~5 µm) and *Halomonas halodurans*, and compared 3 treatments: (1) unpreserved and unstained, (2) unpreserved and stained with LysoTracker Green®, and (3) preserved and stained with LysoTracker Green®. Cultures were grown to mid-logarithmic growth phase before analysis. Unstained samples were not treated prior to analysis on the flow cytometer. Stained samples were incubated for 10 min in the dark with 75 nM LysoTracker Green® (final concentration). Samples were preserved with 1% glutaraldehyde for at least 1 h before staining in the 'Dead' treatment.

Counting cultured heterotrophic protists by flow cytometry. We used 5 species of bacterivorous protists to compare cytometric counts to microscopical counts: *Uronema* sp., *Paraphysomonas butcheri* (4 to 6 µm), *Bodo caudatus*, *Pteridomonas* sp. and *Paraphysomonas bandaiensis*. The marine bacterium *Halomonas halodurans* was used as food for the protists. Bacteria were grown to late stationary phase in 0.1% yeast extract, harvested by centrifugation (4000 rpm for 25 min), rinsed 3 times, and resuspended in 0.2 µm-filtered, sterile seawater. Bacteria were added at a concentration of approximately 10^7 cells ml^{-1} to flasks of 0.2 µm-filtered, sterile seawater. Duplicate flasks were inoculated for each of the 5 protists at starting concentrations of approximately 10^3 protists ml^{-1}. Flasks were sampled throughout the exponential growth phases of the cultures. Samples were analyzed by flow cytometry using LysoTracker Green® to stain the cells and by light microscopy using a Palmer–Maloney counting chamber as described in an earlier subsection.

We used 3 bacterivorous species of protists to test the accuracy of LysoTracker Green® for counting cultures during all phases of growth, including the stationary growth phase, when ingestion rates should be low: *Paraphysomonas vestita* (6 to 8 µm), *Cafeteria roenbergensis* (3 to 5 µm) and *P. imperforata* (5 to 6 µm). Duplicate flasks of 0.2 µm-filtered sterile freshwater were prepared with an unidentified bacterium that served as prey for the freshwater protist *P. vestita*. Duplicate flasks of 0.2 µm-filtered sterile seawater were prepared with *Halomonas halodurans* as described above for the 2 marine protistan species *P. imperforata* and *C. roenbergensis*. Samples were removed every 5 to 12 h for cytometric and microscopical analysis as described above, until the populations reached the late stationary growth phase. Growth rates were calculated from the slopes of the linear portions of plots of ln cell abundance versus time. Growth rates were calculated for each species based on cytometric counts and on microscopical counts, and the 2 rates were compared statistically using an ANCOVA test.

Eukaryotic organisms could accumulate the LysoTracker Green® stain within acidic organelles including chloroplasts. Therefore, phototrophic eukaryotes may interfere with the detection of eukaryotic heterotrophs in natural planktonic communities if the former emit a high green fluorescent signal from the uptake of LysoTracker Green® into chloroplasts. Thus, they need to be eliminated from cytograms used to count heterotrophic protists.

We examined 3 phototrophic species for uptake of the LysoTracker Green® stain, including 2 eukaryotes, *Heterocapsa triquetra* (20 to 25 µm) and *Micromonas pusilla* (1 to 2 µm), and 1 prokaryote, *Synechococcus* sp. Preserved, stained cultures were used as controls. The cultures were grown in sterile seawater f/2 medium (Guillard & Ryther 1962, Guillard 1975), and cells were harvested in late exponential growth phase. A 1 mM stock of LysoTracker Green® was diluted 1:100 with 0.2 µm-filtered sterile seawater and added to cultures at a final concentration of 75 nM. All cultures, live and dead, were stained with LysoTracker Green® for 10 min in the dark before cytometric analysis. Samples to be analyzed as dead cells were preserved using paraformaldehyde (0.5% final concentration) for 1 h prior to treatment. All samples were analyzed on an FACScan flow cytometer. The population of phototrophs was gated based on their red autofluorescent signal (FL3), and the mean green fluorescence (FL1) was determined for all autofluorescent cells.

Heterotrophic protists with ingested fluorescent food particles will themselves have a fluorescent signal that can be detected by a flow cytometer. Therefore, heterotrophic protists with ingested algae will emit red fluorescence and thus may fall within the 'phototroph'

region on a cytogram of red fluorescence (FL3) versus relative size (FSC). The extent to which cytometric counts of heterotrophic grazers may have been affected by the fluorescence of ingested algal prey was investigated using an unidentified chrysophyte and *Uronema* sp. The unidentified chrysophyte (2 to 3 µm) was fed to *Uronema* sp. to examine the effect of algal ingestion on the chlorophyll fluorescence of heterotrophic grazers. The ciliate was fed *Halomonas halodurans* for 4 d prior to the addition of algae in order to eliminate algal fluorescence in the protistan food vacuoles at the beginning of the experiment. The algae and protists were analyzed separately on a FACScalibur flow cytometer prior to mixing the cultures in order to establish baseline red fluorescence values for prey and predators. The samples were acquired on the cytometer until at least 200 events of the population of interest had been recorded. The algae were then added to the culture of heterotrophic protists, and the heterotrophs were allowed to feed for 30 min before subsamples of the mixtures were analyzed on the flow cytometer.

Application of LysoTracker Green® to natural water samples. A FACScan flow cytometer was used to analyze natural water samples from the east coast, and a FACScalibur flow cytometer was used for samples from the west coast. A 1:10 dilution of the 1 mM LysoTracker Green® stock was freshly prepared in 0.2 µm-filtered seawater, and was used as a working stock. An aliquot of natural sample (10 ml) was removed and stained live at room temperature in the dark for 10 min with 7.5 µl of the working stock of LysoTracker Green® (75 nM final concentration). The sample was acquired on the HI flow rate setting for 10 min. The software was configured to record particles with a detectable green fluorescence (FL1), and the threshold was raised until the event rate dropped below 1000 events s^{-1}. An event rate of 1000 events s^{-1} represents the upper limit of the processing speed by the CellQuest software.

A sequential analysis of the resulting light scatter and fluorescence data was employed to determine the number of heterotrophic eukaryotes in the analyzed sample. Eukaryotic phototrophs were first distinguished from eukaryotic heterotrophs on a cytogram of chlorophyll fluorescence (FL3) versus forward scatter (FSC), based on the high relative chlorophyll fluorescence of the phototrophs (Olson et al. 1983, Yentsch et al. 1983). Detritus was then separated from cellular material on a cytogram of side scatter (SSC) versus green fluorescence (FL1), based on the high refractive index of the detritus (Spinrad & Brown 1986, Ackleson & Spinrad 1988). Green fluorescent polystyrene beads (2.5 µm) were then used to establish a size reference on a cytogram of green fluorescence (FL1) versus FSC. The regions of eukaryotic phototrophs, detritus and beads were removed from the cytogram of green fluorescence (FL1) versus size, and all remaining events that had an FSC signal greater than the previously marked 2.5 µm beads were counted as heterotrophic protists.

