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DETECTION OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM FUNDYENSE* (DINOPHYCEAE) WITH OLIGONUCLEOTIDE AND ANTIBODY PROBES: VARIABILITY IN LABELING INTENSITY WITH PHYSIOLOGICAL CONDITION[1]

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The toxic dinoflagellate *Alexandrium fundyense* Balech was grown under temperature- and nutrient-limited conditions, and changes in labeling intensity on intact cells were determined for two probe types: an oligonucleotide probe targeting rRNA and a monoclonal antibody (MAb) targeting a cell surface protein. In nutrient-replete batch culture, labeling with the rRNA probe was up to 400% brighter during exponential phase than during stationary phase, whereas MAb labeling did not change significantly with growth stage at the optimal growth temperature. In cultures grown at suboptimal, low temperatures, there was a significant difference between labeling intensity in stationary versus exponential phase for both probe types, with exponential cells labeling brighter with the rRNA probe and slightly weaker with the MAb. The decrease in rRNA probe labeling with increasing culture age was likely due to lower abundance of the target nucleic acid, as extracted RNA varied in a similar manner. With the MAb and the rRNA probes, slower growing cultures at low, nonoptimal temperature labeled 35% and 50% brighter than cells growing faster at warmer temperatures. Some differences in labeling intensity per cell disappeared when the data were normalized to surface area or volume, which indicated that the number of target antigens or rRNA molecules was relatively constant per unit area or volume, respectively. Slow growth accompanying phosphorus and nitrogen limitation resulted in up to a 400% decrease in labeling intensity with the rRNA probe compared to nutrient-replete levels, whereas the MAb labeling intensity increased by a maximum of 60%. With both probes, labeling was more intense under phosphorus limitation than under nitrogen limitation, and for all conditions tested, labeling intensity was from 600% to 3600% brighter with the MAb than with the rRNA probe. Thus, it is clear that significant levels of variability in labeling intensity can be expected with both probe types because of the influence of environmental conditions and growth stage on cellular biochemistry, cell size, rRNA levels, and the number or accessibility of cell surface proteins. Of the two probes tested, the rRNA probe was the most variable, suggesting that in automated, whole-cell assays, it can be used only in a semiquantitative manner. For manual counts, the human eye will likely accommodate the labeling differences. The MAb probe was less variable, and thus should be amenable to both manual and automated counts.

Key index words: *Alexandrium*; antibody; oligonucleotide; physiology; probe; rRNA

Abbreviations: **FITC, fluorescein isothiocyanate; FLS, forward light scatter; FSB, fetal bovine serum; MAb, monoclonal antibody; PAb, polyclonal antibody; RFU, relative fluorescence units; SET, salt, EDTA, Tris buffer (750 mM NaCl, 5 mM EDTA, 100 mM Tris-HCl [pH 7.8] buffer); SSC, side scatter**

A common problem in phytoplankton field ecology occurs when the species of interest is only a minor component of the mixed planktonic assemblage. Many potentially useful physiological measurements (e.g. chlorophyll, ^{14}C fixation, C:N:P ratios) are not feasible or are extremely difficult to obtain for individual species because of the co-occurrence of other organisms and detritus. Another common problem arises from the difficulty inherent in identifying species or strains, which are so morphologically similar that distinguishing characteristics are difficult to discern under the light microscope. Such fine levels of discrimination are not generally practical in monitoring programs or other studies that generate large numbers of samples for cell enumeration. This situation is encountered frequently in studies of harmful or toxic algae, where toxic and nontoxic varieties of species exist and sometimes even co-occur (e.g. Yentsch et al. 1978, Smith et al. 1990).

A new technology that may well alleviate these problems utilizes molecular "probes" that are specific for certain species of phytoplankton (reviewed in Anderson 1995, Scholin and Anderson 1998). Oligonucleotide probes target particular genes or gene products inside cells using short, synthetic

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DNA segments that bind selectively to sequences specific for a particular organism. For marine systems, this technique has thus far been used primarily on prokaryotes (e.g. DeLong et al. 1989, Amann et al. 1990, Distel et al. 1991), but oligonucleotide (rRNA) probes for toxic phytoplankton, such as *Pseudo-nitzschia* and *Alexandrium,* are emerging (Scholin et al. 1994, Adachi et al 1996, Miller and Scholin 1996, Anderson, unpubl.). An alternative to the rRNA probe approach uses antibodies raised against cell surface proteins. These probes are used to label cells of interest by using indirect immunofluorescence or related procedures for visualizing antibody–antigen binding (e.g. Campbell and Carpenter 1987, Shapiro et al. 1989, Vrieling and Anderson 1996). For harmful or toxic phytoplankton, this work has progressed to the stage where polyclonal and monoclonal antibodies (PAbs and MAbs) are available that identify target species in several different algal classes.

Anderson et al. (1989) developed a PAb specific for the "brown tide" chrysophyte *Aureococcus anophagefferens* that has been used along the northeastern U.S. coastline in order to characterize the distribution of *A. anophagefferens* (Anderson et al. 1993). Several PAbs were developed that recognize toxic and nontoxic varieties of the diatom *Pseudo-nitzschia pungens* (Bates et al. 1993). Antibodies have also been raised against several species of toxic dinoflagellates. A monoclonal antiserum produced by Nagasaki et al. (1991) labeled nine strains of *Gymnodinium nagasakiense* from Japan, and antisera produced against cell surface antigens of *Gyrodinium aureolum* from western Europe have been reported (Vrieling et al. 1994). Additionally, MAbs have been developed for *Prorocentrum micans* (Vrieling et al. 1993) and for toxic *Alexandrium* species (Adachi et al. 1993, Sako et al. 1993). Further promise for the application of antibodies in phytoplankton autecology was demonstrated by the separation of toxic *Alexandrium* cells from mixed plankton assemblages using immunomagnetic bead methodologies, which may eventually be utilized for species-specific physiological measurements (Aguilera et al. 1996).

As work progresses on molecular-probe approaches to phytoplankton identification, enumeration, and separation, one area that requires investigation relates to the variability in labeling intensity caused by changes in the physiological condition of the target species. Both antibody and rRNA probes are potentially affected by environmental effects on the physiological state of the target cells (DeLong et al. 1989, Lee and Kemp 1994, Vrieling et al. 1994, 1996). Although Vrieling et al. (1997) found that the number of antigenic sites on two dinoflagellates did not change in abundance except when cultures were in very late stages of batch culture growth, this might not be the case for all cell surface antigens under different environmental conditions. For RNA, it has been well established for some prokaryotes that rRNA varies systematically with growth rate; faster growing cells have more RNA per cell than do cells growing at slower rates (e.g. DeLong et al. 1989). Efforts are underway to use the variation in rRNA content, measured using oligonucleotide probes, to obtain estimates of bacterial growth rate (Lee et al. 1993, Lee and Kemp 1994). In that instance, variability in labeling intensity is meaningful as an indicator of physiological state, but for other applications, the variability in labeling may lead to ambiguous results. An example of the latter is in flow cytometry, where the intensity of positive labeling must be significantly higher than the background fluorescence of control or unlabeled cells if a particular species is to be identified and gated with electronic thresholds (Vrieling et al. 1994, Anderson 1995). Even if signal enhancement techniques are used for cells with lower antigen-expression levels, flow cytometric gating will introduce estimate errors of the abundance of a target species in mixed algal assemblages, mostly because of the natural dispersion of the parameter distributions for natural populations of cells.

In this study, the toxic dinoflagellate *A. fundyense* was grown under different nutrient and temperature conditions, and changes in labeling intensity were determined for intact cells using two probe types: an oligonucleotide probe targeting ribosomal RNA and a MAb targeting cell surface proteins. The fluorescently labeled probes were detected and quantified with flow cytometry.

MATERIALS AND METHODS

Cultures. All experiments utilized *A. fundyense* (strain GTCA28) isolated from the western Gulf of Maine and rendered clonal by single cell isolation. Inoculum cultures were maintained for several transfers in mid to late exponential growth in modified f/2 medium (Guillard and Ryther 1962) made with 0.2 μM filtered Vineyard Sound seawater (31 psu). The f/2 medium was modified by adding Na_2SeO_3 and by reducing the concentration of $CuSO_4 \cdot 5H_2O$ to a final concentration of 10^{-8} M each. Cultures were grown at the experimental temperatures of 8° and 15° C on a 14:10 h LD cycle (ca. 200 μmol photons·m^{-2}·sec^{-1} irradiance provided by cool white fluorescent bulbs).

