Detection and quantification of alkaline phosphatase in single cells of phosphorus-starved marine phytoplankton

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ABSTRACT: Alkaline phosphatase (AP) activity in marine and freshwater phytoplankton has been associated with phosphorus (P) limitation whereby the enzyme functions in the breakdown of exogenous organic P compounds to utilizable inorganic forms. Current enzyme assays to determine the P status of the phytoplankton measure only the AP activity of the whole community and do not yield information on individual species. A new insoluble fluorogenic substrate for AP, termed ELF (Enzyme-Labeled Fluorescence), yields a stable, highly fluorescent precipitate at the site of enzyme activity and thus has the capability to determine the P status of individual cells. In this study, ELF was utilized for *in situ* detection and quantification of AP in marine phytoplankton cultures and a comparison was made between the insoluble ELF substrate and several soluble AP substrates [3-O-methylfluorescein phosphate (MFP), 3,6-fluorescein diphosphate (FDP) and Attophos]. Non-axenic batch cultures of *Alexandrium fundyense*, *Amphidinium* sp. and *Isochrysis galbana* were grown in different media types using orthophosphate as an inorganic source and sodium-glycerophosphate as an organic source, with final phosphate concentrations ranging from 38.3 to 3 µM (i.e. f/2, f/40, f/80, plus ambient P). Epifluorescence microscopy was used to determine if and where the cells were labeled with ELF, while flow cytometry was used to quantify the amount of ELF retained on individual cells. The detection of the soluble substrates utilized a multiwell fluorescence plate reader (Cytofluor™). Only cells grown in low phosphate concentrations (f/40, f/80) exhibited the bright green fluorescence signal of the ELF precipitate. This signal was always observed for P-starved *Amphidinium* sp. and *I. galbana* cells, but was seen in some *A. fundyense* cells only during the late stationary phase. Cells grown in high phosphate concentrations (i.e. at f/2 levels) showed no ELF fluorescence. Slightly positive soluble substrate assays suggest that these species may have produced small amounts of AP constitutively that were not detected with the precipitable substrate. Similar results were obtained when the cultures were analyzed by flow cytometry. Except for *A. fundyense*, cells grown in low phosphate concentrations showed high ELF fluorescence. However, no positive ELF fluorescence was detected with the Cytofluor for all 3 species due to lack of instrument sensitivity. Comparable analysis using the soluble substrates MFP, FDP, and Attophos™ on the Cytofluor showed little activity for *A. fundyense*, but high fluorescence for P-starved *Amphidinium* sp. and *I. galbana*. Insoluble ELF thus provides a means to detect and quantify AP in individual cells using visual observations or flow cytometry. This technique offers a new level of resolution and sensitivity at the single cell level that can provide insights into the P nutrition of phytoplankton and other microorganisms in natural waters.

KEY WORDS: Phytoplankton · Phosphorus limitation · Alkaline phosphatase · Phosphorus · ELF · *Alexandrium fundyense*

INTRODUCTION

For many years, the role of phosphorus (P) as a limiting nutrient for phytoplankton has been a source of controversy. Many investigators believe that P is the primary limiting nutrient in freshwater ecosystems (Berman 1970, Wynne 1977, Healy & Hendzel 1980), but for marine ecosystems nitrogen (N) is usually

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thought to be the nutrient in lowest supply (Thomas 1970, Goldman et al. 1979). Despite this paradigm, some investigators have argued that P is the limiting nutrient for phytoplankton growth in marine systems such as the central gyre of the North Pacific (Perry 1976) or in estuaries along the northeastern margin of the Gulf of Mexico (Smith 1984). Ryther & Dunstan (1971) maintained that N is limiting in coastal waters, while McComb et al. (1981) suggested that an estuarine system in Australia may shift seasonally between P and N limitation. In spite of these research efforts, the controversy has not been resolved, in part because the various components of the phytoplankton assemblage have different nutrient requirements and uptake capabilities. Thus, there are currently no adequate methods for determining which nutrient limits the growth of individual species of phytoplankton within mixed assemblages (La Roche et al. 1993).

Phytoplankton species in the ocean are thought to obtain the bulk of their P by direct assimilation of soluble inorganic phosphates (in the form of orthophosphate ions). In the presence of sufficient orthophosphate, they are generally unable to directly take up dissolved organic P (in the form of esters such as adenylic, guanylic and cytiylic acids; Parsons et al. 1984). However, laboratory studies indicate that many species are capable of obtaining P from esters in order to sustain growth in the absence of orthophosphate (Jackson & Williams 1985, Cotner & Wetzel 1992). This deficiency is generally expressed in de novo synthesis of phosphatases (Lien & Knutsen 1973). Phosphatases are usually associated with the cell membrane (Cembella et al. 1984) and hydrolyze dissolved phosphate esters outside the cell, allowing the cell to absorb only the phosphate ion, leaving the residual organic moiety in the medium. P-deficient algae can have 25 times more phosphatase activity than cells grown in P-replete conditions (Fogg 1975). These could be acid phosphatases as in Euglena gracilis (Price 1962) or alkaline phosphatases (APs) as in Phaeodactylum tricornutum (Kuenzler & Perras 1965), depending on the pH of their optimum activity. Both forms are capable of hydrolyzing organic phosphates and liberating orthophosphate. AP appears to be more common in phytoplankton than acid phosphatase (Nalewajko & Lean 1980). In addition, some species may not produce APs even while P limited, while some others have constitutive enzymes (Cembella et al. 1984). Thus, the physiological manifestations of P deficiency and the expression of APs are complex and variable among species.

Many methods have been used to measure and detect AP in tissues and in single cells. All use a substrate which produces a colored or fluorescent product when reacted with the enzyme. In general terms, we may distinguish among insoluble and soluble assays

for the detection of this enzyme, the main difference being in whether the product of the enzyme-substrate reaction precipitates at the site of the enzyme activity immediately after its formation or is released in solution (Fig 1; Haugland & Johnson 1993).

The utility of insoluble assays derives from the rapid deposition of a microcrystalline precipitate at the sites of enzyme activity (Horwitz et al. 1966). Substrates that have been used most often for the detection of AP are 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) (Holt 1954, Mc-Gadey 1970). Both are hydrolyzed enzymatically to liberate an insoluble purple/blue dye at the site of the enzyme which can be evaluated visually or spectrophotometrically (Kerkhof 1992). Colorimetric assays are generally regarded as low in sensitivity and not suitable for quantitative measurements (Arakawa et al. 1991). Furthermore, the colored precipitate can sometimes be confused with cell structures (Huang et al. 1993).

Assays yielding fluorescent precipitates have distinct advantages since the signal can easily be analyzed by imaging systems with higher sensitivity and specificity than is possible with chromogenic/colorimetric systems (Guibault 1990, Shotton 1991). Among these AP fluorogenic substrates, the naphthyl phosphate derivatives which form a fluorescent precipitating product (naphthol) at the site of the enzyme (Burnstone 1960) are perhaps the most commonly used (Zyomek et al. 1990, Narisawa et al. 1992, Speel et al. 1992).

AP detection in most phytoplankton studies has been accomplished using soluble products. It is thus not possible to mark the exact localization of the enzymatic activity in the cells or to determine each species' contribution to the overall enzyme signal. Among soluble assays, both colorimetric and fluorogenic substrates have been used. The most common colorimetric substrates are phenyl phosphate derivatives such as p-nitrophenyl phosphate (p-NPP). It is frequently used for identifying phosphatase activity in unicellular algae (Patni et al. 1974), and only a few studies have been conducted with other substrates (Cembella et al. 1984).