RESULTS

Staining conditions

Cell abundances determined by flow cytometric analyses showed decreasing trends with increasing staining time for the 2 species of heterotrophic protists examined (Fig. 1A,C). At each sampling time, a clear population of protists was distinguishable from background fluorescence on a cytogram of green fluorescence (FL1) versus relative size (FSC) (data not shown). The ratio of cytometric counts to microscopical

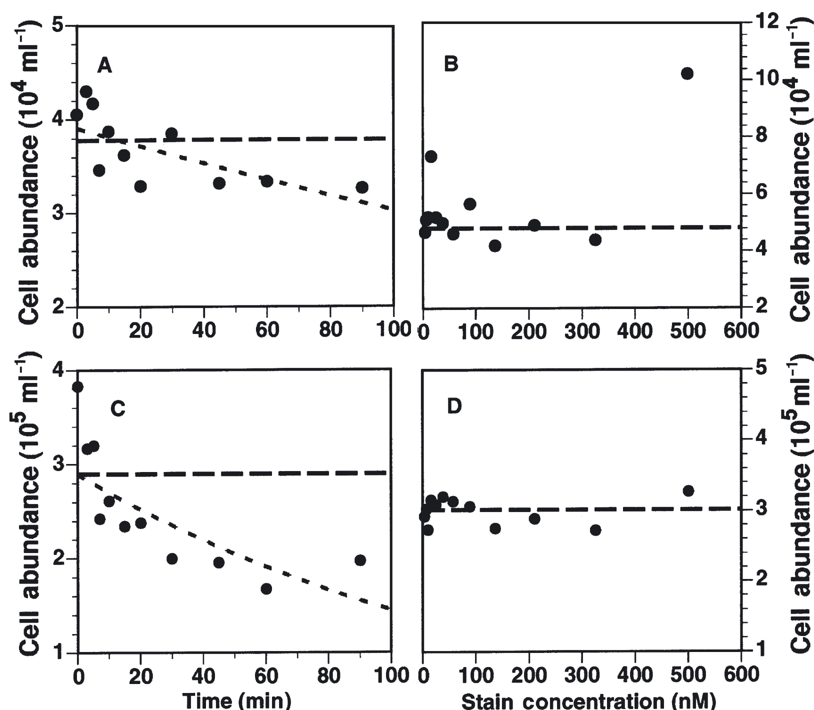

Fig. 1. (A,B) Ciliate *Uronema* sp. and (C,D) heterotrophic flagellate *Bodo caudatus.* Effect of incubation time (A,C) and stain concentration (B,D) on cytometric counts. Thick dashed lines: microscopical count at time zero, (●) cytometric counts; thin dashed lines (A,C): exponential curve fit to protistan counts based on cytometry

counts ranged from 0.86 to 1.14, averaging over all incubation times tested for the ciliate, with an average ratio of 0.97. An exponential curve fit to the cytometric data for the ciliate had the same cell abundance as the microscopical count at approximately 15 min. The ratio of cytometric counts to microscopical counts ranged from 0.68 to 1.32 for the flagellate over all incubation times tested, with an average ratio of 0.86. An exponential curve fit to the cytometric data for the flagellate had the same cell abundance as the microscopical count within the first 5 min. A stain time of 10 min was chosen for all further analyses.

Stain concentration had no noticeable effect on cytometric counts for either protistan species (Fig. 1B,D). The ratio of cytometric counts to microscopical counts for *Uronema* sp. ranged from 0.87 to 1.52 between 4 and 325 nM final stain concentration, with an average ratio of 1.06. The ratio of cytometric counts to microscopical counts ranged from 0.90 to 1.08 for the same range of stain concentrations for *Bodo caudatus*, with an average ratio of 0.99. Within this range, cytometric counts were not consistently higher or lower than microscopical counts with increasing stain concentration. A final stain concentration of 75 nM was used for all subsequent analyses based on these results and the recommendation of the LysoTracker Green® manufacturer.

Analysis of cultured protists

Comparison of unstained, preserved-stained and live-stained cultures of heterotrophic protists showed a marked increase in green fluorescence for the live cells over preserved samples (Fig. 2). The magnitude of the increase in fluorescence appeared to be related to the size of the species. *Paraphysomonas bandaiensis* (~5 µm; Fig. 2A–C) had a mean fluorescence of 18.7 when stained live, but only 5.27 when stained after preservation. *Pteridomonas* sp. (~5 µm; Fig. 2D–F) had a mean fluorescence of 107 when stained live, compared to a mean fluorescence of 4.56 when stained after preservation. *Uronema* sp. (10 to 20 µm; Fig. 2G–I) had a mean green fluorescence of 919 when stained live, compared to a mean fluorescence of 21.4 when stained after preservation. Unstained protists had greatly reduced green fluorescent signals relative to live stained cells. The heterotrophic bacterium (Fig. 2J–L) did not show an increase in mean green fluorescence between live and preserved samples, suggesting that the bacterium did not accumulate LysoTracker Green®.

Uptake of substantial amounts of the LysoTracker Green® stain by heterotrophic protists enabled discrimination of the protists from large numbers of bacteria that were also present in the cultures. Cultures of heterotrophic protists stained with LysoTracker Green® were identified by their very high fluorescent signal and large relative size on a cytogram of green fluorescence (FL1) versus FSC (Fig. 3). The protistan populations were distinct from bacteria on cytograms