Experimental. At the start of the experiment, nutrient-replete cultures were inoculated into six 2.8-L Fernbach flasks containing 1.3 L of temperature-equilibrated f/2 medium for each treatment. Eight 1-L flasks were also inoculated, each with 400 mL of f/40 nitrate or phosphate (identical to f/2, but containing one-twentieth the normal amount of $NaNO_3$ or NaH_2PO_4). The initial cell density in the experimental flasks was calculated to be 200 cells·mL^{-1}. Cell densities of the 8° and 15° C cultures were determined both microscopically, using a Sedgewick Rafter counting chamber, and with a Coulter Counter (Coulter model Z_F with P-128 size distribution analyzer). Both counting methods provided similar results. However, in the low-nutrient treatments, Coulter counts proved to be unreliable, as large numbers of empty thecae interfered with the analysis. Microscope counts and *in vivo* fluorescence measurements made with a Turner model 10-AU fluorometer were therefore used to evaluate the growth of these cultures. Daily triplicate Coulter and single fluorescence measurements were performed on the temperature and low-nutrient experimental flasks, respectively, and triplicate microscope cell-count samples were taken from all treatments when samples for flow cytometric analysis were collected. Cell sizing data for the 15° and 8° C experiments were also collected with the Coulter

Counter. Low-nutrient cell-size information was obtained by measuring a minimum of 100 Utermohl's-preserved cells on an image analysis system composed of a Zeiss Axioskop equipped with a Princeton Instruments MicroMax digital camera. Digitized cell images were quantified with IP Lab Spectrum Software.

Flow cytometry samples for the 15° C batch culture were collected during exponential growth on days 0, 3, 5, 7, and 10. After day 10, the cells entered the stationary phase of growth, and six additional samples were collected over the next 25 days. During the exponential phase of growth of the 8° C cultures, six samples were collected, and an additional four samples were taken during the stationary growth phase from days 20 to 35. Flow cytometry samples were taken by removing a sufficient volume of culture to contain 5×10^5 cells each from two of the six culture flasks. The samples were initially concentrated by reverse filtration through a 13-μm Nitex sieve to a volume of about 40 mL and preserved with formalin (11% v/v, final concentration = 4% formaldehyde). The preserved samples were stored at 4° C for 1 h, centrifuged (5000 × *g*, 5 min, 4° C), and the supernatant aspirated away. The cell pellet was washed three times for 10 min each time with 10-mL volumes of 4° C, 0.2-μm–filtered seawater to remove residual formalin. After the last wash/centrifugation step, the cell pellet was resuspended in approximately 1 mL filtered seawater. The cell slurry was injected through a tuberculin syringe with a 22-gauge needle into 10 mL of ice-cold methanol in order to extract chlorophyll and to desegregate any clumps of cells that may have formed during the sample processing. Methanol-extracted samples were stored overnight at −20° C, concentrated by centrifugation, transferred into 2-mL cryovials, and stored at −70° C until needed for flow cytometric analysis. Samples for biochemical analysis, RNA, and DNA were collected as above, except that only 5000 cells were collected by reverse filtration and centrifugation. The overlying medium was aspirated to about 1 mL above the cell pellet, and the cell concentrate was transferred to a 1.8-mL cryovial, centrifuged, aspirated, and frozen in liquid nitrogen. Frozen samples were transferred to −70° C storage.

Low-nutrient treatments were grown at 20° C in a semicontinuous culture mode by replacing a prescribed volume of culture with fresh medium on a daily basis in order to force the culture into four predetermined growth rates: 0.5, 0.35, 0.2, and 0.1 divisions·day^{-1}. Samples for the low-nutrient component of this experiment were harvested in the same manner as were the batch cultures, except that only one final endpoint sample was taken from duplicate flasks once the cultures were deemed to have achieved steady state. Steady state was defined by stable *in vivo* fluorescence and cell-density measurements for at least 10 consecutive days.

In order to account for any cell loss during sample processing, 10 μL of the −70° C flow cytometry samples were diluted in 1050 μL PBS and counted. Based on the results of these counts, a sufficient volume of sample was removed from each vial to yield 7500 cells for antibody and oligonucleotide probing.

MAbs. Antibody labeling used mouse MAb M8751-1 specific for several strains of *Alexandrium* sp. (Adachi et al. 1993, Sako et al. 1993). This antibody has been shown to cross-react with *A. fundyense* isolate GTCA28 from the Gulf of Maine and has also been shown to label naturally occurring *Alexandrium* sp. from the Gulf of Maine (Anderson, unpubl.). The antibody, which is of the immunoglobulin (Ig)G_1 subclass of Ig proteins, was supplied as a hybridoma culture supernatant from cells that were grown in PRMI1640 medium containing 10% fetal bovine serum (FBS, Nissui, Japan). The supernatant was aliquoted into smaller volumes and frozen at −20° C for later use. Since an unreactive "control" hybridoma supernatant grown under the same conditions as M8751-1 was unavailable, purified mouse myeloma protein (MMP; Sigma Chemical Co. [St. Louis, MO], M9035) of the IgG_1 class was used as the negative control serum. A final concentration of the control serum of 1:100 was used to approximate the specific antibody concentration that might be expected in the final dilution (1:10) of the "positive" M8751-1 supernatant, although the actual specific antibody concentration of the M8751-1 was not determined.

Antibody labeling protocol. Two volumes containing approximately 7500 cells from each methanol-extracted sample were pipetted into separate 1.5-mL microcentrifuge tubes, one each for the positive and negative control samples. The samples were initially washed once with 0.02 M PBS by centrifugation (2000 × *g* for 3 min) in order to remove methanol and methanol/seawater precipitates. The pelleted cells were blocked for 45 min under constant mixing with 1.0 mL of 5% normal goat serum (NGS; Sigma) in PBS. Samples were then pelleted via centrifugation, the NGS/PBS supernatant was discarded, and 0.5 mL of the primary antibody (MAb; M8751-1), diluted 1:10 (v/v with 5% NGS/PBS), was added. After 45 min of incubation with the MAb, the cells were washed three times by centrifugation with 0.02 M PBS in 0.5% NGS. The pelleted cells were then incubated for another 45 min with a goat anti-mouse IgG secondary antibody (heavy + light chain) conjugated to fluorescein isothiocyanate (FITC; Molecular Probes, Inc. [Eugene, Oregon], F-2761; 1:300; v/v with 5% NGS/PBS). After washing two times with 0.5% NGS/PBS, the cells were washed once with PBS only and were then resuspended in 250 μL PBS for analysis on the flow cytometer.

Negative controls for each sample were processed in the same manner as described above, except that a 1:100 dilution of MMP in NGS/PBS was used as a replacement for the specific MAb, M8751-1.

rRNA probes. A large-subunit ribosomal RNA oligonucleotide probe, named NA1 (5′AGTGCAACACTCCCACCA3′; Anderson et al., unpubl.), that is specific for dinoflagellates within the North American *Alexandrium* species cluster (*A. tamarense/fundyense/catenella*) (Scholin et al. 1994) was used to quantify cellular rRNA in these samples. A shipworm bacterium probe (5′GCTGTACTCAAGTTACCCAGTTCTAA3′; Distel et al. 1991) was used as a negative control probe to account for nonspecific labeling and the inherent autofluorescence of cells, which might contribute to the positive NA1 signal. The probes had FITC-conjugated to an ethyl amino group at the 5′ end of the oligonucleotide, as synthesized by Molecular Probes, Inc. The probes were received in a purified, lyophilized form, and a portion of this material was resuspended in sterile distilled water to a final concentration of 50 ng·μL^{-1}, aliquoted in 50-μL volumes, and stored frozen at −20° C.

Oligonucleotide labeling protocol. Subsamples were removed as described in the antibody-labeling protocol. They were then centrifuged (2000 × *g*, 5 min, 23° C), the supernatants were aspirated, and 50 μL of hybridization buffer containing 5× SET (750 mM NaCl, 5 mM EDTA, 100 mM Tris-HCl [pH 7.8]), 0.1 μg·mL^{-1} polyadenylic acid, 0.1% Tergitol NP-40, and 10% formamide were added to the cell pellets. These samples were prehybridized at 50° C for 30 min. Following the prehybridization, 5.0 μL of either the NA1 or shipworm probe (final concentration, 4.5 μg·mL^{-1}) were added and the samples incubated for an additional 2 h at 50° C in the dark. Samples were then centrifuged as described above, and the cell pellets were washed in 50 μL 0.2× SET buffer for 10 min at 50° C. Following the wash, the samples were again centrifuged, the supernatants aspirated, and the hybridized pellets resuspended in 250 μL 5× SET.