Fluorogenic substrates, principally those derived from aromatic ethers, naphthols, or fluoresceins, have been applied in sensitive assays for AP in phytoplankton. The phosphate derivatives of fluorescein were found to be ideal for AP assays due to fluorescein's high extinction coefficient and fluorescence quantum yield (Huang et al. 1992). Other substrates that offer more sensitivity are 3-O-methylfluorescein phosphate (MFP) (Hill et al. 1968, Perry 1972, Healey & Hendzel 1979) and 3,6-fluorescein diphosphate (FDP) (Huang et al. 1992). Another fluorogenic substrate for AP
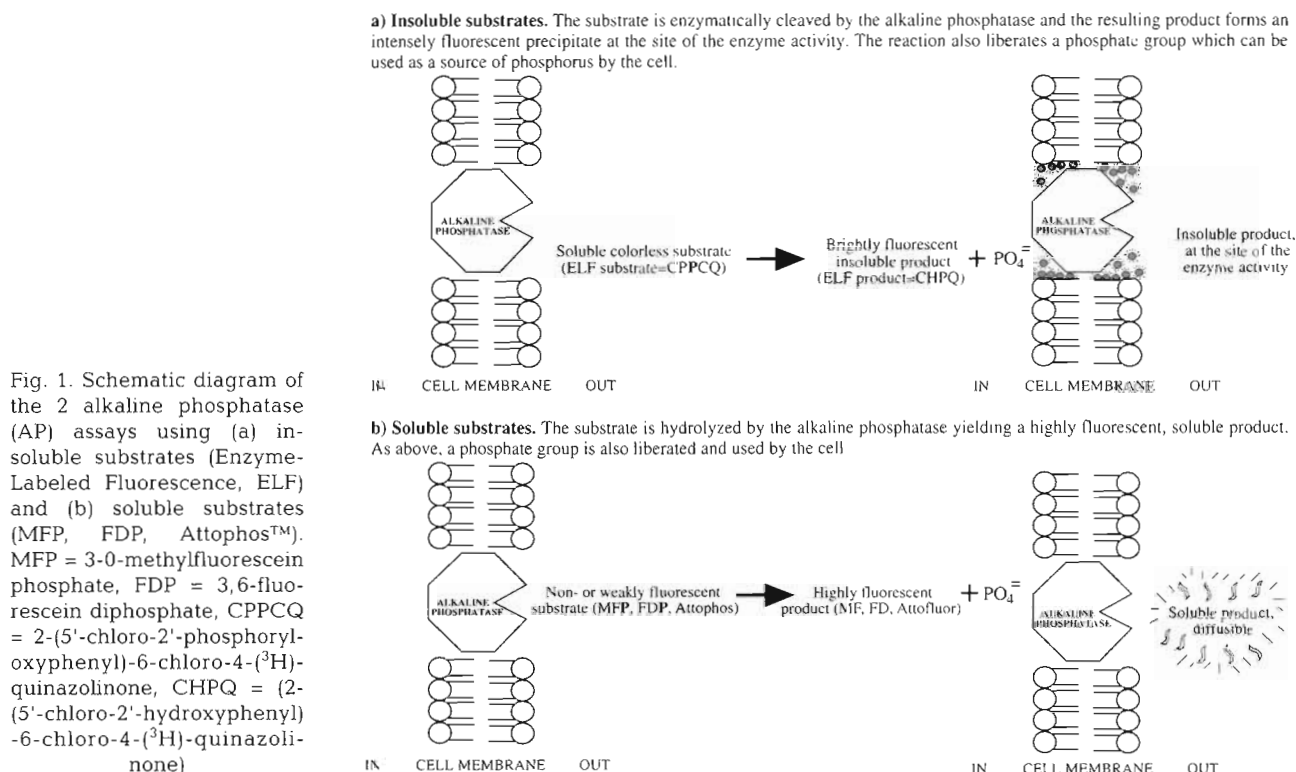
a) Insoluble substrates. The substrate is enzymatically cleaved by the alkaline phosphatase and the resulting product forms an intensely fluorescent precipitate at the site of the enzyme activity. The reaction also liberates a phosphate group which can be used as a source of phosphorus by the cell.

Soluble colorless substrate
(ELF substrate=CPPCQ)

Brightly fluorescent
insoluble product + $PO_4^=$
(ELF product=CHPQ)

Insoluble product,
at the site of the
enzyme activity

IN   CELL MEMBRANE   OUT

IN   CELL MEMBRANE   OUT

Fig. 1. Schematic diagram of the 2 alkaline phosphatase (AP) assays using (a) insoluble substrates (Enzyme-Labeled Fluorescence, ELF) and (b) soluble substrates (MFP, FDP, Attophos™). MFP = 3-0-methylfluorescein phosphate, FDP = 3,6-fluorescein diphosphate, CPPCQ = 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(^{3}H)-quinazolinone, CHPQ = (2-(5'-chloro-2'-hydroxyphenyl)-6-chloro-4-(^{3}H)-quinazolinone)

b) Soluble substrates. The substrate is hydrolyzed by the alkaline phosphatase yielding a highly fluorescent, soluble product. As above, a phosphate group is also liberated and used by the cell

Non- or weakly fluorescent
substrate (MFP, FDP, Attophos)

Highly fluorescent
product (MF, FD, Attofluor) + $PO_4^=$

Soluble product,
diffusible

IN   CELL MEMBRANE   OUT

IN   CELL MEMBRANE   OUT

detection is called Attophos™ (JBL Scientific, San Luis Obispo, CA, USA). Attophos has been mainly used for ELISA (Enzyme-Linked Immunosorbent Assay) and DNA probe applications (Cano et al. 1992) but not yet in phytoplankton studies.

A novel fluorescence-based method for AP detection has been recently developed, based on the use of a colorless molecule, 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(^{3}H)-quinazolinone also called CPPCQ (Huang et al. 1993) or more commonly ELF (Enzyme-Labeled Fluorescence) substrate or reagent (Molecular Probes Inc., OR, USA; Larison et al. 1995). The unreacted ELF substrate is soluble and does not have high fluorescence. When reacted with the AP enzyme, a phosphate is liberated and an insoluble yellow-green product called CHPQ [(2-(5'-chloro-2'-hydroxyphenyl)-6-chloro-4-(^{3}H)-quinazolinone] precipitates rapidly at the site of the enzymatic activity (Huang et al. 1993; Fig. 1). This permits an easy and almost immediate visualization of the sample under fluorescence microscopy.

Thus far the ELF substrate has been employed in histochemistry studies to demonstrate *in situ* localizations of endogenous APs in tissue preparations or fixed cells, especially in organisms such as zebrafish (Haugland & Johnson 1993). To our knowledge it has never been employed in phytoplankton studies.

In this paper we evaluated whether ELF is a suitable substrate for the detection of AP in phytoplankton.

With standard epifluorescence microscopy and ELF's inherent characteristic of forming a precipitate at the site of the enzyme activity, this approach has the potential to indicate which cells or which species are responding to P limitation through the production of AP. Furthermore, we attempted to quantify ELF retained in the cells by measuring the intensity of the green fluorescent signal using flow cytometry and a multiwell fluorescence plate reader. These results were compared to measurements with conventional soluble substrates which utilized the fluorescence plate reader.

MATERIALS AND METHODS

Cultures. Non axenic but clonal cultures of the dinoflagellates *Alexandrium fundyense* Balech (GTCA 29) and *Amphidinium* sp. (AMPHI), as well as the prymnesiophyte *Isochrysis galbana* (TISO), were grown in Pyrex culture tubes (25 × 150 mm) containing 25 ml of f/2-Si media (Guillard & Ryther 1962) at 20°C on a 14 h:10 h light:dark cycle (ca 200 µE m^{-2} s^{-1} irradiance, cool white fluorescent bulbs). To ensure that the ELF signal was not due to bacteria, axenic cultures of *Amphidinium carterae* Hulburt (CCMP 1314) and *Isochrysis* sp. (CCMP 1324) were obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton (Boothbay Harbor, ME, USA).

Local seawater from Vineyard Sound, MA, USA, (salinity 31 psu) was filtered through a 0.22 μm filter, autoclaved in Teflon bottles, and used within 1 wk. All nutrients, vitamins and trace metals (except the iron/chelator mix) were also heat-sterilized separately in Teflon bottles and then added to the seawater shortly before the start of the experiments. All glassware was cleaned with a phosphate-free detergent, rinsed well, placed for several days in 2 N HCl, and then rinsed again with distilled water several times. Cultures were checked for the presence of bacteria weekly using phase contrast microscopy.

The cultures were grown under both nutrient-replete and P-starved conditions by varying only the phosphate concentration in the medium. Three concentrations of added phosphate were chosen (at the levels of f/2, f/40 and f/80, i.e. 36.3, 1.7 and <1 μM, respectively) based on earlier observations of P starvation in dinoflagellates (Anderson & Lindquist 1985). Two different phosphate sources were used for the media, $NaH_2PO_4 \cdot H_2O$ as inorganic phosphate, and Na_2-glycerophosphate as an organic source. The concentrations of the other nutrients were maintained at f/2 levels. Background inorganic phosphate concentrations in the natural seawater used to prepare the media were measured colorimetrically by the standard molybdenum blue method. The values were consistently ≤2 μM, yielding final P concentrations for each media type of 38.3, 3.7 and <3 μM, respectively.

Growth of the cultures was monitored using *in vivo* chlorophyll fluorescence (Turner Designs Model 10 AU). The cultures grown in low phosphate media were maintained for at least 3 transfers before the analysis of AP. When populations reached the mid-stationary phase of growth (see Fig. 2), usually around Day 20, the cells were removed for analysis.