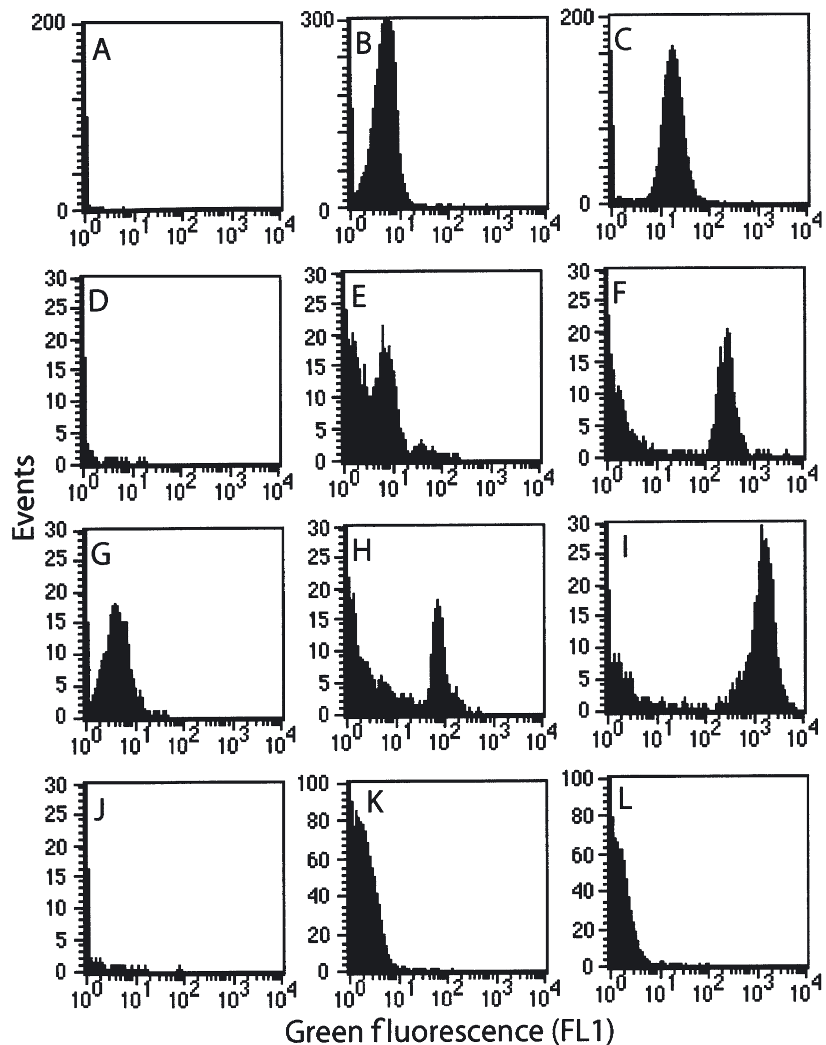

Fig. 2. (A–C) *Paraphysomonas bandaiensis*, (D–F) *Pteridomonas* sp., (G–I) *Uronema* sp. and (J–L) *Halomonas halodurans*. Effect of LysoTracker Green® on green fluorescence of 3 heterotrophic protists and 1 heterotrophic bacterium in unstained cultures (A,D,G,J), in cultures preserved with 1% glutaraldehyde and then stained (B,E,H,K) or in cultures stained live (C,F,I,L)

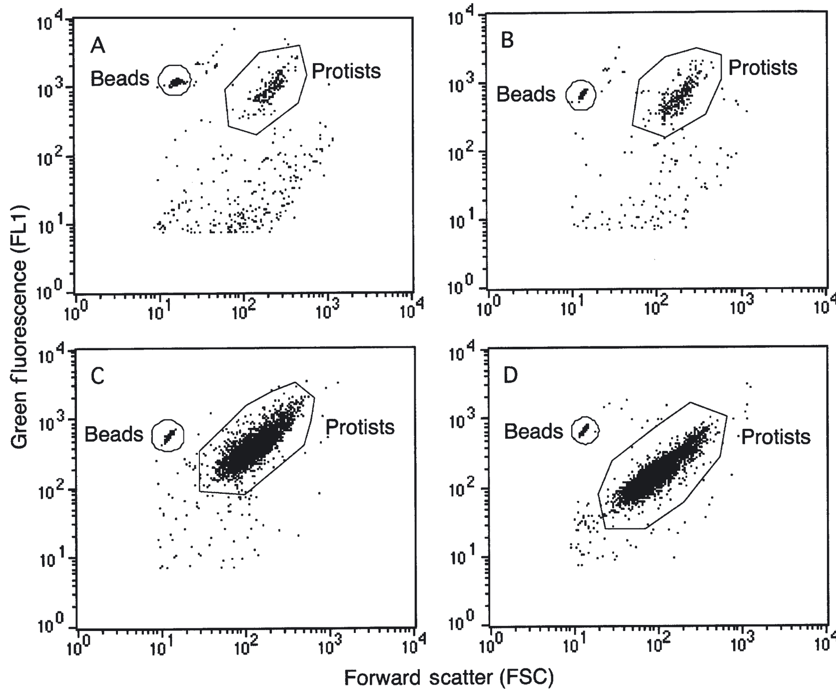

Fig. 3. *Pteridomonas* sp. Example showing detection and discrimination of a heterotrophic protistan species in a bacterized culture. Cytograms demonstrate population growth over time. All samples were stained live with LysoTracker Green® before cytometric analysis. Population growth is shown by steadily increasing events within the protistan polygon at (A) 24 h, (B) 48 h, (C) 72 h and (D) 96 h

4.3 d^{-1} based on microscopical counts (Fig. 5B). Cytometric and microscopical counts of *P. imperforata* also matched closely during the lag, log and early stationary growth phases. During the late stationary growth phase, however, the cytometric counts of *P. imperforata* remained relatively constant, while microscopical counts decreased (last 2 samples in Fig. 5C). *P. imperforata* had a growth rate of 2.7 d^{-1} based on cytometric counts, and 3.1 d^{-1} based on microscopical counts (Fig. 5C). No statistically significant differences were detected between growth rates calculated based on cytometric or microscopical counts for any of the species when tested at α = 0.01. A comparison of microscopical and cytometric counts for the 3 protists in all phases of population growth yielded a regression of slope 0.99 (r^2 = 0.96) (Fig. 5D). The ratio of cytometric counts to microscopical counts was 1.14 averaged over all species and growth phases.

Live phototrophic eukaryotes had higher green fluorescence than preserved cultures, but prokaryotic phototrophs showed no increase in green at all ranges of protistan and bacterial abundances tested. As the protistan cultures grew, the number of events registered within the previously identified 'protistan' region on the flow cytometer increased accordingly (Fig. 3B–D).

A comparison of microscopical counts to cytometric counts showed a close agreement between the 2 methods of enumeration for 5 species of heterotrophic protists examined (Fig. 4). All species were in exponential growth phase during the sampling period. A regression of the flow cytometric versus microscopical counts for all species yielded a slope of 0.95 (r^2 = 0.98). The ratio of cytometric counts to microscopical counts was 0.84 averaged over all species and growth phases.

We examined 3 small flagellates over their full growth cycle to examine the effect of growth phase on the ratio of cytometric counts to microscopical counts (Fig. 5). The cytometric and microscopical counts closely mirrored each other throughout the entire growth cycle for *Paraphysomonas vestita* and *Cafeteria roenbergensis* (Fig. 5A,B). The growth rate of *P. vestita* was 1.2 d^{-1} based on cytometric counts, and 1.1 d^{-1} based on microscopical counts (Fig. 5A). The growth rate of *C. roenbergensis* was 4.7 d^{-1} based on cytometric counts, and

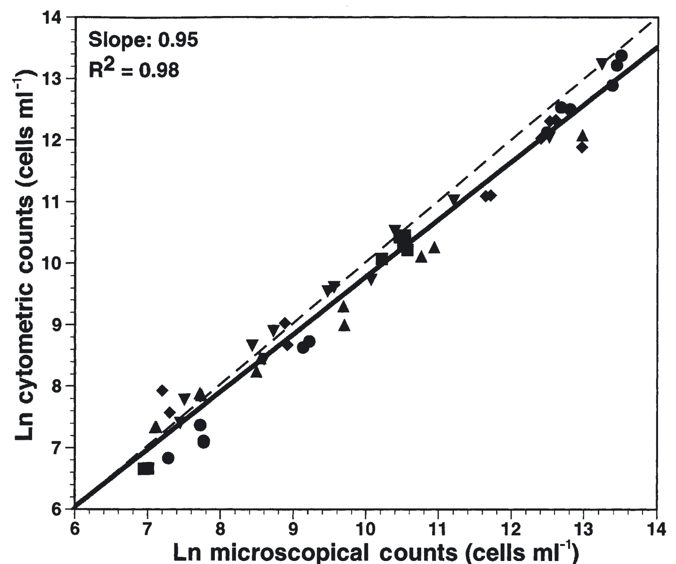

Fig. 4. Heterotrophic protists. Comparison of microscopical counts and cytometric counts of 5 actively growing populations over a range of abundances. Continuous line = regression of data; dashed line = 1:1 ratio; (■) *Uronema* sp., (▲) *Paraphysomonas butcheri*, (◆) *Bodo caudatus*, (●) *Pteridomonas* sp., (▼) *Paraphysomonas bandaiensis*