These samples, as well as the antibody-labeled samples, were packed in ice and sent via overnight mail for flow cytometric analysis. The samples were refrigerated (4° C) upon arrival. Analyses were all performed within 24–48 h of the labeling procedures.

Flow cytometric analysis. All analyses were performed on a Becton Dickinson FACScan flow cytometer that is housed at the MacIsaac Center for Particle Analysis, Bigelow Laboratory for Ocean Sciences. The instrument was equipped with a 15-mW, 488-nm argon laser for excitation. Emission detection for green (FITC) fluorescence used a 515–525-nm bandpass filter, and detection for red (chlorophyll) fluorescence used a 650-nm longpass filter. Detectors for forward scatter (FLS) and side scatter (SSC) were also in place to collect information relative to cell density and size/composition, respectively.

The antibody- and the oligonucleotide-labeled samples contained in microcentrifuge tubes were transferred to plastic vials, and the samples were run to near dryness with occasional mixing in order to insure that the maximum number of cells was analyzed and that the cells remained in suspension. A 10-μm external

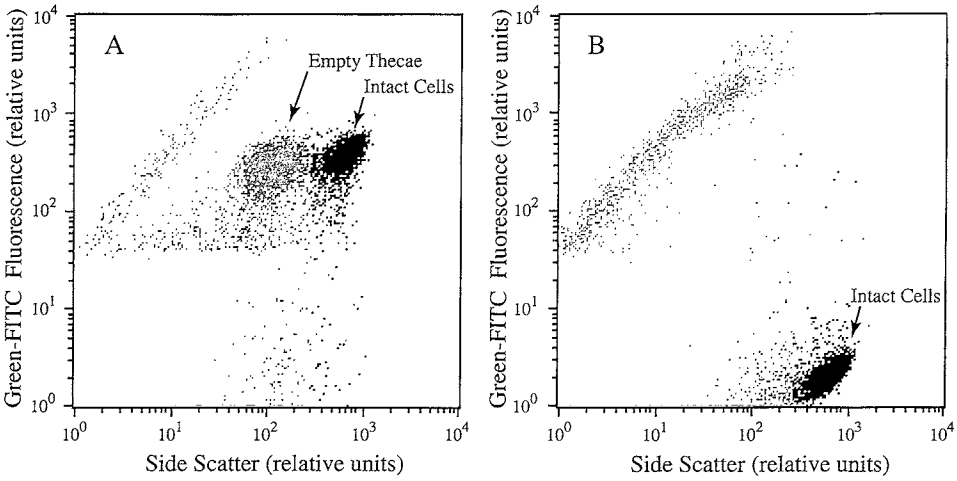

FIG. 1. Typical scatter plots of flow cytometry data relating SSC (a measure of cellular refractive index) and green-FITC fluorescence of MAb-labeled *Alexandrium* cells. Positively labeled cells and empty thecae were observed as separate distributions in panel (A) (black = intact cells; grey = empty thecae). Both populations had similar green fluorescence intensities, but the empty thecae had lower SSC values. A single distribution of unlabeled intact cells (black) in the negative control sample is shown in panel (B). Because of the lack of antibody (FITC) and autofluorescence signals, the empty thecae were below baseline and thus were not detected in the negative control.

bead standard (DNAcheck fluorospheres, Coulter Corp., Miami, Florida; 6603488) that fluoresces at all wavelengths was run after every 10 samples to verify instrument stability.

Four parameters (green fluorescence, red fluorescence, FLS, and SSC) were collected on a logarithmic scale, whereas cell-density information (number of particles counted) was collected linearly. In order to eliminate the enumeration of particles from culture debris, we gated on chlorophyll (red) fluorescence, thus analyzing only intact cells (containing residual chlorophyll after methanol extraction) and larger particles that scatter enough light into the red channel to be above threshold. Flow cytometric data are typically presented in the form of scatter plots, in which two parameters are plotted against one another. For instance, SSC, which is related to cell size, is plotted as the independent (X) variable, and labeling intensity from the green fluorescence emission of FITC is plotted as the dependent (Y) variable (e.g. Fig. 1A, B). The fluorescence information derived from the flow cytometer is in relative fluorescence units (RFU) for both positive and negative controls.

Nucleic acid analysis. Cellular RNA and DNA were analyzed with the CyQuant™ Cell Proliferation Assay Kit (Molecular Probes, Inc.). Using nuclease-free techniques, frozen cell pellets were resuspended in 1.06 mL of the lysis buffer and mixed. Three 20-μL subsamples were then diluted to 1 mL for cell counts to quantify the nucleic acid values on a per-cell basis. An additional 300 μL were placed in a 2-mL screw-cap tube, filled with 500 μm zirconium beads, and shaken on a high setting for 90 s in a Mini-Beadbeater™ (Biospec Products, Bartlesville, Oklahoma). Four 200-μL aliquots of cell lysate were added to separate wells of a 96-well tissue culture plate. Three serial dilutions of the samples were made by adding equal amounts of lysis buffer to the original sample in wells adjacent to the undiluted sample. One set of sample dilutions was left untouched, while the other three sets were reacted with either DNase I (RNase free, 45 Kunitz units·mL^{-1} final concentration; Boehringer Mannheim, 776 785) or RNase A (DNase free, 1.35 Kunitz units·mL^{-1} final concentration; United States Biochemical, 21210) or a combination of the two nucleases. Serial dilutions of salmon testes DNA (Sigma, D-9156) and bovine liver RNA (Sigma, R-5502) were made at the same time in order to quantify *Alexandrium* nucleic-acid concentrations. The samples were incubated in the dark at 37° C for 1 h and then were reacted with 2× Gr/lysis buffer for 5 min in the dark. The plate was read on a Millipore Cytofluor™ 2300 Fluorescence Measurement System with 480-nm emission and 520 excitation filters in place. DNA concentrations were calculated by subtracting the fluorescence of the DNase/RNase treatment from the RNase-only treatment, whereas the RNA concentration was calculated by subtracting the DNase/RNase treatment from the DNase-only treatment. These products were compared to DNA and RNA standard values in order to determine the nucleic-acid concentration in the algal extracts.

RESULTS

Saturation of antibody and rRNA probe labeling. Experiments were performed to determine the probe concentration necessary to achieve uniform (saturated) labeling of the cells regardless of the cell densities in the samples. A MAb dilution of 1:10 in 0.5 mL of 5% NGS/PBS was sufficient to label up to 20,000 cells in a sample without a significant decrease in labeling intensity (data not shown). Similarly, the amount of rRNA probe needed to saturate the target rRNA in a given number of cells was determined by keeping the concentration of probe and the volume of hybridization buffer constant at 4.5 μg·mL^{-1} and 50 μL, respectively, whereas the number of cells to be hybridized was varied between 7000 and 68,000. A decrease in labeling intensity was observed at concentrations above 23,000 cells per sample (data not shown), so a probe concentration of 4.5 mg·L^{-1} was deemed adequate for the densities used in this study, which were always below 20,000 cells per sample. All experiments presented here were completed with the same batch of antibody (both primary and secondary) and rRNA probes. If the need to use different batches does arise, it would be necessary to retitrate in order to determine the titer of probes so that variability in labeling intensity due to this factor can be avoided.

Cultures. The 15° C cultures in nutrient-replete f/2 medium maintained a growth rate of 0.65 divisions·day^{-1} for approximately 10 days, after which the cells entered stationary phase for the next 25 days (Fig. 2A). The exponential growth phase was defined as the interval between days 3 and 10, based on the sustained, steady growth rate observed during that time. Stationary phase was defined as the interval between days 20 and 35 and thus did not