Insoluble ELF assays. *Microscopic analysis:* About 5 ml of live cells of each strain (GTCA29, AMPHI, TISO, CCMP 1314 and CCMP 1324) were centrifuged and the pellet placed in a siliconized microcentrifuge tube. One ml of 70% ethanol was then added to each of the samples and removed 30 min later by aspiration. Just before use, the ELF substrate was diluted 1:20 in ELF Detection Buffer (supplied with the ELF substrate as part of the kit), and then filtered (0.2 μm) to eliminate possible precipitates of the substrate. 100 μl of this diluted ELF solution was added to each sample. The mixture was incubated for 30 min in the dark at room temperature, and then the samples were washed 5 times with 10 mM phosphate buffered saline (PBS) to stop the reaction. For negative controls, samples were treated identically as above except that ELF Detection Buffer was added without any addition of ELF substrate. For long term storage (e.g. months), ELF-stained cells were washed with both 10 mM and 100 mM PBS (Molecular Probes pers. comm.).

Samples were observed under a Zeiss Axioscope microscope using a Zeiss MC 100 camera equipped with a long-pass DAPI (4',6-diamidino-2-phenyl-indole) filter set (excitation 365 ± 8 nm; emission >420 nm). Typical exposure times were ca 15 to 30 s for the negative samples (controls and non-stained cells) and 1 to 2 s for the positive samples (ELF-stained cells).

Flow cytometry analysis: To quantify the ELF signal from the cells, samples were prepared in the same manner as for microscope examination. The ELF-stained samples were stored at 4°C until the samples could be analyzed by flow cytometry the following day. Flow cytometry analysis was performed on a Becton-Dickinson FACS Vantage equipped with an Innova 305 argon ion laser set to 100 mW UV (333 to 364 nm excitation). Emission of ELF was quantified using a 530 ± 30 nm band pass filter. Approximately 2000 cells of *Alexandrium fundyense*, 55 000 of *Amphidinium* sp. and 80 000 of *Isochrysis galbana* were analyzed for each sample. Based on the flow cytometry measurement of the fluorescence of each individual cell, the mean relative fluorescence was calculated for the sample population of each species grown in the phosphate replete or deplete media.

Cytofluor analysis: Fluorescence measurements were performed using a 96-well plate (Costar) read in a Cytofluor™ 2300 Fluorescence Measurement System (Millipore Corp., Bedford, MA, USA). It is a spectro-fluorometer capable of reading up to 96 wells in a microwell plate (Danks et al. 1992). Filters used for ELF detection were 360 ± 40 nm for excitation and 530 ± 25 nm for emission, with the sensitivity set at 5 (maximum).

Before analysis, the cell concentrations of each tested species from each media type were diluted using the media in which the cells were grown to attain approximately the same number of cells for each species (i.e. for any given species the number of cells well^{-1} was the same) and each repetition of the experiment. A 50 μl suspension of the nutrient-replete and P-starved test cells was added to each well of the microplate. This volume was equivalent to 2340 ± 175 cells well^{-1} for *Alexandrium fundyense*, 4211 ± 158 cells well^{-1} for *Amphidinium* sp. and 101 600 ± 11 500 cells well^{-1} for *Isochrysis galbana*. To the cells in each well, 50 μl of the diluted ELF solution was added. Three replicates of each sample were run with the ELF substrate present, accompanied by 3 replicate controls without the ELF substrate (i.e. treatments and negative controls). The microplate was read in the Cytofluor at 10 min intervals for 60 min, but 30 min of incubation with the ELF substrate was chosen as an endpoint for the analysis to represent the total fluorescence from the cells in each well. The data were then normalized to reflect fluorescence on a per cell basis.

Soluble fluorescent assays. *Substrates:* Three soluble substrates were tested: 3-O-methylfluorescein phosphate (MFP) (Sigma Chemical Co., St. Louis, MO, USA), 3,6-fluorescein diphosphate (FDP) (Sigma Chemical Co.) and Attophos™ (JBL Scientific). For the preparation of MFP and FDP we followed established procedures (Perry 1972). In summary, a stock solution of each substrate was prepared in cold 0.1 M sterile Tris buffer (Trizma Base, Tris[Hydroxymethyl]aminomethane) pH 7.8 (adjusted with 1 N HCl) to a concentration of 50 µg ml⁻¹ and stored frozen in small aliquots. The stock solution was then diluted in the same buffer to 1:50, 1:100, 1:200 and 1:400 to obtain working concentrations of 1, 0.5, 0.25 and 0.125 µg ml⁻¹, respectively. The Attophos was reconstituted following the manufacturer's instructions, i.e. by dilution in Attophos buffer (2.4 M Diethanolamine, pH 10, 0.057 mM MgCl₂), yielding a concentration of 0.6 mg ml⁻¹. Working solutions of all substrates were made immediately before the experiments were performed.

Procedure: Reactions were performed directly in the 96-well plates (Costar) by adding 50 µl of the harvested cells from each of the media used to each well (equivalent to 1200 ± 63 cells well⁻¹ for *Alexandrium fundyense*, 3750 ± 353 cells well⁻¹ for *Amphidinium* sp. and 100 000 ± 9870 cells well⁻¹ for *Isochrysis galbana*). As above, the concentrations of the cells were adjusted so that a given species contained the same number of cells well⁻¹ for each treatment and repetition of the experiment. 50 µl of test substrate solution [MFP (1 µg ml⁻¹), FDP (1 µg ml⁻¹), or Attophos (0.6 mg ml⁻¹)] were then added to the cells to start the reaction. For the negative controls, the buffers in which the substrates were diluted were used instead of the substrates themselves (i.e. 0.1 M Tris for MFP and FDP, and 2.4 M Diethanolamine for Attophos). As above, 3 replicates were tested for each sample and for the controls. Plates were incubated with the different substrates for 60 min, during which the fluorescence was measured every 10 min. As with ELF above, a 30 min endpoint was chosen for the comparative analysis, which reflects the total fluorescence from the cells in each well at a given time. The data were then normalized to reflect fluorescence on a per cell basis.

Cytofluor analysis: Fluorescence measurements were performed using the Cytofluor™ with the following excitation-emission filters: excitation 485 ± 20 nm and emission 530 ± 25 nm for both MFP and FDP (sensitivity settings at 3 and 2 respectively); and 450 ± 50 nm excitation and 530 ± 25 nm emission (sensitivity setting at 2) for the Attophos substrate.

Standards: Primary standards were used for the initial calibration of the instrument prior to the experiments to ensure that the fluorescent response from each substrate was linear. The primary standard of

MFP was 3-0-Methylfluorescein (Sigma) prepared with absolute methanol to a concentration of 0.5 mg ml⁻¹ and stored at –5°C. It was then diluted 1:100 with 0.05 N NaOH to a concentration of 5 µg ml⁻¹ for use as a stock solution. Finally, several dilutions with distilled water to 1:5, 1:10, 1:20, 1:30, 1:40 and 1:50 yielded concentrations of 1, 0.5, 0.25, 0.166, 0.125 and 0.1 µg ml⁻¹ respectively of standard. The same procedure was followed for fluorescein (Sigma), the standard used for FDP. We did not use any standard for the Attophos, as the Attophos itself was used for calibration. In all the cases, a linear relationship exists between the intensity of fluorescence of the standards and their concentration (data not shown). The standard calibration curves also permitted the proper selection of the filters and the optimization of substrate concentrations, which were determined to be 1 µg ml⁻¹ for MFP and FDP.

RESULTS

Cultures

Typical batch culture growth curves of each of 3 species grown with the different media types are presented in Fig. 2. When the cells were incubated under nutrient-replete conditions using either f/2 inorganic phosphate media or f/2 organic phosphate media, they grew exponentially for approximately 10 to 15 d, after which they reached the stationary phase. When less P was added to the media at concentrations below 2 µM (i.e. f/40 and f/80 media), the stationary phase was reached several days earlier and the maximum *in vivo* chlorophyll fluorescence was lower than in the f/2 grown cultures. Cell counts confirmed that the P-starved cultures had lower biomass than P-replete ones (data not shown). In all media types, the stationary phase continued for 10 to 15 d before a slow decline in chlorophyll fluorescence was observed. To ensure that all cells were nutrient-starved, cells were harvested for enzyme analysis during the stationary phase but before any decline (i.e. at about Day 20 of growth).