Table 1. Uptake of LysoTracker Green® by 2 phototrophic eukaryotes and 1 phototrophic prokaryote. Samples were preserved with 0.5% paraformaldehyde in 'Dead' treatment

Phototrophic culture	Green fluorescence (FL1) Mean	SD	CV (%)	Ratio live:dead
Heterocapsa triquetra CCMP 448				
Live	437	126	29	128
Dead	3.4	1.3	37	
Micromonas pusilla CCMP 494				
Live	9.5	5.2	54	4.3
Dead	2.2	1.0	46	
Synechococcus sp. CCMP 837				
Live	2.0	1.0	51	1
Dead	2.0	1.0	50	

fluorescence between live and preserved samples (Table 1). After staining with LysoTracker Green®, the live cultures of *Heterocapsa triquetra* emitted a much stronger green fluorescence than the dead cultures. The fluorescence ratio of live:dead cultures was 128. The eukaryotic alga *Micromonas pusilla* also showed an increase in fluorescence between live and dead cultures (ratio of 4.3). The live *Synechococcus* sp. cultures showed no difference in green fluorescent signal relative to dead cultures.

Some heterotrophic grazers with ingested phototrophic prey may potentially be counted as phototrophs in the cytometric analysis of a natural water sample. The ingestion of algae by heterotrophic protists had a noticeable effect on red (chlorophyll) fluorescence of the heterotrophs (Fig. 6). The ciliate *Uronema* sp. had a very low red fluorescent signal when fed bacteria (Fig. 6B). The chrysophyte prey had a strong red fluorescent signal relative to its size, and formed a distinct region within the cytogram of chlorophyll fluorescence (FL3) versus FSC (gated areas in Fig. 6A). After the heterotrophic protists had fed for 30 min on the algae, the red fluorescent signal of the heterotrophs increased substantially and the culture displayed a fairly wide range of chlorophyll fluorescence (Fig. 6C). This range of red fluorescent signals of the heterotrophic protists partially overlapped the algal region on the cytogram of FL3 versus FSC.

Analysis of natural water samples

The accurate cytometric enumeration of heterotrophic protists within mixed microbial communities was accomplished by distinguishing them from phototrophic eukaryotes and detritus on the cytogram of green fluorescence (FL1) versus relative size (FSC) (Fig. 7). This discrimination was accomplished by sequential processing of light scatter and fluorescence

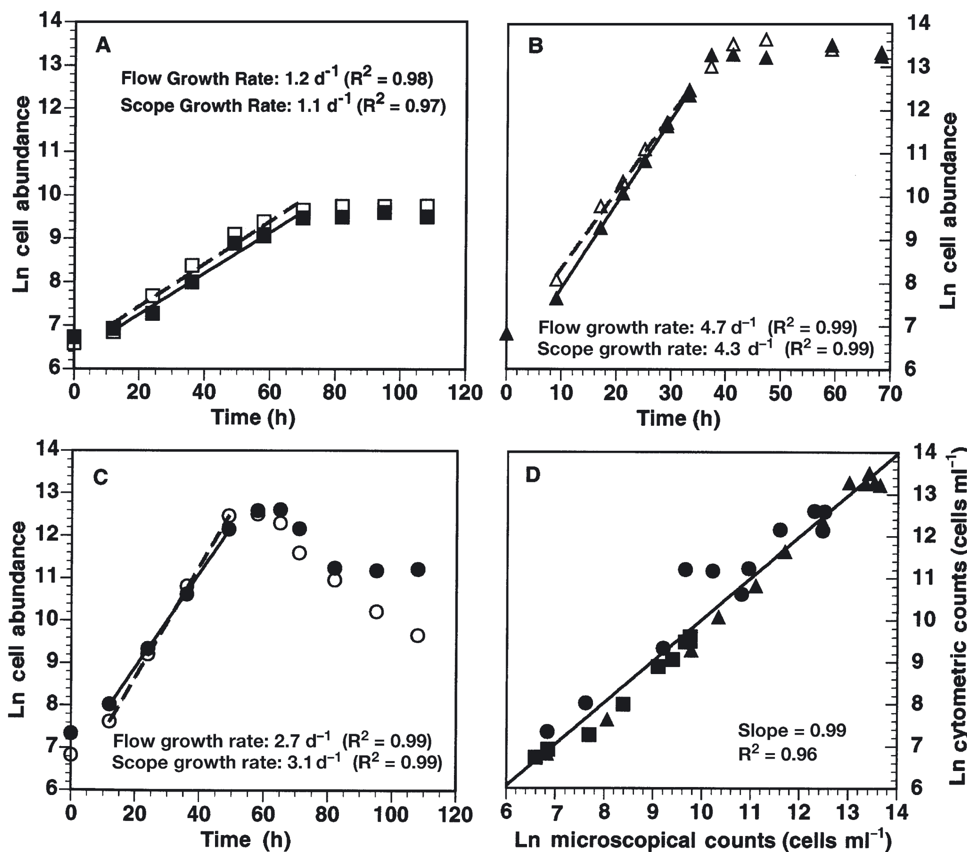

Fig. 5. Heterotrophic protists. (A–C) Comparison of growth curves of 3 species, enumerated by cytometric counts (filled symbols) and microscopical counts (open symbols). Growth rates calculated from slope of the line of population growth during exponential growth phase. Continuous lines: growth rates calculated from cytometric counts; dashed lines: growth rates calculated from microscopical counts. (D) Comparison of microscopical counts and cytometric counts during all phases of population growth from (A–C). (□, ■) *Paraphysomonas vestita*; (△, ▲) *Cafeteria roenbergensis*; (○, ●) *P. imperforata*