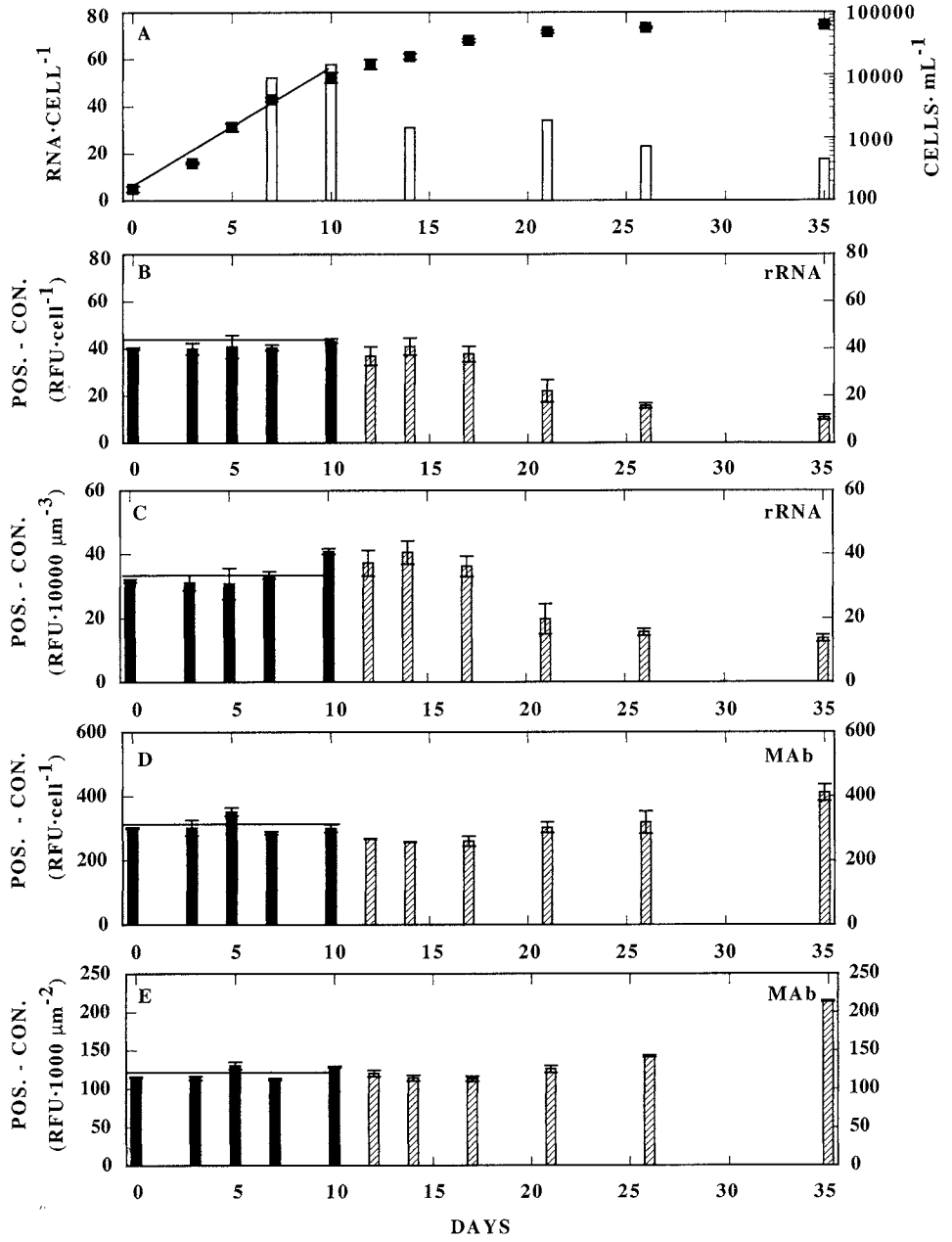

FIG. 2. Changes in RNA concentration, cell density, and probe labeling intensities in 15° C batch culture. Labeling intensities (panels B–E) depict positive (POS) cell fluorescence (i.e. with species-specific rRNA or MAb probes) minus control (CON) cell fluorescence (i.e. negative rRNA probe or nonspecific primary antibody). Solid line indicates data average during exponential growth. Error bars denote ±1 SD. (A) Extracted RNA concentration per cell, left axis, open bars; *Alexandrium fundyense* cell density, right axis, filled boxes; (B) rRNA-probe labeling intensity (RFU·cell^{-1}); (C) rRNA-probe labeling intensity (RFU·10000 μm^{-3}); (D) Mab-probe labeling intensity (RFU·cell^{-1}); (E) Mab-probe labeling intensity (RFU·1000 μm^{-2}).

include the transition phase following exponential growth. The 8° C cultures grew at 0.32 divisions·day^{-1}, about half as fast as the 15° C cultures (Fig. 3A). Exponential phase was defined as days 3 to 17 and stationary phase as days 24 to 35, for that culture. For both the 15° C control and the 8° C low-temperature treatments, replicate cultures grew at similar rates and achieved similar cell densities.

The nitrogen (N) and phosphorus (P) nutrient-limited cultures (Figs. 4, 5) were equilibrated to a semicontinuous mode of growth for 12 days before steady state was achieved. Steady state was then maintained for at least 10 consecutive days before cells were harvested for analysis.

In all treatments, the cells were in good morphological condition throughout the experiments; however, there were large numbers of empty thecae in the cultures during the stationary phase of growth and in the nutrient-limited treatments, which is typical for *A. fundyense* under these conditions. The low-temperature and the N-limited cultures both resulted in larger cells than did the nutrient-replete, warm-temperature treatments. Average surface areas and volumes for the N-limited cells were 6% and 9% larger, respectively, than the P-limited cells, whereas the 8° C cells were 26% and 40% larger than the 15° C control cells.

Epifluorescent microscope observations. Aliquots taken from the samples intended for flow cytometer analysis were examined under an epifluorescence micro-

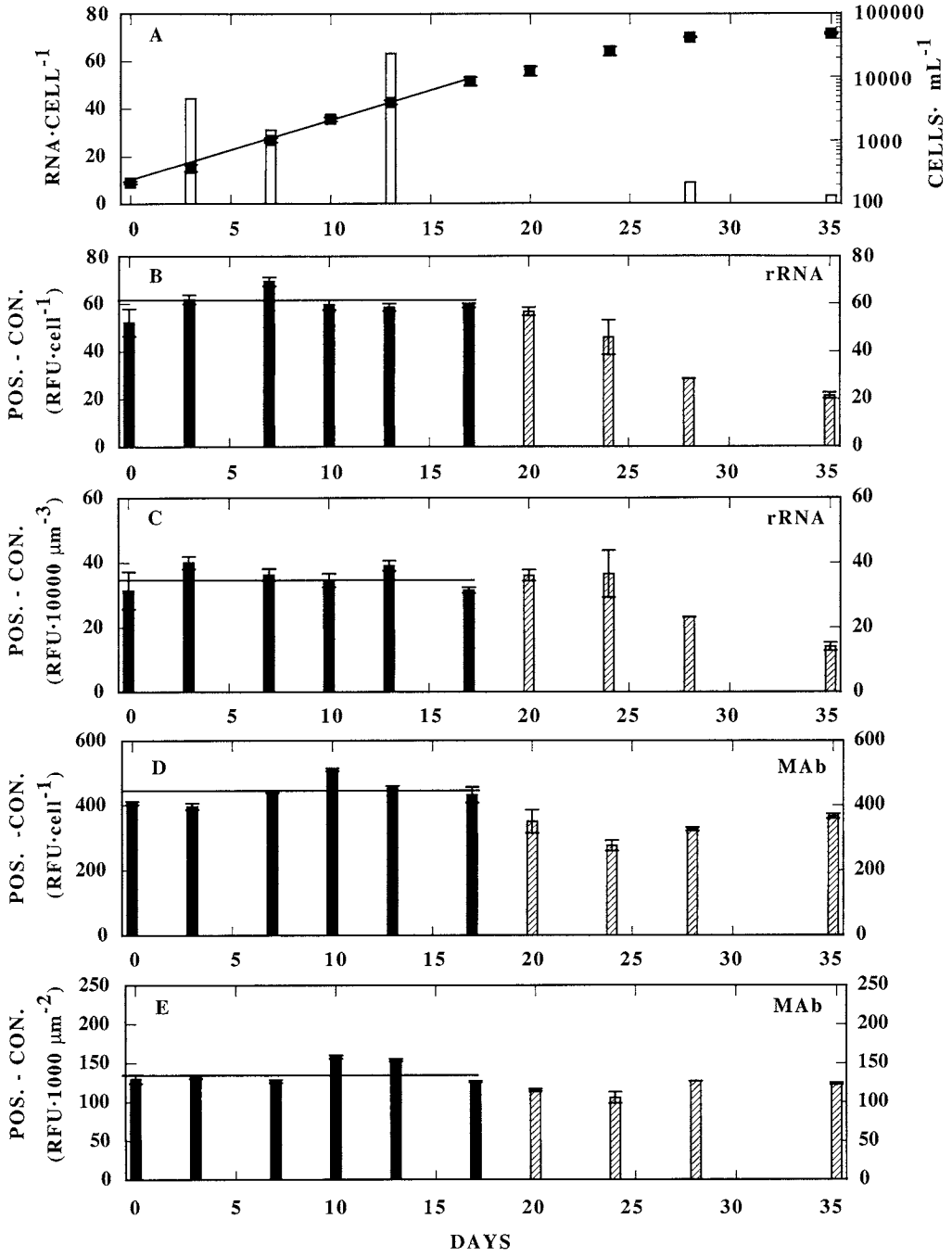

FIG. 3. Changes in RNA concentration, cell density, and probe labeling intensities in 8° C low-temperature batch culture. Details as in Figure 2.

scope equipped with a long bandpass FITC filter set to verify that the stains worked properly. The MAb-labeled *Alexandrium* cells appeared bright green around the periphery of the cell, indicating that the antibody recognized cell surface antigens (Sako et al. 1993). Negative control samples using MMP instead of the M8751-1 MAb did not show the bright green FITC peripheral staining. Because the cells were methanol extracted, red autofluorescence due to chlorophyll was minimal. Cells labeled with the rRNA probe showed green fluorescence throughout the cytoplasm. The negative shipworm controls showed only a faint yellow–orange autofluorescence in the cytoplasm.