Insoluble ELF assays

Microscope observations. When ELF-treated *Alexandrium fundyense* cells were examined microscopically using the DAPI filter set, bright green fluorescence characteristic of the precipitate was not observed for any of the media types (Table 1, Fig. 3A). All cells exhibited only autofluorescence. Results were identical when control samples of *A. fundyense* were examined (i.e. no ELF substrate present; Table 1). In a few very limited cases, bright green fluorescence was
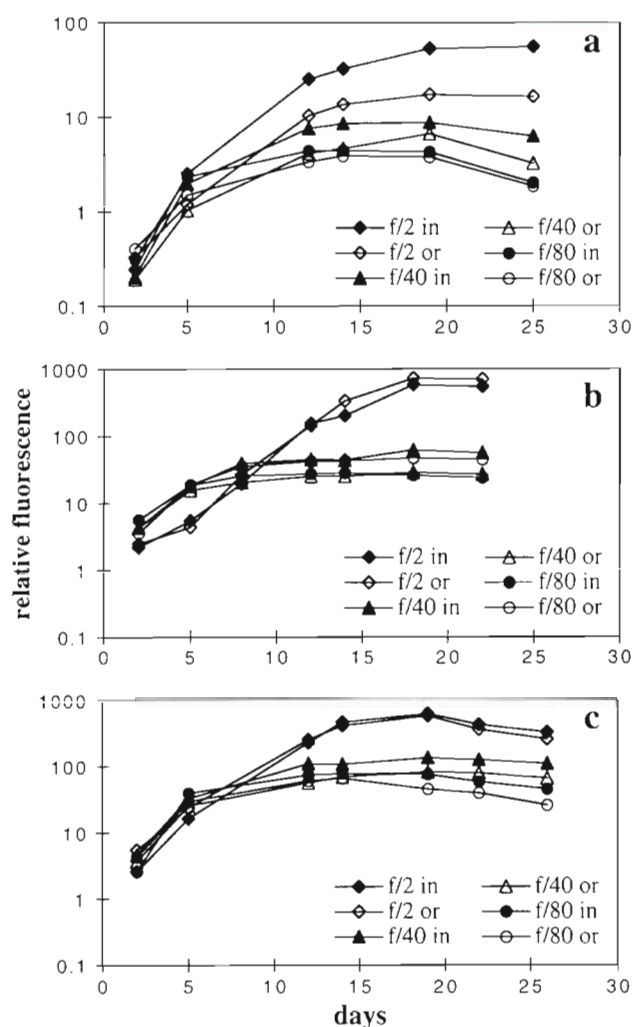
Fig. 2. Representative growth curves for clones of (a) *Alexandrium fundyense* (clone GTCA29), (b) *Amphidinium* sp. (clones AMPHI) and (c) *Isochrysis galbana* (clone TISO), using *in vivo* fluorescence. Final phosphate concentrations (additions + background) were 38.3 µM (f/2 media), 3.7 µM (f/40), and <3 µM (f/80). in = inorganic phosphate, or = organic phosphate

Table 1. Epifluorescent microscopy observations for 3 phytoplankton genera that were tested with ELF. ELF treated: cells treated with ELF substrate; control: cells not treated with ELF substrate, but with detection buffer only; +: positive green fluorescence observed; −: no green fluorescence observed; ±: sometimes positive, usually negative

Strain	f/2 in	f/2 or	f/40 in	f/40 or	f/80 in	f/80 or
Alexandrium fundyense						
GTCA29						
ELF treated	−	−	±	±	±	±
Control	−	−	−	−	−	−
Amphidinium spp.						
AMPHI						
ELF treated	−	−	+	+	+	+
Control	−	−	−	−	−	−
CCMP 1314[a]						
ELF treated	−	−	+	+	+	+
Control	−	−	−	−	−	−
Isochrysis spp.						
TISO						
ELF treated	−	−	+	+	+	+
Control	−	−	−	−	−	−
CCMP 1324[a]						
ELF treated	−	−	+	+	+	+
Control	−	−	−	−	−	−

[a] Axenic strains

observed in some *A. fundyense* cells, but only when the cells were grown for very long periods of time, well into the late stationary phase of growth (Fig. 3B).

When ELF-stained *Amphidinium* sp. and *Isochrysis galbana* were observed under the epifluorescence microscope, cells grown in f/40 and f/80 media showed a very bright green fluorescence (Table 1, Fig. 3D, F). This appeared as green deposits in the membrane of the cells presumably due to the precipitation of the ELF product at the site of the AP activity. The small green spots were easily distinguished at 100× magnification for *Amphidinium* sp. and at 200× for *I. galbana*, usually with a clean, black background. When the cells grown in f/2 were examined, no ELF fluorescence was

observed (Table 1, Fig. 3C, E). There was no apparent difference between organic and inorganic media types. As with the *A. fundyense* controls, no green fluorescence was observed in either the *Amphidinium* sp. or the *I. galbana* control treatments for any of the media types. The ELF staining patterns of the axenic strains of *Amphidinium* (CCMP 1314) and *Isochrysis* (CCMP 1324) were identical to the non-axenic strains (Table 1).

Flow cytometry analysis. The bright green ELF signal on individual cells was quantified using flow cytometry (Fig. 4). No differences were found between the treatments and the controls of *Alexandrium fundyense* for the different media types (Fig. 4a).

For *Amphidinium* sp., ELF fluorescence was detected in each of the P-starved treatments. Cells with the highest fluorescence were those cultured in f/80 inorganic and in f/40 organic media, for which the fluorescence of the treatments was 3 to 4 times brighter than the negative controls (Fig. 4b). However, the mean ELF fluorescence of the cells did not show any differences between the ELF-treated samples and the controls for either the f/2 inorganic or f/2 organic media.

The detection of ELF by flow cytometry was most distinct when P-starved cells of *Isochrysis galbana* were analyzed (Fig. 4c). When the cells were grown in P-deficient media, the green fluorescence signal was high
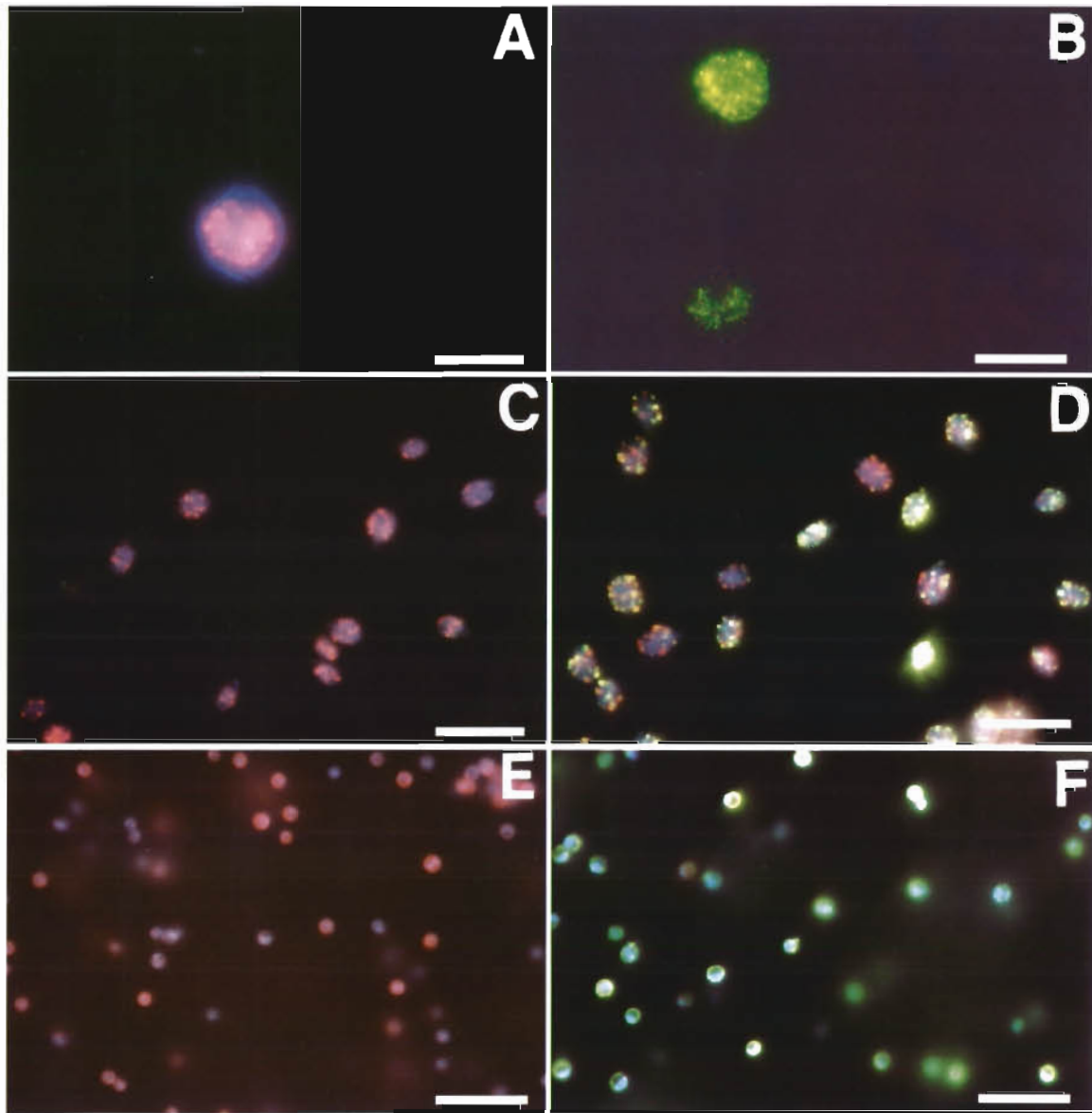
Fig. 3. Microscope observations of *Alexandrium fundyense*, *Amphidinium* sp. and *Isochrysis galbana*. Two sets of samples were observed for each species and for each treatment: cells grown in P-replete media and treated with ELF (left panels), and cells grown in P-depleted media and also ELF-treated (right panels). Samples were observed under a long-pass Hoechst/DAPI filter set (excitation 365 ± 8 nm, emission >400 nm). Scale bars = 30 µm. (A) *A. fundyense* cell grown in f/2 inorganic medium and treated with ELF. Similar images were obtained for all media analyzed, i.e. f/2, f/40 and f/80, with organic or inorganic phosphate. No labeling differences were observed between the controls and ELF-treated cells. (B) *A. fundyense* cells grown in f/80 inorganic medium and treated with ELF. Green fluorescence corresponding to ELF precipitate at the site of AP activity is shown within the cells. This, however, was only observed in a few cells in f/40 and f/80 media. (C) *Amphidinium* sp. cells grown in f/2 inorganic medium and treated with ELF. No green fluorescence due to ELF is observed. Similar observations were made for f/2 organic medium and for all controls. (D) *Amphidinium* sp. cells grown in f/40 organic medium and treated with ELF. The bright green spots correspond to the ELF precipitate near the surface of the cells. Similar patterns were observed for cells grown in f/40 inorganic or f/80 (organic or inorganic) media. (E) *I. galbana* cells grown in f/2 inorganic medium and treated with ELF. No green ELF fluorescence is apparent. This same pattern was observed when cells were grown in f/2 organic medium or control cells were analyzed. (F) *I. galbana* cells grown in f/80 organic medium and treated with ELF. Green fluorescence due to the ELF precipitate is visible near the surface of the cells. Similar results were observed for cells grown in f/80 inorganic or f/40 (organic or inorganic) media