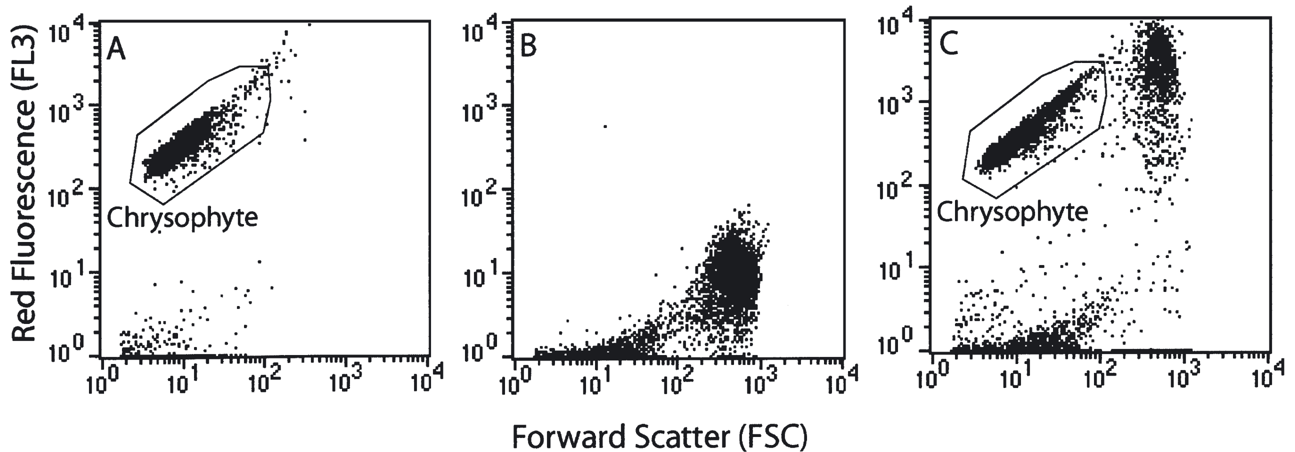

Fig. 6. *Uronema* sp. Effect of ingestion of algal prey on fluorescent signal of a heterotrophic protist. A small unidentified chrysophyte was used as prey. Cytograms show (A) red fluorescence (FL3) versus forward scatter for the chrysophyte, (B) late exponential cultures of *Uronema* sp. and (C) cultures of *Uronema* sp. 30 min after addition of the chrysophyte. Regions were drawn around the algal population in (A) to include at least 97 % of the events, and this gate was then applied to (C)

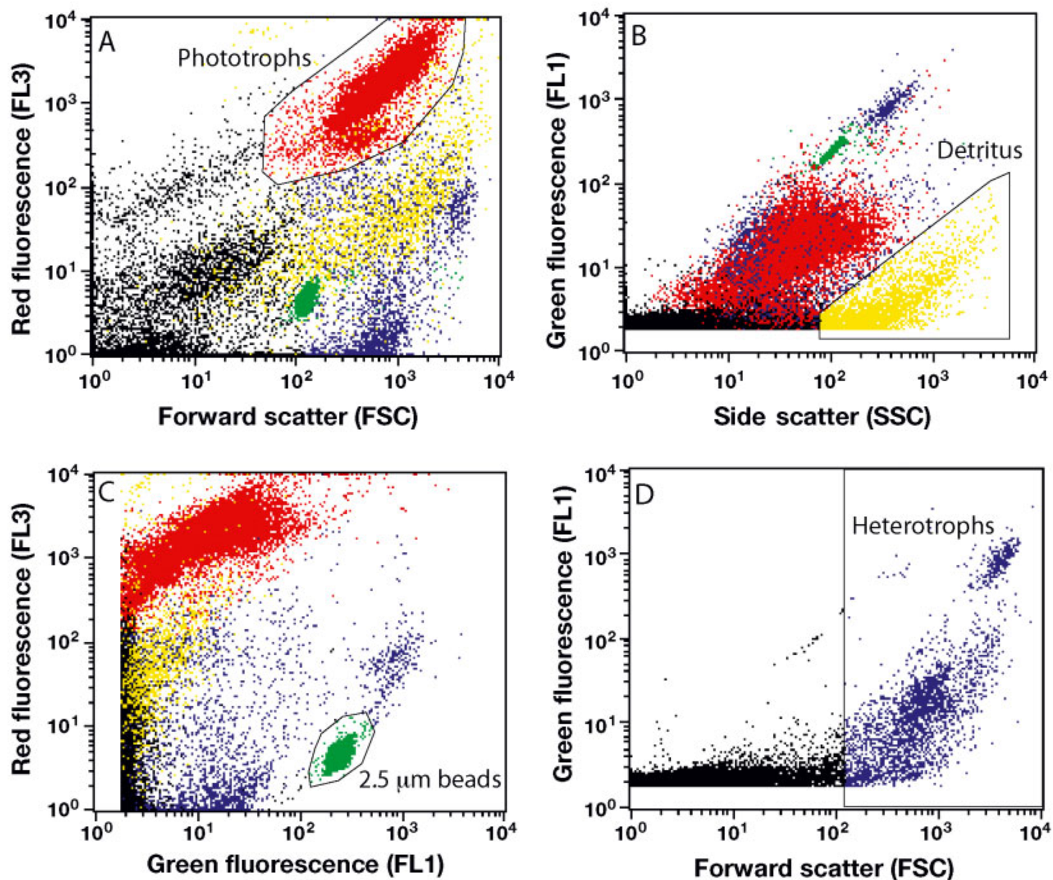

Fig. 7. Cytometric analysis of a natural water sample from Boothbay Harbor, Maine, for enumeration of heterotrophic nanoplanktonic protists. (A) Phototrophs detected based on high relative chlorophyll fluorescence in cytograms of red fluorescence (FL3) versus forward scatter (FSC) (red events gated in A). (B) Detrital particles identified based on relatively high side scatter and relatively low LysoTracker Green® fluorescence in cytograms of green fluorescence (FL1) versus side scatter (SSC) (yellow events gated in B). (C) 2.5 µm beads used for size estimation; identified based on their high green fluorescence and relatively low red fluorescence in cytograms of red fluorescence (FL3) versus green fluorescence (FL1). (D) Populations identified in (A–C) were removed from the plot of LysoTracker Green® fluorescence versus forward scatter, and remaining events larger than 2.5 µm were counted as heterotrophic nanoplanktonic protists (blue events gated in D)

data acquired for each sample. Phototrophic eukaryotes had to be eliminated from the counts of heterotrophic eukaryotes because phototrophic eukaryotes also accumulated the LysoTracker Green® stain (Table 1). Phototrophic eukaryotes were detected based on their high chlorophyll fluorescence relative to size, using a cytogram of FL3 versus FSC (red events in Fig. 7A). Detrital particles were identified based on their high side scatter and low green fluorescence, using a cytogram of FL1 versus SSC (yellow events in Fig. 7B). Polystyrene beads (2.5 µm; gated green events in Fig. 7C) were used to establish a minimum size for protists on the cytogram of green fluorescence (FL1) versus forward scatter (FSC) (vertical line in Fig. 7D). Subsequently, the phototrophic eukaryotes, detritus and beads were removed from the cytogram of green fluorescence (FL1) versus FSC using logical gates, and all particles greater than the minimum size (to the right of the vertical line in Fig. 7D) were counted as eukaryotic heterotrophic nanoplanktonic protists (blue events in Fig. 7D).

A comparison of microscopical counts and cytometric counts (determined as described in the previous paragraph) of 26 natural water samples showed close agreement between the 2 methods of quantification (Fig. 8). A regression of the data resulted in a line of slope 1.16 (r^2 = 0.95). The ratio of cytometric counts to microscopical counts was 1.09 averaged for all 26 samples. The locations sampled included a wide range of coastal environments, from relatively isolated beaches with a strong oceanic influence (Leo Carrillo Beach, Malibu) to highly eutrophic harbors (Newport Beach Harbor and Los Angeles Harbor) and a town dock near a treated sewage outfall (East Greenwich).