ANTIBODY LABELING

Empty thecae. The presence of empty thecae in samples harvested during the stationary phase and in the nutrient-starved cultures interfered with flow cytometric analysis of the MAb-probed samples, since both empty thecae and intact cells had similar levels of green fluorescence (Fig. 1A). This was due to the MAb recognition and FITC labeling of surface antigens on cell membranes found on intact

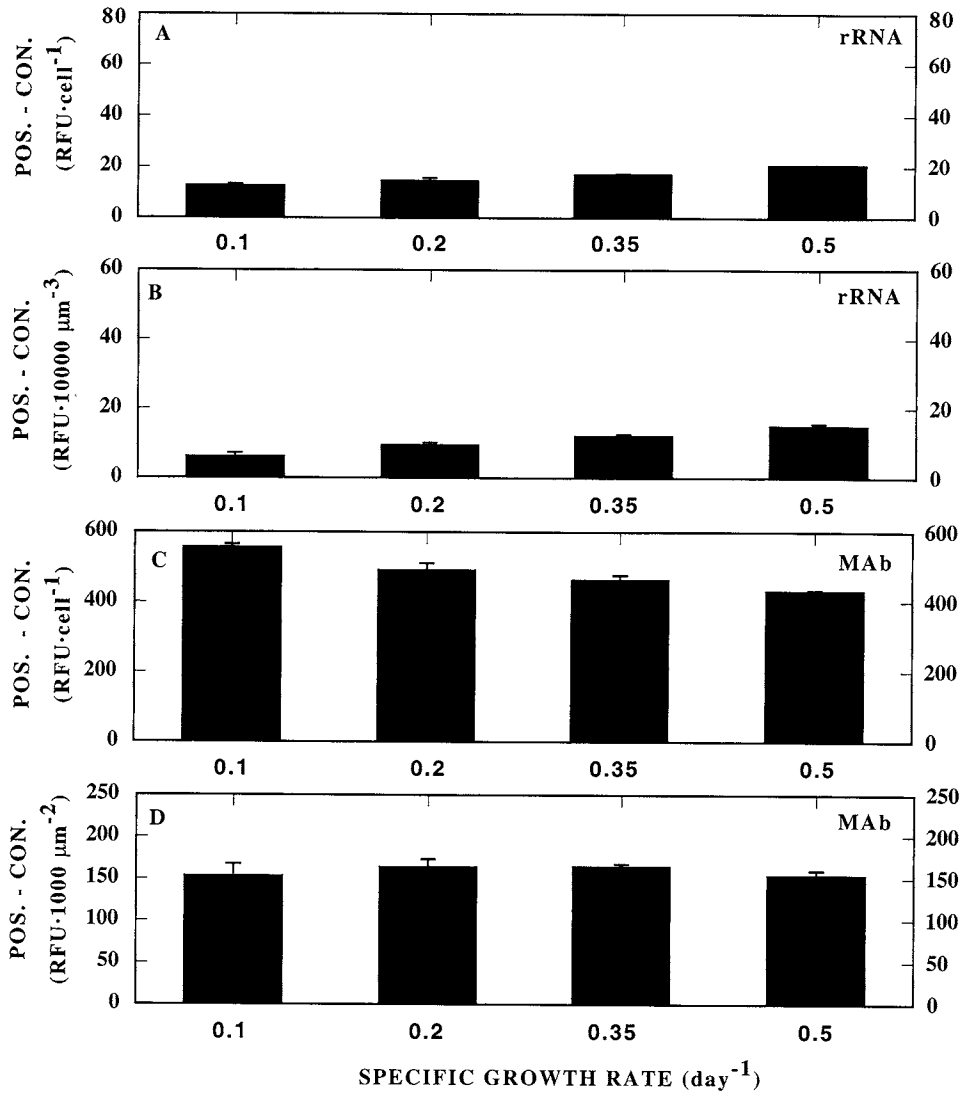

FIG. 4. Changes in rRNA- and Mab-probe labeling intensities in N-limited, semicontinuous culture. (A) rRNA-probe labeling intensity (RFU·cell^{-1}); (B) rRNA-probe labeling intensity (RFU·10000 μm^{-3}); (C) Mab-probe labeling intensity (RFU·cell^{-1}); (D) Mab-probe labeling intensity (RFU·1000 μm^{-2}).

cells as well as the empty thecae. Samples probed with the rRNA probe did not suffer from interference by empty thecae, since that probe is specific for rRNA found in the cytoplasm of intact cells. In order to exclude empty thecae during quantification of the intact cells labeled with the MAb, it was necessary to separate particles on the basis of autofluorescence in the green channel and light-scattering characteristics of the unlabeled control samples (Fig. 1B) compared with probed samples (Fig. 1A). In the unlabeled sample, the empty thecae were not detected in either green or red fluorescence channels, because there was no antibody or chlorophyll signal. Unlabeled intact cells showed normal background amounts of green fluorescence (Fig. 1B) and high red fluorescence because of residual chlorophyll (data not shown). However, in the labeled sample, two populations were observed, both of which had similar green fluorescence intensities but which differed in SSC signals (Fig. 1A). Since SSC can be a measure of refractive index for particles (Spinrad and Brown 1986), the population that had the lower SSC signal in the labeled sample would intuitively be the empty thecae. Even though we gated on chlorophyll, the labeled empty thecae were observed because of spectral bleed of the FITC into the red channel. Observations of a subsample by epifluorescent microscopy showed that the empty thecae had bright green fluorescence on their surfaces that were attributable to the antibody, but they lacked any chlorophyll fluorescence. Intact cells, on the other hand, had both green fluorescence on the surface of the cells and residual chlorophyll fluorescence. Green autofluorescence and SSC of the controls (Fig. 1B) were thus used to define the domain of the intact cells in labeled samples (Fig. 1A), so all RFU values reported here do not include empty thecae.

Temperature and growth-stage effects. For the 15° C cultures, background fluorescence values of all unlabeled control samples (e.g. no primary antibody) were very low and stable throughout the growth