for all ELF-stained samples, typically 30 to 50 times brighter than the controls. As with *Amphidinium* sp., the cells with the highest ELF fluorescence were those cultured in the f/80 inorganic media. The fluorescence in the negative controls (i.e. no addition of the ELF substrate) was very low and uniform for all the treatments.
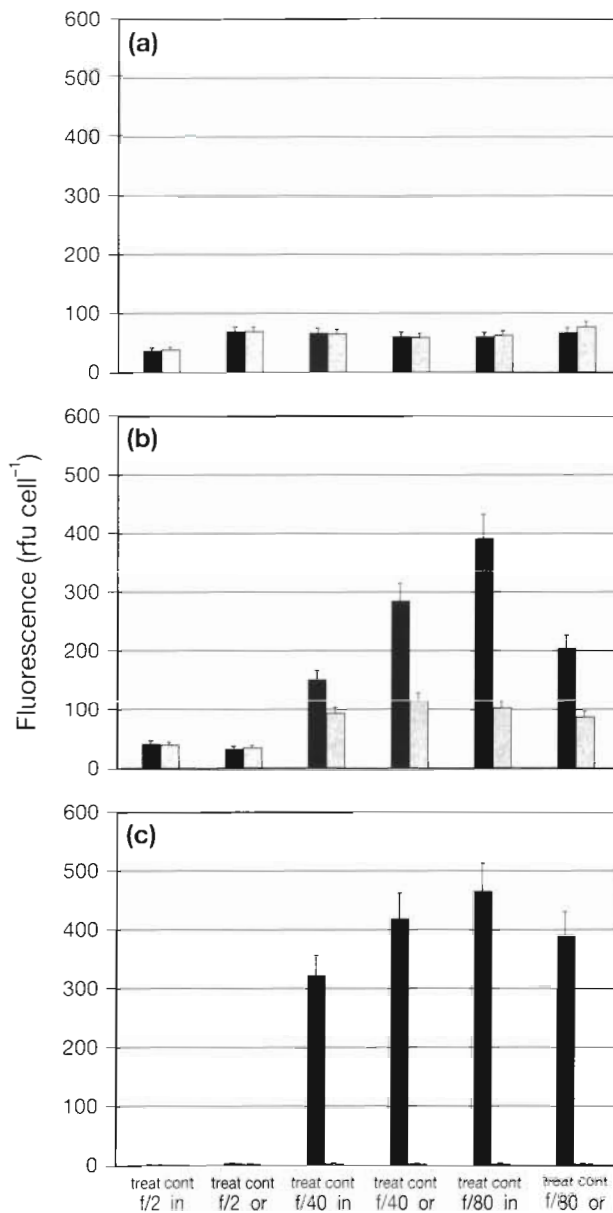
Fig. 4. Flow cytometry data for ELF analysis in (a) *Alexandrium fundyense*, (b) *Amphidinium* sp. and (c) *Isochrysis galbana*. Fluorescence cell⁻¹ [mean relative fluorescence units (rfu cell⁻¹) + SD] for all media are presented. in = inorganic phosphate, or = organic phosphate, treat = treatment (ELF substrate added), cont = control (ELF Detection Buffer added without ELF substrate)

Cytofluor analysis. Because the ELF signal was found to be very bright microscopically, an attempt was made to quantify the ELF signal for each species and each media treatment using the Cytofluor fluorescence plate reader (Fig. 5). The fluorescence data was normalized on a per cell basis to account for differences in cell numbers between the species, so the scale changes shown between the different species generally reflects difference in the size of the cells, not dif-

ferences in number. Based on the microscope observations, we did not expect to measure ELF fluorescence using the Cytofluor for *Alexandrium fundyense* in any of the media types, yet a relatively high fluorescence was observed for all media treatments (Fig 5a). The fluorescence level was not different from the controls, however, so the signal was attributed to autofluorescence and not to ELF. *Amphidinium* sp. and *Isochrysis galbana*, on the other hand, were expected to show
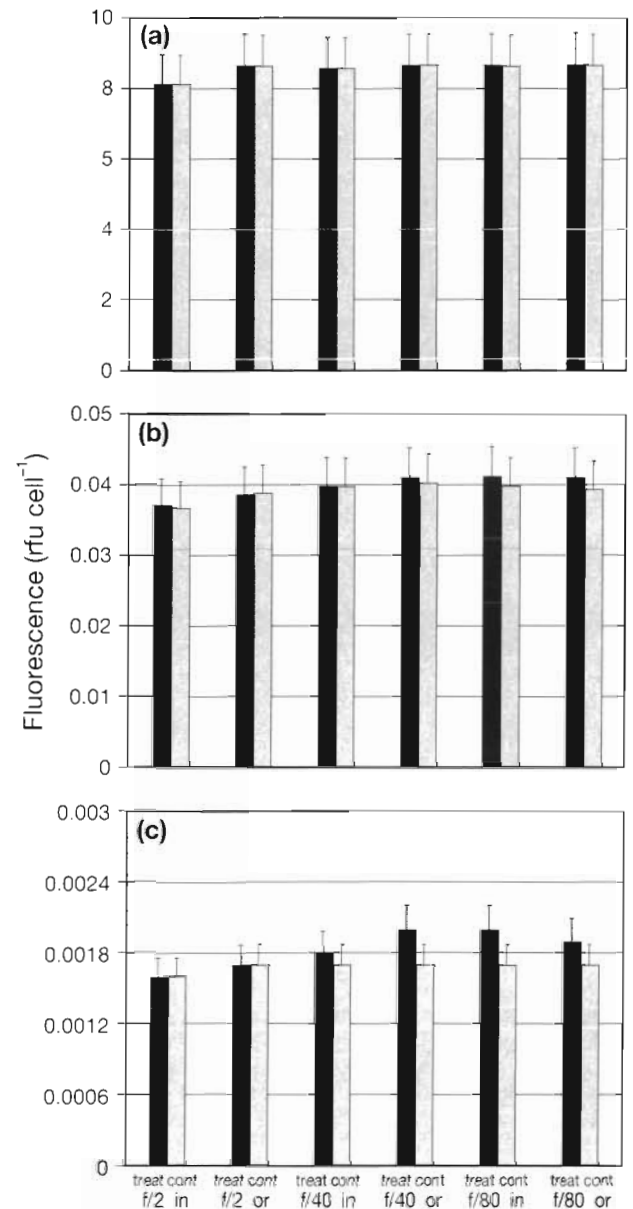