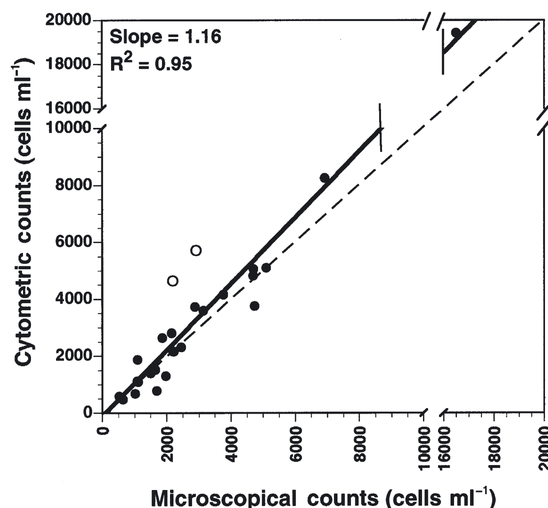

Fig. 8. Comparison of microscopical and cytometric counts for 26 natural water samples. Continuous line = regression of data, dashed line = 1:1 ratio. (O) samples from highly eutrophic locations (Newport Beach Harbor and Los Angeles Harbor)

DISCUSSION

Cytometric analysis of live protists

LysoTracker Green® can only be used with live samples because preservation destroys membrane potential, resulting in the rapid loss of fluorescent stain (Fig. 2, Table 1). There are benefits as well as drawbacks to the requirement for conducting this procedure with live cells. There is an obvious inconvenience associated with the inability to analyze preserved samples, and there will undoubtedly be situations where field samples are collected too far away for return to the laboratory for live analysis. If the samples are to be transported to a laboratory prior to analysis, measures must be taken to insure their integrity during transport. Samples should be kept in the dark and on ice (but not frozen) during transport, and processed as soon as possible after return to the laboratory. In addition, the salinity of the sheath fluid should be matched approximately to the salinity of the sample to avoid distortion of the forward scatter signal (Cucci & Sieracki 2001).

On the positive side, this method was developed using commercially available benchtop flow cytometers (FACSCalibur and FACScan, Becton Dickinson) that are relatively portable and can be used aboard ships and at field stations with electrical power. Another advantage of acquiring cytometric information on live samples is the avoidance of sample preservation that might cause lysis of some species of protists and the egestion of food vacuole contents (Sieracki et al. 1987). Moreover, the stain is not strongly accumulated by prokaryotes, which do not possess intracellular vacuoles. This ability to distinguish between prokaryotic and eukaryotic cells is a primary advantage of LysoTracker Green® for detecting and counting heterotrophic protists.

Staining time and stain concentration

Bodo caudatus appeared to show more dramatic decreases in observed cell abundance with increasing staining time than *Uronema* sp. (Fig. 1A,C), although both cultures showed a trend of decreasing cytometric counts with increasing time of staining. These results were not a consequence of changing physiological state of the protists, because both cultures remained in the exponential growth phase throughout the analyses. A probable explanation for this trend is the nature of the stain molecule itself, which consists of a fluorophore linked to a weak base that accumulates in intracellular locations with a low pH (such as food vacuoles). We speculate that this accumulation could

eventually raise the pH of the acidic organelles in which it accumulates, thereby reducing the affinity of the stain for these organelles over time. Thus, the organelles themselves may have the potential to become neutralized, resulting in diffusion of the stain out of the vacuoles and loss of fluorescence. The exponential curve fit to the cytometric counts for the *B. caudatus* culture indicated a steeper initial decline in cytometric counts. A more rapid decrease in apparent cell abundance for *B. caudatus* would be consistent with more rapid neutralization of acidic vacuoles in this smaller species.

We examined whether LysoTracker Green® actually caused cell death as a result of the presumptive neutralization of acidic vacuoles. A culture of *Uronema* sp. was stained with LysoTracker Green®, cytometrically sorted into a bacterial culture, and observed for 1 wk. Some of the sorted cells were dead immediately after sorting (approximately 15% of the culture), but most were still alive and actively swimming. The culture continued to grow for at least 1 wk after staining and sorting, indicating that most of the cells were not adversely affected by the combination of LysoTracker Green® staining and cytometric sorting.

Surprisingly, cytometric counts of *Uronema* sp. and *Bodo caudatus* exceeded microscopical counts for both species during the first several minutes of staining (Fig. 1A,C). We speculate that the initial cytometric counts were higher than the microscopical counts due to nonspecific staining of small acidic pockets within detrital particles or perhaps in bacterial aggregates, which were neutralized within the first few minutes of staining. The exponential curve fit to the data indicated that the cytometric counts were equivalent to the microscopical counts after approximately 15 min of staining for the ciliate, and within the first 5 min for the flagellate. Based on our results with these 2 species, we chose a staining time of 10 min for subsequent analyses of protistan cultures and natural water samples as a compromise between ensuring that all eukaryotes would emit a strong fluorescent signal on the one hand, and minimizing nonspecific staining on the other.

Stain concentration did not have a consistent effect on cytometric counts when stained for 10 min (Fig. 1B,D). Cytometric counts of *Bodo caudatus* generated from all stain concentrations compared well with the microscopical counts of the culture (Fig. 1D). Only the highest stain concentration resulted in a cytometric count of the ciliate that greatly exceeded the microscopical count (Fig. 1B). We chose 75 nM as a final working concentration based on our results and the recommendation of the manufacturer (Molecular Probes, Eugene, Oregon, Technical Bulletin #07525).

Application of LysoTracker Green® to cultured heterotrophic protists

Flow cytometry has been used successfully to enumerate heterotrophic protists in culture. Lindström et al. (2002) observed a close correlation between microscopical and cytometric counts for 2 ciliates fed *Cryptomonas* sp. and stained with TO-PRO-1®. Bratvold et al. (2000) enumerated heterotrophic flagellates in cultures using flow cytometry on cytograms of relative size (FSC) and relative refractive index (SSC) to distinguish protists from bacteria. The latter authors selected their study species based on substantial size differences between protists and bacteria that allowed unequivocal separation of the 2 populations. This strategy is effective for some protistan species, but can greatly constrain the protist–prey combinations that can be examined. For example, Bratvold et al. (2000) reported that 2 of the 4 protistan species considered for their study had cytometric patterns that overlapped with those of the bacteria and therefore were not employed in their experiments. Our method expands the number of small bacterivorous protistan species that can be effectively differentiated and counted separately from their bacterial prey using flow cytometry, because bacteria do not exhibit a substantial LysoTracker Green® (FL1) fluorescent signal.