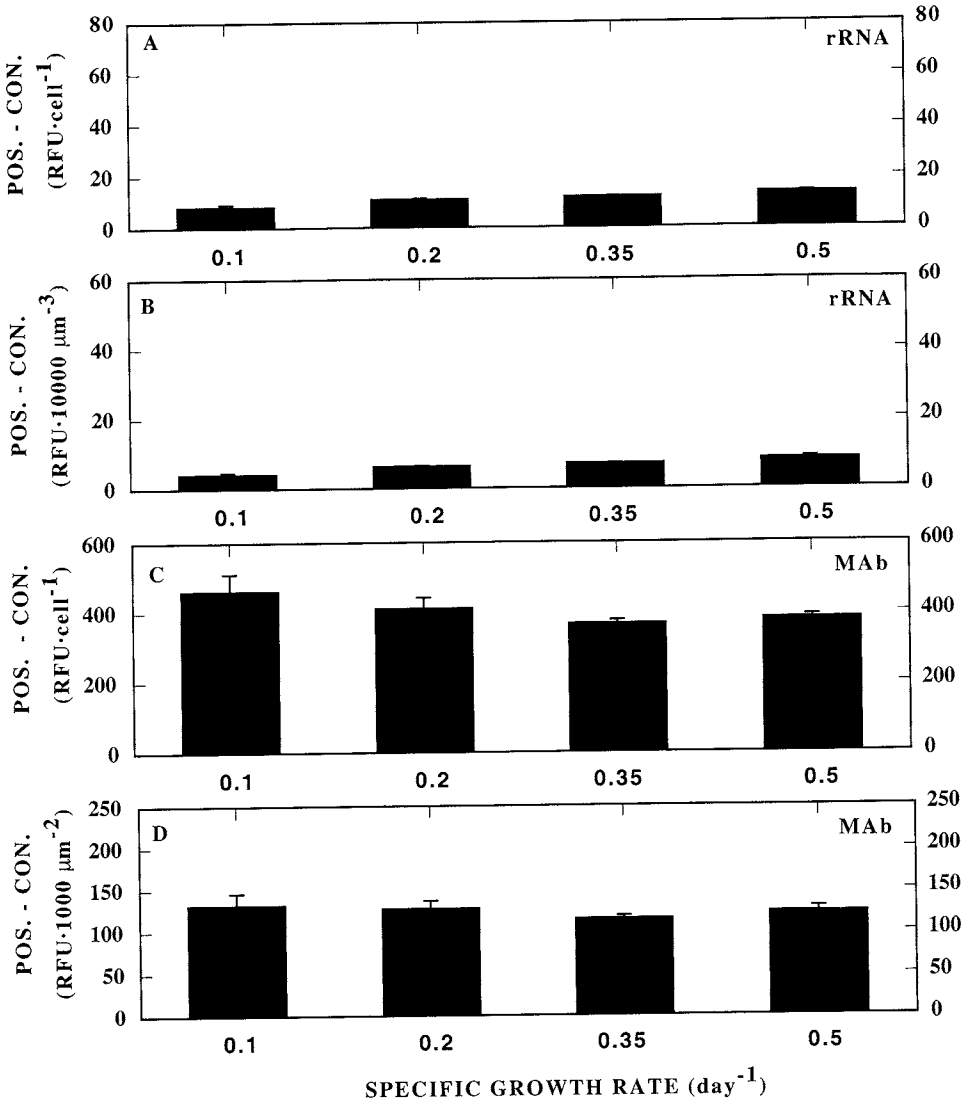

FIG. 5. Changes in rRNA- and Mab-probe labeling intensities in P-limited, semicontinuous culture. Details as in Figure 4.

curve, with an average RFU·cell^{-1} of 2.4. Positive labeling was at least two orders of magnitude higher, typically 260–410 RFU·cell^{-1} (Fig. 2D). Labeling intensity was relatively uniform through time, with only the last sample, which was harvested on day 35 during late-stationary phase, showing a major departure from the overall mean (by increasing 33% to about 411 RFU·cell^{-1}) (Fig. 2D). Overall, there was no significant difference between the fluorescence intensity of cells during exponential growth and those in stationary phase ($P = 0.217$; Kruskal-Wallis one-way analysis of variance). These conclusions hold whether the data are expressed on a per cell basis (Fig. 2D) or are normalized to cell surface area (Fig. 2E).

Antibody-labeling intensities for the low-temperature (8° C) cultures (Fig. 3D) were generally higher and more variable than were the 15° C treatments (Fig. 2D). Values ranged from over 500 RFU·cell^{-1} during mid-exponential growth to a low of 280 during stationary phase (Fig. 3D). The mean was approximately 400 RFU·cell^{-1}, about 30% higher than for the faster growing 15° C cultures. Background fluorescence of the negative controls was very low, at 2.8 RFU·cell^{-1}. Stationary-phase intensities were significantly different from those in exponential phase ($P < 0.0001$; Kruskal-Wallis one-way analysis of variance).

When these data were normalized to cellular surface area, the differences between exponential- and stationary-phase cells in the 8° C treatment as well as those witnessed between the 8° and 15° C treatments almost completely disappeared, with cells at nearly all stages of growth averaging 130 RFU·1000 μm^2 (Figs. 2E, 3E), regardless of growth temperature or growth stage.

Nutrient effects. Antibody-labeling intensities at the four different N-limited growth rates are shown in Figure 4C and D. Mean intensities decreased from 461 to 366 RFU·cell^{-1} as growth rate increased from 0.1 to 0.5·d^{-1}. Consistent with this trend, the intensity obtained during exponential phase in the nu-

trient-replete batch culture (Fig. 2D) growing at 0.65·d^{-1} was approximately 300 RFU·cell^{-1}. These data were consistent with a significant inverse correlation between labeling intensity and growth rate ($P < 0.05$; Spearman coefficient of rank correlation). When normalized to surface area, however, there were no significant differences between the labeling intensities of cells at different growth rates, and the intensities per unit of surface area were similar to the average batch culture values; 124 versus 130 RFU·1000 μm^{-2}, respectively.

P-limited cells decreased in labeling intensity as growth rate increased ($P < 0.01$; Spearman coefficient of rank correlation) and had overall intensities (Fig. 5B) that were about 20% higher than those for N-limited cells. Mean intensities varied from 556 to 431 RFU·cell^{-1} as growth rate increased from 0.1 to 0.5·d^{-1}. The intensity during exponential growth in the fast-growing (0.65·d^{-1}) nutrient-replete batch culture was 300 RFU·cell^{-1}, which was consistent with decreasing labeling as growth rate increased. When these data were normalized to cell surface area, there was no significant difference between growth rates (Fig. 5D).

rRNA PROBE LABELING

Temperature and growth-stage effects. Cells labeled with the shipworm (negative control) rRNA probe had very low labeling intensities, averaging 2.9 RFU·cell^{-1} in the 15° C control cultures (data not shown). This typically represented 10% or less of the positive labeling intensity (Fig. 2B). The intensities of cells labeled with the NA1 rRNA probe varied with growth phase in the 15° C cultures (Fig. 2B). During exponential growth, labeling intensity averaged 40.8 RFU·cell^{-1}, whereas cells in the stationary phase averaged 27.3 RFU·cell^{-1}. During the transition period between exponential and stationary phase (days 12–17), labeling intensities remained relatively constant and were not significantly different from exponential levels, even though most cells were no longer actively dividing. In the late-stationary phase, the intensity decreased dramatically. The decrease became most apparent on day 21, 11 days after the cessation of exponential growth. Thereafter, the labeling intensity was approximately 10 RFU·cell^{-1}, or one-fourth of the exponential level. The difference between exponential- and stationary-phase labeling intensities was significant ($P < 0.009$; Kruskal-Wallis one-way analysis of variance).

For the slower growing, 8° C cultures, labeling intensities were, on average, 53% higher than those of the 15° C control cultures growing near optimal rates (Fig. 3B). At 60 RFU·cell^{-1}, these were the highest intensities obtained using the rRNA probe in this study. Stationary-phase intensities averaged 32 RFU·cell^{-1}. Negative control cells had low fluorescence, averaging 4 RFU·cell^{-1} (data not shown). There was a significant difference between the labeling intensities in exponential versus stationary phase at 8° C ($P < 0.001$; Kruskal-Wallis one-way analysis of variance).

Normalizing rRNA-probe labeling intensity data to cell volume did not significantly alter the trends observed on a per-cell basis within each temperature treatment; exponential-phase cells had 24% and 45% higher intensity per unit volume than did stationary-phase cells grown at 15° and 8° C, respectively (Figs. 2C, 3C). Volume normalization did, however, remove most of the differences between the two temperatures; exponential-phase cells were only 7% brighter per unit volume at 8° C than they were at 15° C, compared to a 53% difference on a per-cell basis. The 8° C cells were substantially larger than were the 15° C cells, and, therefore, they were proportionally brighter.

Nutrient effects. N-limited cells labeled with the rRNA probe had relatively low fluorescence, ranging from less than 10 to 13.3 RFU·cell^{-1} as growth rate increased from 0.1 to 0.5·d^{-1} in semicontinuous cultures (Fig. 4A). There were significant differences between the fluorescent intensities at these growth rates ($P < 0.01$; Spearman coefficient of rank correlation). If we include results from the nutrient-replete batch culture grown at this same temperature, which peaked at approximately 40 RFU·cell^{-1} at 0.65·d^{-1} (Fig. 2B), differences in labeling due to N limitation can vary by a factor of four, with highest intensities at the higher growth rates. Normalization to cell volume did not change this factor (Fig. 4B).

With P limitation, positive labeling was brighter than with N limitation (by almost 50%, ranging from 13 to 21 RFU·cell^{-1} at growth rates of 0.1 to 0.5·d^{-1}) (Fig. 5A). Normalization of labeling intensities to cell volume increased this difference to 67% (Fig. 5B). Positive labeling intensity thus increased significantly with increasing growth rate ($P < 0.01$; Spearman coefficient of rank correlation). Here again, nutrient-replete batch cultures (Figs. 2B, 3B) suggest an upper limit of approximately 40 RFU·cell^{-1} at 0.65·d^{-1} or higher when cells are not nutrient limited, so rRNA labeling intensities per cell varied by a factor of three under different degrees of P starvation and varied twofold when expressed on a cell volume basis. Control or background intensities for the N- and P-limited treatments averaged 2.5 and 3.3 RFU·cell^{-1}, respectively.