Fig. 5. Fluorescence cell⁻¹ (rfu cell⁻¹ + SD) of samples treated with the ELF substrate and measured in the Cytofluor for (a) *Alexandrium fundyense*, (b) *Amphidinium* sp. and (c) *Isochrysis galbana* is presented for all media types. The values shown were recorded after a 30 min incubation with the ELF substrate. Abbreviations as in Fig. 4

high fluorescence in both organic and inorganic f/40 and f/80 phosphate media based on the microscope observations. However, for both species, the Cytofluor signal was no different from the controls for all the media treatments (Fig. 5b, c).
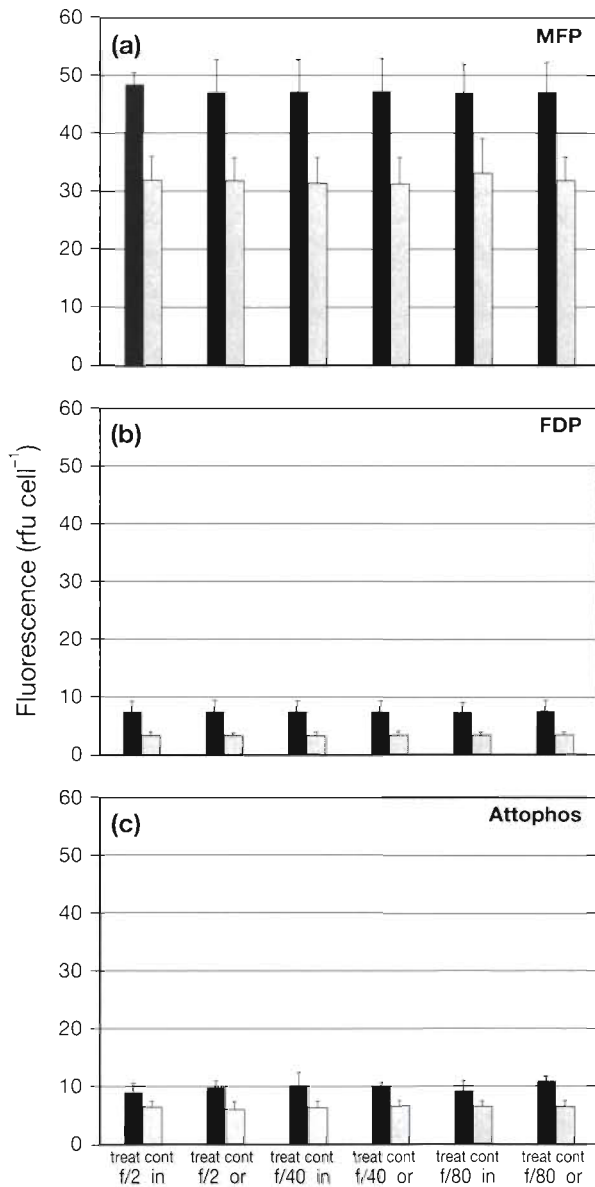

Fig. 6. Fluorescence cell^{-1} (rfu cell^{-1} + SD) of *Alexandrium fundyense* treated with the soluble substrates and measured in the Cytofluor. All the growth media types are shown for each soluble substrate tested. The values shown were recorded after a 30 min incubation with each substrate. MFP = 3-0-methylfluorescein phosphate, FDP = 3,6-fluorescein diphosphate. Both MFP and FDP measurements used filters with 485 ± 20 nm excitation, and 530 ± 25 nm emission, and sensitivity settings at 3 and 2 respectively. Attophos™ measurements used filters with an excitation of 450 ± 50 nm and emission of 530 ± 25 nm, and the sensitivity set at 2. in = inorganic phosphate, or = organic phosphate, treat = treatment (substrate added), cont = control (buffer added without substrate)
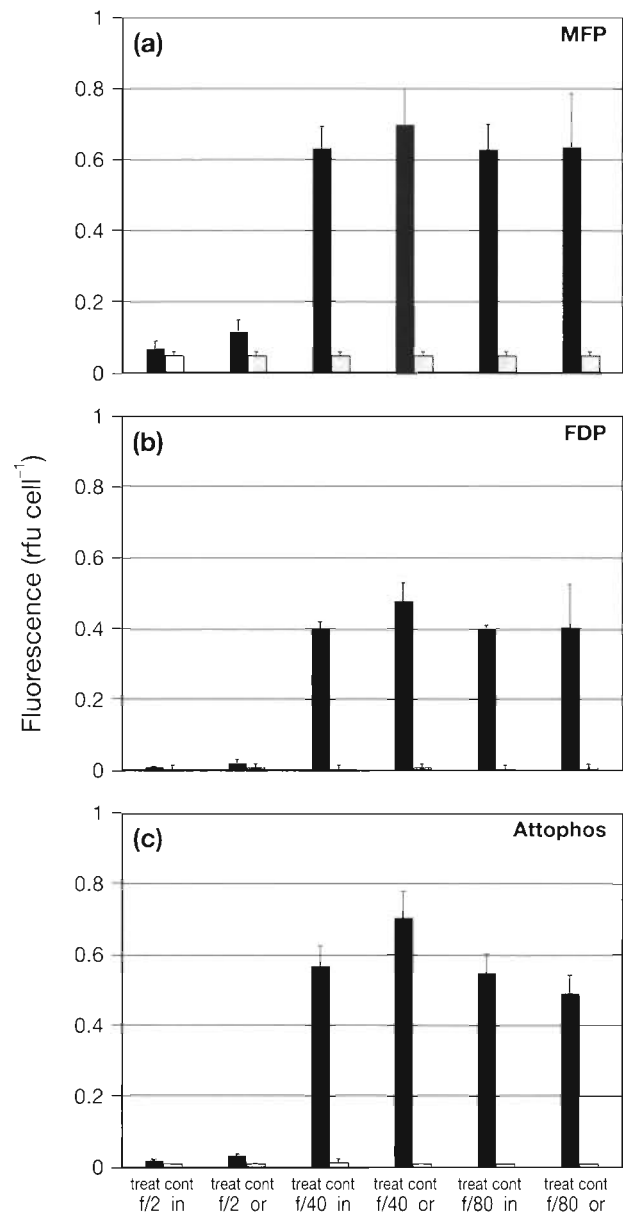

Fig. 7. Fluorescence cell^{-1} (rfu cell^{-1} + SD) of *Amphidinium* sp. treated with the soluble substrates and measured in the Cytofluor. Abbreviations as in Fig. 6

Soluble fluorescent assays

Each of the 3 species grown with the different media types were also treated with substrates which released soluble fluorescent products when hydrolyzed by AP enzymes (Figs. 6, 7 & 8). The Cytofluor was used to measure the released fluorescent product, which was distributed throughout the microwell.

When *Alexandrium fundyense* cells were measured using the 3 soluble substrates, differences were observed between the MFP-treated samples and the untreated controls for each of the media types (Fig. 6a).
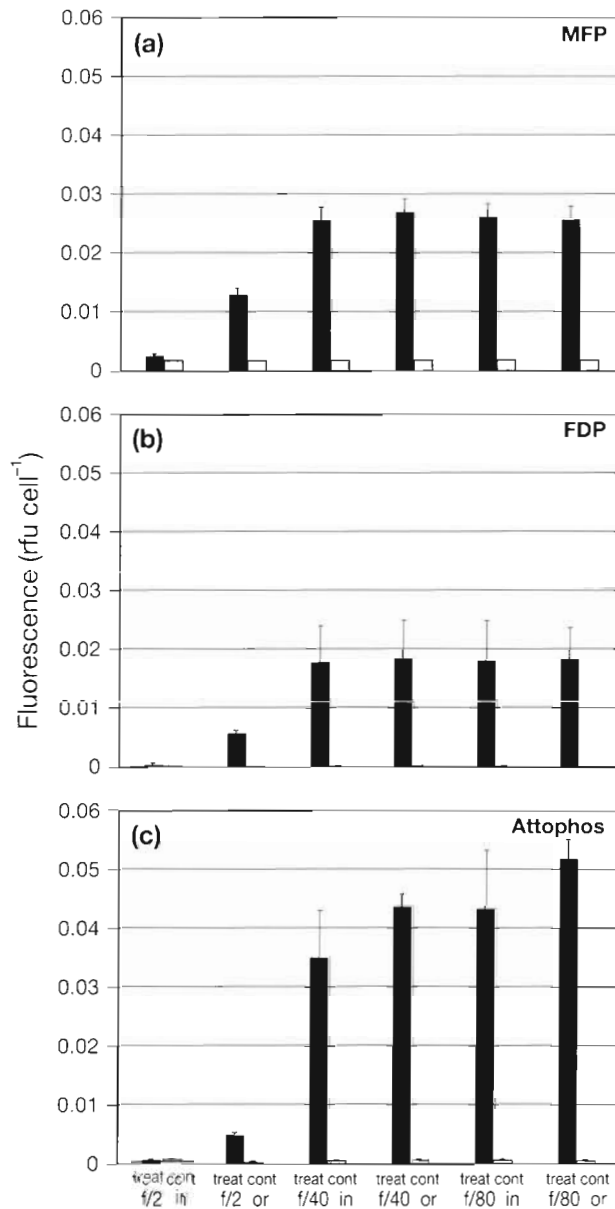
Fig. 8. Fluorescence cell^{-1} (rfu cell^{-1} + SD) of *Isochrysis galbana* treated with the soluble substrates and measured in the Cytofluor. All the growth media types are shown for each soluble substrate tested. The values shown were recorded after a 30 min incubation with each substrate. Abbreviations as in Fig. 6