Heterotrophic bacteria showed no strong increase in mean green fluorescence when stained live or preserved with LysoTracker Green® (Fig. 2J–L), indicating that heterotrophic bacteria did not take up the stain to any appreciable degree. This result is consistent with microscopical observations, which showed no visually detectable fluorescence (Zeiss standard microscope equipped with a 450 to 490 nm excitation filter, a 510 nm dichroic beam splitter, and a 520 nm barrier filter). Live heterotrophic protists, in contrast, showed a clear increase in mean green fluorescence over preserved or unstained samples (Fig. 2A–I). The increase in fluorescence for live cells was related to the size of the cells. The largest species, *Uronema* sp., had the largest increase in mean fluorescence between dead and live samples, while the smaller flagellate species, *Paraphysomonas bandaiensis* and *Pteridomonas* sp., showed smaller increases in mean fluorescence between dead and live samples. The stained, preserved heterotrophic protistan cultures still had some green fluorescence relative to unstained controls, and the larger preserved cells again showed a higher signal than the smaller preserved cells. Since the dead and live cells within the uniprotistan cultures had distinct fluorescent signals, it was possible to distinguish them on a cytogram (Fig. 2A–I). However, since the green fluorescent signal of the preserved and live cells varied based on the size of the protistan cells, the method may

not be able to distinguish live small protists from large dead ones in a mixed protistan assemblage.

The use of LysoTracker Green® and relative size provided information on some cultures of heterotrophic protists beyond simple enumeration. Of the 9 protistan species examined in this study, 8 formed tight clusters of events within the cytogram of green fluorescence (FL1) versus relative size (FSC). The mean green fluorescence for these populations dropped slightly when food became limiting and the cultures entered stationary growth phase (presumably due to a reduced number of food vacuoles), but the population as a whole remained distinct and unified. *Paraphysomonas imperforata* formed a tight cluster of events during lag and exponential growth, but the population split into 2 somewhat distinct subpopulations on cytograms during early stationary growth phase. These subpopulations had the same relative green fluorescence (FL1), but distinctly different relative sizes (FSC). The culture again formed a tight cluster of events within the cytogram after approximately 20 h in stationary phase. These findings corroborate the microscopical observations of Goldman & Caron (1985), in which a subpopulation of *P. imperforata* apparently resorted to cannibalism during the stationary growth phase, when bacterial abundance was low in the culture. The authors reported a shift to a bimodal size distribution during the stationary growth phase of the flagellate, as observed by us. They described an initial wide range of cell sizes (5.5 to 10 µm) shifting to a narrow range of small cells (3.5 to 6 µm) in early stationary phase (as prey became limiting). The species then formed 2 distinct subpopulations in late stationary phase that was presumably a result of feeding by some of the individuals on smaller protistan cells within the population.

The cytometric counts of 5 species of heterotrophic protists in exponential growth were highly correlated to microscopical counts of these cultures (Fig. 4). Further analysis of 3 species of heterotrophic protists indicated excellent correlation between cytometric and microscopical counts during active protistan growth (Fig. 5). Growth rates of these 3 heterotrophic protistan cultures were calculated from cytometric and microscopical counts while the populations were in exponential growth. These paired measurements yielded rates that were in close agreement for each species (Fig. 5A–C). The rates were compared using an ANCOVA test ($\alpha = 0.01$). No significant differences were detected between the growth rates calculated based on cytometric counts or microscopical counts. These results indicate that our flow cytometric technique has wide applicability for experimental studies to examine the growth rates of cultured protists.

Populations in exponential growth were actively feeding, and would be expected to have both lysosomes and food vacuoles (for which LysoTracker Green® should have high affinity). Cells in the stationary growth phase, however, had few prey available, and presumably the number of acidic vacuoles would have been reduced during this growth phase. We examined whether populations in stationary phase were accurately quantified by our method. Cytometric counts were similar to microscopical counts during the stationary growth phase for *Paraphysomonas vestita* and *Cafeteria roenbergensis* (Fig. 5A,B). Microscopical counts of *P. imperforata*, however, were somewhat less than cytometric counts as the culture of this protist entered late stationary growth phase (2 data points in Fig. 5C). This result was unexpected because we hypothesized that cells in late stationary growth would stain less intensely and thus yield lower counts than microscopical estimates. We speculate that this result might be explained by the substantial amounts of aggregated detritus formed by this species as it feeds (Caron et al. 1985, Goldman & Caron 1985). It is possible that the detritus was colonized by bacteria remaining in the culture and formed microzones of low pH that accumulated LysoTracker Green®. Thus, the cytometric counts may have overestimated the number of heterotrophic protists in these samples. Conversely, detrital material may have obscured heterotrophic protists in these samples and thus caused an underestimation in the microscopical counts. Neither *P. vestita* nor *C. roenbergensis* formed significant amounts of detrital aggregates, and neither culture showed a discrepancy between cytometric and microscopical counts during late stationary growth. A comparison of cytometric and microscopical counts from all stages of the growth cycle resulted in a regression of slope 0.99 ($r^2 = 0.96$) (Fig. 5D). These results indicated that LysoTracker Green® should provide accurate counts of heterotrophic protists for most situations.

Uptake of LysoTracker Green® by phototrophic prokaryotes and eukaryotic algae

LysoTracker Green® caused both heterotrophic and phototrophic eukaryotes to emit a green fluorescent signal (Table 1, Fig. 2). LysoTracker Green® stains all acidic vacuoles, so chloroplasts should accumulate stain as well as lysosomes and food vacuoles. Thus, phototrophic eukaryotes must be distinguished from heterotrophs during cytometric analyses using gating based on the red autofluorescence of chlorophyll *a*. Live cultures of 2 phototrophic eukaryotes (*Heterocapsa triquetra* and *Micromonas pusilla*) demonstrated increased fluorescence relative to preserved controls (Table 1). Preserved cells were used as controls because membrane potentials are lost upon cell death,

the acidic pH of vacuoles is neutralized, and LysoTracker Green® fluorescence dissipates rapidly (based on microscopical examination). Cell abundances of the phototrophic eukaryotes before and after staining with LysoTracker Green® did not vary, suggesting uniform staining of the chloroplasts. Both cultures of eukaryotic algae showed increased mean green fluorescence in live cells relative to preserved cells, indicating active uptake of the stain by the eukaryotic algae. The magnitude of the increase in fluorescence differed markedly for the 2 species. *H. triquetra* is a much larger species than *M. pusilla*, with many more chloroplasts, which explains the higher fluorescent ratio between live and dead cultures of *H. triquetra*. In contrast, *Synechococcus* sp., a prokaryote, stained poorly with LysoTracker Green® (Table 1). This result indicated that no accommodation was necessary to account for the photosynthetic prokaryote community within the cytometric analyses.

Application of LysoTracker Green® to natural water samples

The efficacy of counting heterotrophic nanoplankton in natural samples by flow cytometry was predicated on the differentiation of these cells from detrital particles and other groups of co-occurring microorganisms, including archaea, heterotrophic bacteria, phototrophic prokaryotes and phototrophic eukaryotes. These populations and particles were sequentially eliminated from cytograms of natural samples stained with LysoTracker Green® to obtain counts of heterotrophic protists.