Extracted RNA. Patterns of extracted, total cellular RNA were very similar to the RFU·cell^{-1} values observed in cells labeled with the rRNA probe. During exponential growth, high levels of RNA, about 50 pg·cell^{-1} on average, were measured in nutrient-replete 15° and 8° C cultures (Figs. 2A, 3A). As these cultures entered stationary phase, cellular RNA quotas decreased, on average, to about 16 pg·cell^{-1}. Equally low total RNA values, on average about 20 pg·cell^{-1}, were also observed at all growth rates in the two nutrient-limited experiments (data not shown). These cultures had extracted RNA concentrations comparable to those seen in the stationary

phase of cultures grown under nutrient-replete conditions (Figs. 2A, 3A).

DISCUSSION

Identification of cells using MAb and rRNA probes has been accomplished with a variety of phytoplankton species in several different classes. For MAbs, specificity has been demonstrated at the genus and species levels (e.g. Campbell et al. 1983, Hiroshi et al. 1988, Anderson et al 1989, Shapiro et al. 1989, Uchida et al. 1989, Bates et al. 1993, Vrieling et al. 1993). Separation at the strain or variety level has been reported for the dinoflagellate *Alexandrium* (Adachi et al. 1993, Sako et al. 1993) and for the diatom *Pseudo-nitzschia* (Bates et al. 1993), although the strains involved in the latter study have since been reclassified as separate species (Manhart et al. 1995). Likewise, superb sensitivity has been demonstrated for rRNA probes, some of which can differentiate between species of *Pseudo-nitzschia* that appear to be morphologically identical at the light microscope level (Scholin et al. 1994, Miller and Scholin 1996). Oligonucleotide (rRNA) and antibody probes thus have great promise as tools to facilitate the rapid identification, enumeration, and separation of target species in a mixed plankton sample. Here we demonstrate that care must be taken in the application of these probes because of environmentally induced variability in labeling intensity. Our results supplement those reported in a study that used antibody probes to the dinoflagellates *Gyrodinium aureolum* and *Gymnodinium nagasakiense* by Vrieling et al. (1996) but are the first reports of physiologically linked labeling variability in phytoplankton using rRNA probes.

General issues. For some applications, the objective is simply to label the cells of interest so that they are easy to visually distinguish from co-occurring species. In such cases, variability in the absolute level of labeling on each cell may not be a critical parameter, as would be the case with manual microscopic examination of samples or flow cytometric analysis of a mixed population treated with a species-specific MAb or rRNA probe. With the flow cytometer, instrument settings could be adjusted so as to specify positive identification across a suitably broad range of labeling intensities, as long as cross-reactions with non–target species are unlikely, and other parameters, such as light scatter, can be used to provide secondary confirmation of species identification (Anderson 1995, Vrieling et al. 1996).

Other applications, such as automated enumeration, are more sensitive to the intensity of labeling. For example, in sandwich hybridization or enzyme-linked immunosorbent (ELISA) assays, the amount of probe bound to target antigens or nucleic-acid sequences should be directly proportional to the number of cells in that sample (i.e. the number of target sites for probe binding per cell should be invariant). If this is not true, abundance can only be estimated with a precision determined by the probe-binding variability that might occur because of changes in cell physiology.

Of the two probe types tested in this study, the rRNA probe was most variable in this regard. Labeling intensity was 3–4 times higher in exponential growth than in late stationary phase in batch culture and varied threefold between nutrient-replete and nutrient-limited cultures. The MAb labeling was more uniform under these same conditions, but significant differences (33%) were observed between exponential- and stationary-phase cells in the slower growing 8° C batch culture. MAb-labeling intensity also changed with the degree of nutrient limitation, decreasing with increasing growth rate under both N and P limitation, but these differences were smaller than those associated with the rRNA probe. These results and their significance with respect to the use of these two probe types in natural waters are discussed in more detail below.

Autofluorescence and background effects. The ability to recognize probe-labeled cells depends in part on the difference in fluorescence between the cells of interest (positives) and the background fluorescence of unlabeled (control) cells. Several factors contribute to this background fluorescence, including the type of fixation and the degree of nonspecific binding. Our experience and that of others (e.g. Shapiro et al. 1989, Lin et al. 1994) is that autofluorescence varies dramatically with fixation procedures. Glutaraldehyde fixation produced intense autofluorescence at all wavelengths examined, even following borohydride reduction. In our hands, formalin fixation followed by chlorophyll extraction in ice-cold methanol and storage at −20° C preserved cell morphology the best and gave the lowest autofluorescence readings. The methanol extracts most of the chlorophyll, so it is easier to visualize internal probes, such as the rRNA oligonucleotide, which otherwise would be partially obscured by red fluorescence. Cells preserved with this procedure and stored for several years retained excellent morphology. No changes in antibody labeling were seen in formalin-preserved, methanol-extracted cells over the course of 2 months, and the rRNA-probe labeling remained stable for at least 6 months, although autofluorescence appeared to increase slightly depending upon the storage temperature of the samples (data not shown). Autofluorescence and storage effects are also discussed by Lin et al. (1994).

If samples are to be run on the flow cytometer and target cells distinguished from co-occurring species, chlorophyll fluorescence can be a useful supplementary marker. In such instances, fixation with formalin alone followed by dark storage at 4° C can provide good samples for MAb labeling after storage for weeks to months. Although others (e.g. Vrieling et al. 1994) contend that paraformaldehyde provides the best preservation of their target phytoplankton species, our results with *Alexandrium* sug-

gest that commercially available formalin works just as well, if not better, than the time-consuming and hazardous paraformaldehyde preparations.

Signal/noise. The data from this study could be presented in several different forms: a) as the difference between the positive labeling intensity and negative control intensity (i.e. positive–control) for each time point or growth rate on a per-cell, per–cell volume, or per–cell surface area basis and b) as the ratio of the positive signal to the negative control signal (positive/control). (These alternatives could also be viewed as "signal–noise" or "signal/noise.") Signal/noise ratios are often used to quantify the validity of a detection strategy, but in our studies, subtracting the control or background signal from the positive signal for that treatment expressed on a per-cell or per–cell volume/area measurement gave a difference that was more meaningful than the ratio of these two values. This is because a small change in the control fluorescence (from 2.5 to 5 RFU·cell^{-1}, for example) could cause a twofold decrease in the positive/control ratio, even though positive labeling may have remained unchanged or may have even increased from earlier time points. In both the rRNA-probe and MAb experiments, the background or control fluorescence was always very low—typically 2–3 RFU·cell^{-1}. For these reasons, interpretation of our experimental data is based entirely on the difference between the positive and control intensities.

Growth stage variability. Cells grown in batch culture experience a rapidly changing environment because of the depletion of major nutrients and the buildup of excreted metabolites. It is logical to expect that the nature and abundance of cell surface proteins and rRNA would vary under these conditions. It follows that antibodies targeted to those proteins or oligonucleotides targeting rRNA would label cells with differing intensities. It is also possible that cell permeability or the accessibility of the target protein or nucleic acid would vary with physiological condition, again resulting in variability in labeling intensity.

Our data demonstrate that significant variability in labeling of cells can occur with both probe types in a single culture during different growth stages. The MAb-probe intensity was essentially constant in the control culture at 15° C, but it decreased significantly (33%) from exponential levels to late stationary phase at 8° C. Vrieling et al. (1996) also saw a small but significant difference in labeling intensity with growth stage when their MAb was used on two other dinoflagellates (*G. aureolum* and *G. nagasakiense*). The nature and function of the cell surface antigen for the MAb M8751-1 that we used are not known (Sako et al. 1993), so the extent to which they vary with physiology could not be predicted in our experiments. If our growth-stage data are normalized to cell surface area, the antibody-labeling differences drop to 10% or less, well within the range of experimental error. Thus, our studies and a related set of observations by Sako et al. (1996) demonstrate that for this antibody and this species, antibody labeling remains relatively constant in nutrient-replete, light-saturated batch cultures grown at optimal temperatures, even after division slows because of carbon limitation, which is presumed to be the cause of growth cessation (Anderson et al. 1990). However, there is no way to predict how different antibodies will behave on different organisms.