However, no differences were apparent between the samples grown in different media. The fluorescence signals for FDP (Fig. 6b) and Attophos (Fig. 6c) were also uniform in AP activity for all media types with the values significantly higher than the controls. AP activity was thus present in the GTC-A29 strain of *A. fundyense*, but the level did not change with growth conditions. The use of different substrates required different filter and sensitivity settings and thus the values are not directly comparable.

The results for *Amphidinium* sp. (Fig. 7) were much different than those obtained for *Alexandrium fundyense* since the fluorescence measurements obtained using the substrates MFP (Fig. 7a), FDP (Fig. 7b), and Attophos (Fig. 7c) all showed much higher values for cells that were cultured in the f/40 or f/80 media (organic or inorganic) than for those grown in f/2, indicating that there was significant AP activity in the P-starved treatments. Although there was some variability in the fluorescence responses with each substrate, there were no differences in enzyme activity among the P-starved treatments. However, it appears that there may be a very slight amount of AP activity present in the f/2 organic P treatment because the substrate-treated samples were consistently higher than the controls for all 3 substrates.

A similar pattern was obtained for *Isochrysis galbana* cells treated with the different substrates (Fig. 8), although *Amphidinium* sp. had fluorescence cell^{-1} values 10 times higher than for *I. galbana*. All the cells cultured in f/40 or f/80 (inorganic or organic) media exhibited the highest values of fluorescence, regardless of the soluble substrate treatment, while the f/2 nutrient-replete conditions were significantly lower. Substrate-treated fluorescence was 10 to 15 times higher than the controls for MFP and 80 to 90 times higher for FDP. There were no differences between the fluorescence of organic and inorganic f/40 or f/80 treatments with both MFP and FDP. Similar to the *Amphidinium* sp. cells, *I. galbana* also appeared to maintain a low level of AP activity when grown in f/2 organic P, whereas AP activities in the f/2 inorganic P treatments were negligible.

DISCUSSION

ELF has tremendous potential as a tool for detection of endogenous AP activity in a variety of organisms and tissues. The principal advantage of ELF over other substrates is its ability to form a stable fluorescent precipitate at the site of enzyme activity, providing information about the source of the phosphatase activity within the sample. Based on the results obtained here, ELF was found to be a suitable substrate for the detection of AP activity in 2 of the 3 marine phytoplankton species tested. The source of AP activity in samples of marine and freshwater phytoplankton has been elusive, and now can be explored at the species level to determine which organisms are responsible for the 'whole water' activity. This demonstration of the utility of ELF for phytoplankton is not insignificant and promises to enhance our knowledge of the role of phosphorus in the ecology of aquatic microorganisms. Furthermore, studies now in progress indicate that

ELF also can detect AP activity in bacteria (P. Carlsson pers. comm.).

The bright green signal due to the ELF precipitate reproducibly showed that 2 of the 3 analyzed genera (*Amphidinium* spp. and *Isochrysis* spp.) synthesized this enzyme when they were cultured in P-deficient media using either inorganic or organic phosphates. No signal was observed when they were grown in nutrient-replete media. The inorganic and organic phosphate-replete treatments reached stationary phase at relatively high biomass levels where they were most likely limited by carbon (Anderson et al. 1990), while the lower biomass observed when all 3 species were grown on the P-deficient media (Fig. 2) confirmed that P starvation occurred in both the inorganic and organic f/40 and f/80 treatments. Thus, ELF can identify cells (at least in the case of *Amphidinium* sp. and *Isochrysis galbana*) that are P starved regardless of whether the source of available P is organic or inorganic.

ELF was negative (i.e. not different from the controls) for the f/2 organic treatments and was not different from the f/2 inorganic treatments (Table 1, Fig. 4) for both *Amphidinium* sp. and *Isochrysis galbana*. High ELF fluorescence was seen in the f/40 and f/80 glycerophosphate treatments. Using the soluble assays, a low level of AP activity was detected in the f/2 organic treatments with *I. galbana*, and possibly *Amphidinium* sp. (Figs. 7 & 8). This unexpected result suggests that the cells did not need to produce significant amounts of AP to break down the high concentrations of glycerophosphate to meet their P requirements. Alternatively, ELF may not be capable of detecting all of the phosphatases produced by the cells, or perhaps the cells do not need to cleave phosphate from glycerophosphate prior to uptake. The absence of ELF at the high concentrations of organic P remains a mystery.

In contrast to the positive ELF results for *Amphidinium* sp. and *Isochrysis galbana*, *Alexandrium fundyense* did not demonstrate AP activity to any great extent in any of the media types. Subsamples of *A. fundyense* cells grown under the same media conditions and tested using soluble fluorescent substrates (Fig. 6) indicate that there was low-level AP activity present in *A. fundyense*, but those levels did not increase under P-starved conditions, as was the case with *Amphidinium* sp. (Fig. 7) and *I. galbana* (Fig. 8). Since AP activity was not enhanced by P starvation in our experiments for *A. fundyense* (strain GTCA29), the low levels observed may reflect constitutive expression. It is also possible that *A. fundyense* possesses acid phosphatases or other enzymes which will not react with our soluble or insoluble substrates under the conditions (e.g. pH) used in our experiments.

This result is in conflict with Boni et al. (1989), who detected AP in *Protogonyaulax tamarensis* (= *Alexandrium tamarense*) from the Adriatic Sea using the colorimetric soluble substrate p-NPP. Their experiments suggest that AP in this closely related *Alexandrium* species was induced by P starvation and that the levels were inversely proportional to the initial P concentration in the medium. Boni et al. grew *A. tamarense* cells at a slightly lower inorganic phosphate concentration (1.8 µM P) than the final concentrations used in our P-deficient experiments (the final inorganic P concentrations of our f/40 and f/80 media types were 3.7 and <3 µM, respectively). The concentration differences are not likely to be significant, however, since our growth curves (Fig. 2) and cell counts (data not shown) indicate that the reduced P in the f/40 and f/80 cultures resulted in growth limitation. Alternatively, Boni et al. used late-stationary phase cells instead of mid-stationary as in our experiments. They also found an apparent diurnal fluctuation in AP activity for those cells. Diurnal fluctuation in AP acitivity has previously been noted for both marine and freshwater dinoflagellates from natural populations, apparently due to an endogenous circadian rhythm in these organisms (Rivkin & Swift 1979, Wynne 1981). Although our experiments were performed at different hours of the day, the vast majority of the *A. fundyense* population never exhibited an ELF response, so this explanation is also unlikely. Another possibility is that the GTCA29 strain of *A. fundyense* used in these experiments has been maintained in culture for over 10 yr using f/2 medium with inorganic P, and thus may have lost its ability to produce APs under P-deplete conditions. Of all the possible explanations, the simplest invokes strain or species differences.

The ELF staining pattern observed under the epifluorescent microscope shows that AP is localized in the outer membrane, or at least in the region closest to the periphery of the cells for both *Amphidinium* sp. (Fig. 3D) and *Isochrysis galbana* (Fig. 3F). When the microscope was focused up and down on the specimens, the green signal was always localized at the periphery of the cells, although it was not clear whether the signal was in the cell membrane or in the cell wall. This generally agrees with other workers who have noted localization of the enzyme in the outer surfaces of the cell (Kuenzler 1965), at the cell wall (Brandes & Elston 1956), at the plasma membrane (Patni et al. 1974) or in the periplasmic region (Matagne et al. 1976). This surface localization of AP has been observed in many different groups of phytoplankton as well, including the marine diatoms *Chaeotoceros affinis*, *Skeletonema costatum* and *Phaeodactylum tricornutum* (Kuenzler & Perras 1965, Moller et al. 1975), the chlorophyte *Chlorella* (Brandes & Elston 1956), the prymnesiophyte *I. galbana* (Kuenzler & Perras 1965) and the dinoflagellate *Peridinium*

cinctum (Wynne 1977). However, in some other species such as the dinoflagellate *Prorocentrum micans* (Uchida 1992) or the chlorophyte *Scenedesmus quadricanda* (Overbeck 1962), this enzyme is apparently not localized in the outer membrane.