Separation of stained phototrophic eukaryotes from heterotrophic eukaryotes was possible on the flow cytometer by gating phototrophs based on their high red autofluorescent signal (Fig. 7A). The CellQuest software allowed for their subsequent removal from cytograms of green fluorescence (FL1) versus relative size (FSC) (Fig. 7D). Detrital particles emitted a low level of green fluorescence, most probably due to the accumulation of LysoTracker Green® in microzones of low pH associated with bacteria. Conveniently, most detritus can be identified by its high side-scatter properties and relatively low green fluorescence (Spinrad & Brown 1986, Ackleson & Spinrad 1988). Based on these characters, detritus could be identified in cytograms of green fluorescence (FL1) versus side-scatter (SSC) (Fig. 7B), gated and subsequently eliminated from cytograms of green fluorescence (FL1) versus FSC (Fig. 7D).

The effectiveness with which detritus was identified in cytograms was somewhat dependent on the total amount of detritus present in a natural water sample. Large amounts of detritus in highly eutrophic samples were more difficult to accurately gate because the range of side-scatter from the detrital particles somewhat overlapped that of live cells. Most coastal sites did not pose a problem, but problems were observed for a few highly eutrophic harbors (Newport Beach Harbor and Los Angeles Harbor). Samples from oceanic waters, while not specifically tested, should not pose a problem, because of the normally low levels of detritus present in these ecosystems.

The minimal size of protists in the cytograms was established by gating 2.5 µm fluorescent beads that were added to all natural water samples. Beads were used to set a lower limit on the size of events accepted as 'protists' on a cytogram of green fluorescence (FL1) versus relative size (FSC) (Fig. 7D). A rectangular gate was placed through the center of the bead population and extended to include all events with a greater FSC signal in order to gate protists. The beads were then removed from the cytogram of green fluorescence (FL1) versus FSC so that they were not counted as protists (Fig. 7C). In some samples, the heterotrophic protists could overlap with bacteria in size, and a size gate smaller than 2.5 µm could be used. Since LysoTracker® appears to stain small eukaryotes much better than prokaryotes, it should allow adequate discrimination of protists from bacteria.

Cytometric counts of events remaining after (1) elimination of phototrophic cells, (2) elimination of detrital particles and (3) using beads to establish a minimum size were recorded as heterotrophic nanoplanktonic protists in natural water samples (Fig. 7D). Cytometric counts of heterotrophic nanoplankton determined in this manner agreed closely with counts based on DAPI staining and epifluorescence microscopy (Fig. 8). Samples from a wide range of coastal environments, comprising 26 different locations on the east coast and west coast of the USA were analyzed to test the applicability of this method. The regression of the resulting data yielded a line of slope 1.16 (r^2 = 0.95). The average ratio of cytometric counts to microscopical counts (1.09) indicated a very close agreement between the 2 counting methods.

Cytometric counts of heterotrophic nanoplankton might have been expected to provide an overestimation of the abundance of heterotrophic protists compared to epifluorescence microscopy due to misidentification of detrital particles as heterotrophic nanoplankton in the cytogram. The incomplete removal of detrital particles from the region gated to count heterotrophic nanoplankton (Fig. 7B,D) would have artificially increased cytometric counts. As noted above, this only appeared to be a problem in a few extremely eutrophic environments with high concentrations of particulate material (Newport Beach Harbor

and Los Angeles Harbor). However, these samples were also problematic to count using epifluorescence microscopy. The presence of large amounts of detritus in the water samples may have obscured some cells on slide preparations, resulting in underestimation of the abundances of heterotrophic nanoplankton. The combination of a potential underestimation of heterotrophic nanoplankton by epifluorescence microscopy and potential overestimation by flow cytometry most probably caused the slight discrepancies observed between these 2 counting methods in our study (ratio of cytometric:microscopical counts of 1.09; Fig. 8). Nevertheless, we found a close relationship between the cytometric quantification of heterotrophic nanoplankton and the commonly employed microscopical method of enumeration.

As previously noted, eukaryotic phototrophs accumulated LysoTracker Green® within their chloroplasts, and emitted a strong green fluorescent signal (Table 1). These species were separated from eukaryotic heterotrophs on the flow cytometer based on the strong red autofluorescence in the phototrophs. It has been shown that the fluorescent particles within heterotrophic protists can be detected by the flow cytometer, giving the heterotrophs a fluorescent signal (Gerritsen et al. 1987, Cucci et al. 1989, Keller et al. 1994, Weisse & Kirchoff 1997). One potential complication of our methodological approach is that heterotrophic grazers with ingested algae would also exhibit red fluorescence and might fall within the 'phototroph' region on a cytogram of red fluorescence (FL3) versus relative size (FSC). Therefore, the abundance of heterotrophic nanoplankton would be underestimated if heterotrophs with ingested phototrophs were counted as algae.

To examine the possibility of this artifact, we measured the change in red fluorescence after the addition of algae to a culture of herbivorous, heterotrophic protists (Fig. 6). Regions were gated encompassing the phototrophs on cytograms of the algal culture alone (Fig. 6A); then, observations were made with respect to how many heterotrophs exhibited substantial chlorophyll fluorescence after feeding for 30 min on the algae (Fig. 6C). The heterotrophs displayed a wide range of red fluorescent signals, which was expected because each individual heterotroph might vary in the number of ingested algae at any one time and the degree of digestion of the prey. We observed that some of the heterotrophs did fall within the 'phototroph' region after feeding on algae, but most were located outside the gated region (Fig. 6C). This result indicated that the cytometric quantification of heterotrophic nanoplankton may underestimate the total number of heterotrophic protists in a natural water sample if a substantial portion of the phagotrophic assemblage is actively involved in herbivory. Many of these herbivorous cells, however, would still be included in cytometric counts of heterotrophic nanoplankton because of the low red autofluorescence of these cells relative to their size.

It should be noted that this bias (i.e. mistaken classification of herbivorous protists as phototrophic protists) can also be a problem with the established method of counting heterotrophic protists by epifluorescence microscopy. The microscopical method differentiates phototrophic nanoplankton (PNAN) from heterotrophic nanoplankton (HNAN) based on the presence or absence of red autofluorescence under blue light excitation. Some differentiation between heterotrophic protists with ingested algae and phototrophic protists is possible using microscopy, but this task can be difficult for some species of protists.

Similarly, ciliates and heterotrophic dinoflagellates that retain functional chloroplasts obtained from ingested algal prey (Stoecker 1998) might be eliminated from cytograms based on their red fluorescence 'acquired' from the chloroplasts. Elimination of these cells could result in underestimation of the abundance of heterotrophic nanoplankton by cytometry. However, most of these latter cells are larger than the cells that can be effectively counted by flow cytometry. In any event, our cytometric counts compared very well to microscopical counts of natural samples (Fig. 8), implying that either herbivorous and mixotrophic protists did not contribute significantly to the samples analyzed in this study, or that our method of enumeration does not exclude any more heterotrophs than the commonly used microscopical method.

CONCLUSIONS

LysoTracker Green® is a very promising tool for the semi-automated enumeration of nanoplanktonic heterotrophic protists by flow cytometry. Heterotrophic protists in cultures and in natural water samples were accurately quantified using a standard benchtop flow cytometer. The flow cytometer dramatically decreased the time for sample processing relative to traditional microscopical methods, and yielded results for natural samples that were very comparable to counts obtained using epifluorescence microscopy.

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