In contrast, fluorescence from the rRNA probe varied significantly between exponential- and stationary-phase growth at both 15° and 8° C, respectively. It should be emphasized, however, that the major decrease in labeling intensity with the rRNA probe occurred after more than 3 weeks in culture. The cells were clearly old and dense and had experienced prolonged carbon limitation. It is reasonable to question whether this level of stress will ever occur in natural waters. If an earlier date were selected as the marker for stationary phase, the rRNA-probe variability with growth stage would be much smaller and more acceptable—about 10% between exponential- and early stationary-phase cells. Variability due to nutrient limitation remains an issue, however.

When the rRNA-probe data are normalized to cell volume, there is still a significant difference between exponential-phase cells and those in late stationary phase. A decrease is not apparent during the transition interval between exponential- and stationary-phase growth. The decrease in rRNA-probe labeling in the late stages of batch culture growth is not surprising, since rRNA levels are known to decrease with declining growth rate in other microorganisms (DeLong et al. 1989, Lim et al. 1993). Alternatively, the differences could be due to changes in cell permeability, which might decrease in older cells, thus hindering the hybridization process. However, the decreases in rRNA revealed by rRNA-probe intensities were verified by direct RNA measurements in cell extracts. Since rRNA is presumed to comprise the vast majority of the total RNA in our *Alexandrium* cells, this comparison of the two different measurement methods is valid. The results suggest that when *Alexandrium* cells are not nutrient limited and are growing exponentially, high cellular rRNA levels are found, whereas cells in late stationary phase or those that are nutrient limited have RNA levels that are reduced by at least 50%. This follows the generally accepted paradigm for prokaryotic organisms (e.g. DeLong et al. 1989). These results also suggest that it may be possible to estimate the physiological condition or growth status of *Alexandrium* cells in the field by quantifying rRNA values, perhaps using the rRNA probe described here to make species-specific measurements within mixed plankton assemblages.

Growth conditions and nutrient effects. The foregoing compares cell labeling intensities within the same

culture at different stages of growth, but comparisons among cultures grown under different conditions are needed as well. For example, cells growing slowly at 8° C and labeled with the MAb or the rRNA probes were 30%–50% brighter than those growing faster at 15° C. This was clearly a cell-size effect, since the differences between temperatures diminished dramatically when label intensity was calculated on a cell-volume or cell-surface area basis. The number of cell surface antigens and rRNA molecules remained relatively constant per unit of surface area or cell volume, respectively. This agrees with the results of Vrieling et al. (1996), who demonstrated that the number of cell surface antigens detected by his antibody to *G. aureolum* and *G. nagasakiense* remained stable during batch culture growth.

For the rRNA probe, a decrease in growth rate due to nutrient limitation reduced labeling intensity, regardless of whether the limitation was due to N or P. Fast-growing cells labeled 1.6 times brighter than did the slowest growing cells or up to three times brighter if the nutrient-replete batch cultures growing at $0.65 \cdot d^{-1}$ were considered. For the MAb, the trend was just the opposite—labeling intensity increased as growth rate decreased. Slow-growing, P-limited cells labeled 20%–30% brighter than did fast-growing cells (or 60% brighter if the intensity of the fast-growing batch culture was considered as well). The reason that this trend is opposite that of the rRNA probe is not known but clearly relates to the nature of the cell surface antigen for MAb M8751-1. If it were a protein involved in nutrient uptake, for example, its abundance might be expected to increase as cellular demand for the depleted nutrient is enhanced. However, when the labeling data are expressed on a surface area basis for the nutrient-limitation experiments, the discrepancies between fast-growing and slower growing cells are dramatically decreased. This indicates that the number of antigens per unit of surface area did not increase—there was just more surface area on the larger cells, and, thus, more antigen presented.

The trends observed with the semicontinuous cultures (i.e. increasing rRNA-probe intensity and decreasing MAb intensity with increasing growth rate) are consistent and significant on their own, but they are only valid over the growth-rate range of 0.1–$0.5 \cdot d^{-1}$. In our analyses, we chose to extend these data using the labeling intensities from the exponential phase of the nutrient-replete batch culture at the same temperature, which grew at $0.65 \cdot d^{-1}$. The batch culture intensities for both probe types are entirely consistent with the intensity trends in the semicontinuous cultures.

Comparisons between probe types. For all treatments, the fluorescent intensity of the MAb-labeled cells was considerably higher than for the rRNA probe. Since control or background fluorescence was similar for the two probe types, the positive/control or signal/noise value for the MAb was always higher than for the rRNA probe by an order of magnitude or more. This, coupled with lower labeling variability across a range of growth stage and growth conditions, suggests that the MAb is the preferred probe for distinguishing *A. fundyense* from co-occurring species in whole-cell assays. However, even though the rRNA-probe labeling intensity was low, there was still sufficient signal strength for *A. fundyense* cells to be recognized in cultures. Recent analyses of field samples from the Gulf of Maine using epifluorescence microscopy showed that the signal intensity of oligonucleotide-probed cells is indeed bright enough that labeled *Alexandrium* cells are easily distinguished from other phytoplankton (Anderson, unpubl.). In order to improve detection of rRNA-probed cells in field populations, signal-enhancement techniques would be needed. This could be accomplished using probes end-labeled with biotin, with detection by fluorescein-labeled avidin (e.g. Lim et al. 1993) or with the use of multiple probes that target different sites on the rRNA molecule. Modifications in the hybridization protocol may also enhance the signal. Scholin (pers. comm.) reports very bright fluorescence in *Alexandrium* cells treated with the NA1 probe following short hybridization with minimal washing.

With the MAb, maximum intensity differences were in the 20%–30% range across all treatment types except for P limitation, which suggests that "automated" or instrumented enumeration methods utilizing this probe (such as ELISA plate assays) could have a counting precision comparable to that of manual cell counts (Guillard 1973). The higher variability observed with P-limited cells is more problematic, however, as the highest labeling intensities were 60% brighter than the lowest. These extremes reflect realistic situations, as the highest intensities were obtained for P-limited cells that were still growing, albeit slowly, and the lowest values were for nutrient-replete batch cultures growing rapidly at environmentally relevant temperatures. It follows that an error factor of about 2 will be inevitable with automated detection methods of field populations based on the MAb. The rRNA probe was even more variable (up to 4 times across reasonable growth conditions), which suggests that automated whole-cell assays may be semiquantitative when nutrient limitation is involved. For manual cell counts, the human eye is able to accommodate the variability in labeling intensity and still allow accurate identification and enumeration (Anderson, unpubl.).

One key issue involves the calibration of automated field measurements made with these probes. If cultured cells are used to generate a standard curve, the variabilities described above will create uncertainties when that curve is used to enumerate cells in field samples. It may therefore be necessary to perform *in situ* calibrations during a field study in order to enumerate target cells, both manually and with probes, for a sufficient number of samples

to generate a calibration curve that can then be used with the remainder of the samples collected at that time and location. There are clearly problems with this approach, but it seems preferable to calibration using laboratory cultures.

The extent to which field populations actually experience the extremes in growth conditions forced upon cultures in this study remains to be demonstrated, as the rRNA and MAb probes for *A. fundyense* have not yet been applied to natural populations to any significant degree. Insights can be gained from studies by Miller and Scholin (1996) and Scholin et al. (1997), who used rRNA probes in a sandwich hybridization assay for toxic diatoms in the genus *Pseudo-nitzschia.* The sandwich assay uses one probe to capture the rRNA from lysed cells and a second probe to detect it. The assay worked well for cultured cells, but in tests of field populations, it consistently overestimated the density of *P. australis* by a factor of 2 (on average) when compared with manual counts from filter-based, whole-cell hybridizations (Scholin et al. 1997). One of the reasons given to explain this discrepancy is that naturally occurring *P. australis* contained more rRNA per cell than did those in pure culture, an understandable conclusion in light of our data. Other possibilities are that cells in nature may be lysed more efficiently than those in culture or that there are cell fragments that are detected by the sandwich assay that are not visible in the whole-cell counts (Scholin and Anderson 1998).

Overview. Antibody and rRNA probes are just two of several new techniques being evaluated in the search for ways to identify, enumerate, and separate species of interest from natural plankton assemblages. The methods are relatively simple, and in some instances, they show remarkable specificity. They can be used both manually and in an automated fashion and thus have great potential to enhance the accuracy and efficiency of field studies or monitoring programs. The variability in labeling intensity documented here and by others (e.g. Vrieling et al. 1996) indicates areas in which caution will be needed in terms of applying these methods. Further studies are required to characterize in full the factors that can influence the degree of probe labeling of target species under environmentally relevant conditions. The differences between whole-cell and lysed-cell formats are critical in this regard.

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