When the ELF staining pattern was occasionally observed in *Alexandrium fundyense* (Fig. 3B), AP was not near the outer membrane but rather showed a more general distribution within the cell. This again suggests that the AP observed in *A. fundyense* may not have been induced by P starvation. Experiments with the dinoflagellate *Peridinium cinctum*, which also has a cellulose wall or theca, showed that AP was localized within the cellular interior (Messer & Ben-Shaul 1969, Wynne 1977). However, when the cell needed the enzyme, it was transported to the cell wall and secreted through its pores. If *A. fundyense* was producing AP in response to P starvation, then we would expect to observe more ELF-stained cells in the population and the localization should have been peripheral. Perhaps our observations of a few labeled cells are more related to enzymatic breakdown of dead or dying *Alexandrium* cells than to P starvation. Schmitter & Jurkiewicz (1981) found that both *Gonyaulax polyedra* and *Gonyaulax tamarensis* (= *A. tamarense*) cells contained acid phosphatases and suggested that they play a role in autophagous processes. ELF may thus have detected phosphatases that are unrelated to phosphorus nutrition in *A. fundyense*.

Since bacteria are also known to produce AP, it was essential to eliminate the possibility that the particulate ELF signal was due to surface-bound bacteria on our algal cells. Bacteria were detected in very low numbers only in non-axenic cultures by both phase contrast and epifluorescent microscopy using DAPI-staining. The ELF signal was always detected within the periphery of the algal cells and not in the background. To remove all doubt of the source of the ELF, axenic cultures of *Amphidinium carterae* and *Isochrysis* sp. were tested for bacterial contamination in several types of marine broth by the Provasoli-Guillard Center before shipment to our laboratory. They were retested in our hands and examined under phase contrast microscopy for the presence of bacteria. All results were negative; the ELF labeling of the axenic cultures was identical to the non-axenic ones (Table 1). Thus, the observed labeling pattern was due to algal AP and not attached bacteria.

This is the first report of the use of flow cytometry to quantitatively estimate cell-bound AP activity from ELF in single phytoplankton cells. In all 3 species tested, our flow cytometry results (Fig. 4) agreed with microscope observations (Fig. 3, Table 1). ELF fluorescence in the green channel was higher in the P-starved

treatments compared to the nutrient-replete conditions for *Amphidinium* sp. and *Isochrysis galbana*, and well above control levels. *A. fundyense* did not show a response to P starvation that was significantly different from controls. For both *Amphidinium* sp. and *I. galbana*, there was a direct relationship between the intensity of ELF labeling and the level of P starvation, although the f/80 organic treatments did not have as high an intensity as f/80 inorganic medium (Fig 4). Flow cytometry is thus a useful tool to detect phytoplankton cells that are P starved and react to ELF-staining of their APs. However, it remains to be demonstrated whether flow cytometry will be capable of detecting discrete populations of P-limited cells in mixed plankton assemblages.

It is of note that ELF intensity was higher on a per cell basis for *Isochrysis galbana* than for *Amphidinium* sp. (Fig. 4b, c). *I. galbana* is much smaller than *Amphidinium* sp., with a diameter of 5 μm compared to 9 to 13 μm and thus has a surface area one-fourth as large. This small size probably accounts for the lower background autofluorescence in controls for *I. galbana* relative to *Amphidinium* sp., but smaller size is inconsistent with the higher positive ELF fluorescence of *I. galbana* The explanation for this inconsistency may lie in the quantity of AP per cell, which may have been higher with *I. galbana* than *Amphidinium* sp. If this were true, however, the same elevated fluorescence would have been seen for *I. galbana* using the soluble product assays, but this was not the case. A comparison of Figs. 7 & 8 shows that *Amphidinium* sp. consistently gave a higher fluorescent signal than *I. galbana* in the soluble assays. The high ELF intensity for *I. galbana* may thus be better explained in terms of a higher affinity of this species' AP for the ELF substrate compared to the AP in *Amphidinium* sp. This is similar to our observation that AP in *Alexandrium fundyense* was not detected with ELF, but was observed at low levels with the 3 soluble assays. Clearly, the ELF substrate is not hydrolyzed equally by all APs.

Although ELF can distinguish P starvation in cells by microscopic analysis and can be quantified by flow cytometry, its signal was below the sensitivity of the Cytofluor plate reader. Even at the highest sensitivity setting on the Cytofluor, only very low values of fluorescence were detected for each of the 3 species, and none of the readings were different from the controls (Fig. 5). While this result was expected for *Alexandrium fundyense*, the lack of signal for the very bright ELF-labeled cells of *Amphidinium* sp. and *Isochrysis galbana* was surprising. To increase the signal intensity, the reaction was allowed to proceed for several more hours, but the results remained unchanged. This is in agreement with other unpublished results from our lab which suggest that antibody-labeled FITC

(fluorescein isothiocyanate)-stained or phycoerythrin-stained *A. fundyense* cells cannot be measured easily on the Cytofluor unless a large number of cells are present. The labeled cells settled in a layer on the bottom of the plate and did not yield enough signal for detection in this format, even when the *I. galbana* concentration was ca 100 000 cells well^{-1}. The soluble substrates yielded products which diffused throughout the well and were easily detectable. Therefore, the Cytofluor is not recommended for the quantification of AP activity in phytoplankton cells using ELF.

For each of the 3 species grown with the different media types, low levels of AP were detected with each of the soluble substrates that we could not detect with ELF using either visual observation, the epifluorescent microscope or the flow cytometer. Background levels of AP in *Alexandrium fundyense* were detected with the 3 soluble substrates but there was no increase of AP production with P starvation (Fig. 6). Low levels of AP activity were also detected in nutrient-replete *Amphidinium* sp. and *Isochrysis galbana*, notably in the f/2 organic treatments, that were not observed with ELF (Figs. 7 & 8). When more AP activity was apparent, as in the f/40 and f/80 P-starved conditions of *Amphidinium* sp. and *I. galbana*, the soluble substrates also yielded much higher fluorescence cell^{-1} than with ELF. Thus, soluble substrates are more sensitive than ELF.

These substrates cannot, however, identify the source of the AP activity in a sample. Furthermore, the ELF precipitate is approximately 500-fold more photostable than soluble fluorescein (Larison et al. 1995), and can be observed under the microscope for more than an hour without significant fading (Haugland & Johnson 1993). In the cultured phytoplankton samples, fluorescence signals were detected even after 3 mo of storage. Samples should be washed with 10 mM PBS to stop the ELF reaction, and then kept in the dark at 4°C.

ELF is thus a useful tool for the detection of endogenous AP activity in phytoplankton. Its signal is stable, easily visualized using an epifluorescent microscope, and can be quantified by flow cytometry. For species that produce significant amounts of AP when phosphorus-stressed, ELF can be used to assay the nutrient status of the organism. One noteable aspect of this study is that ELF represents an 'off the shelf' biological probe or indicator of P starvation that provides the same type of information that can be obtained using molecular probes or antibodies, such as those to cell-surface proteins involved in P uptake (Dyhrman & Palenik 1995). In this case, however, ELF is useful for multiple species, is available commercially, and does not require extensive protein purification, characterization, and antibody production.

Significant ELF-detectable AP activity was induced only when P was deficient in the media, but other types

of nutrient limitation or physiological stress (e.g. nitrogen starvation or light effects) must now be tested to determine the 'uniqueness' of the response. Further testing of ELF must also include various nutrient-limited continuous or semi-continuous culture conditions to insure that ELF-detectable AP activity is present when cells are grown in 'balanced' conditions that are more representative of field populations (Eppley 1981, Cullen et al. 1992). The distinction between nutrient-limited and nutrient-starved cells is significant in this regard. Finally, it is also necessary to screen many more species among the different algal classes from both marine and freshwater systems to determine the extent to which the observations reported here are valid for other organisms. Since the method only requires about 30 min for staining, rapid screening of multiple cultures using either epifluorescent microscopy or flow cytometry can easily be accomplished. If the method proves to be useful for many other microorganisms, as we expect, the procedures described here will provide unique insights on P limitation at the species level in natural plankton communities.

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