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Note

A method for determining alkaline phosphatase activity in marine phytoplankton using spectrofluorometry

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ABSTRACT

A method for determining relative percent intensity alkaline phosphatase activity (APA) using enzyme labeled fluorescence coupled with spectrofluorometry is presented. Compared to traditional microscopy and flow cytometry, we increase statistical power and reduce sample-handling issues. Combined with a biological standard, our method can quantify APA of natural plankton assemblages.

Enzyme-labeled fluorescence (ELF) is a method used to detect extracellular alkaline phosphatase activity (APA) with the commercially available substrate ELF-97 (Invitrogen #E6601). Most ELF-APA studies use epifluorescent microscopy for eukaryotic organisms or flow cytometry for prokaryotes. We propose that spectrofluorometry can be used to augment or replace these methods as spectrofluorometers measure intensity rather than presence/absence of ELF-APA. Spectrofluorometry increases sensitivity (Dignum et al., 2004; Telford et al., 1999) and decreases time and labor relative to microscopy (Nedoma et al., 2003) while simultaneously broadening the size spectrum of organisms that can be analyzed.

Herein we develop a spectrophotometric method, compare this with standard epifluorescent microscopy and flow cytometry (Dyhrman and Palenik, 1999; González-Gil et al., 1998), and demonstrate the use of a biological standard to quantify relative ELF-APA intensity from field samples.

The AP-producing (González-Gil et al., 1998; Lomas et al., 2004) dinoflagellate *Amphidinium carterae* (CCMP strain 1332) was cultured with sterile-filtered artificial seawater (ASW) under P-replete (L1 nutrients, CCMP) and P-deplete conditions. Cells were transferred at least twice to fully deplete residual P, and used as standards when they reached stationary phase. *A. carterae* was selected based on its fast growth and small size, making it amenable for use with flow cytometry. Similar results were obtained with *Akashiwo sanguinea* (data not shown).

Field samples were collected from the Santa Cruz Municipal Wharf (36 57.48′ N, 122 81.02′ W). Ancillary measurements included macronutrients (Knepel and Bogren, 2001; Smith and Bogren, 2001a, 2001b), temperature, chlorophyll-a (Welschmeyer, 1994), ammonium (NH_4; Holmes et al., 1999), and urea (Mulvenna and Savidge, 1992; Price and Harrison, 1987). Phytoplankton was preserved for species identification using 4% buffered formalin.

ELF-APA samples were filtered (<30 mm Hg) onto 1 or 5 μm polycarbonate filters (Poretics) to minimize the contribution of heterotrophic bacteria, and stored at −80 °C or in 1 mL reagent-grade 70% ethanol at 4 °C in the dark. A subset of samples (n = 6) was analyzed immediately. Cells were gently scraped off filters and placed in epitubes for ELF-labeling following modified procedures (González-Gil et al., 1998; Dyhrman and Palenik, 1999) by substituting ASW for sterile seawater and component B (Invitrogen #E6601) for P-buffer.

Ten μL of the prepared sample was suspended in two drops of mounting medium component C (Invitrogen #E6601) on Corning glass slides. Samples were examined with a Zeiss Axio Imager manual A1 microscope equipped with Chroma Technology filter set #31000v2. Cells with ELF-labeling were considered positive for APA (Dyhrman and Palenik, 1999; González-Gil et al., 1998). Field samples were enumerated as percent labeled cells relative to all enumerated cells (>100 per sample).

A Cytopeia Influx (Cytopeia Inc., Seattle, WA, USA) flow cytometer with a blue (488 nm) laser was used for a subset of samples. Each sample was characterized by forward angle light scatter (FSC; relates to particle size), and three fluorescence parameters: ELF (525 +/− 40 nm), red fluorescence for chlorophyll-a (692 +/− 20 nm), and SYBR Green I (520 +/− 25 nm) for heterotrophic bacteria. Samples

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were enumerated using ELF-fluorescence intensity versus FSC with a minimum of 45,000 particles per sample. FlowJo software was used to obtain a volumetric estimate of cell density (cells mL^{-1}; Goebel et al., 2008). Histograms were used to determine the geometric mean of the fluorescent peak of each sample (Meseck et al., 2009) and frequency statistics were computed in FlowJo.

Heterotrophic bacteria were counted with flow cytometry after being labeled with SYBR Green I to increase the intensity of the DNA signal. Autotrophic phytoplankton and heterotrophic bacteria were enumerated with red fluorescence versus FSC and green fluorescence versus FSC respectively.

For spectrofluorometric analysis, 70–100 µL samples were analyzed on a 96-well opaque plate using an M2e SpectraMax spectrofluorometer internally standardized with fluoroscein isothiocyanate (FITC; 3 fmol $well^{-1}$). Excitation/emission was set to 365 nm and 400–700 nm at 5 nm resolution. Automatic mixing (20 s) was used to minimize particle sinking. Temperature control (+/−0.5 °C) was used during the analysis. The 525 nm emission peak corresponds to the emission used for ELF-microscopy (González-Gil et al., 1998). For our intensity analysis we determined the area under the emission curve from 470 to 620 nm. Intensity was measured in arbitrary units (A.U.).

A. carterae cultured under P-replete and deplete conditions was used to establish a standard curve by serial dilution. The standard curve was created using known percent labeling (from microscopy) versus A.U. (spectrofluorometry) after normalizing A.U. to 450 nm to account for differences in biomass. Field sample ELF-APA intensity was expressed as percent-labeled intensity by applying the standard curve.

APA from epifluorescent microscopy is performed based on presence or absence of labeling (Fig. 1; Dyhrman and Palenik, 1999; González-Gil et al., 1998). This does not account for intensity of ELF-labeling (i.e., how many AP sites are labeled based on fluorescence), intercellular labeling (Dyhrman and Palenik, 1999; Díaz-de-Quijano and Felip, 2011), or the inability to 'count' the number of cell markers when the entire cell wall is not visible (Dyhrman and Ruttenberg, 2006; González-Gil et al., 1998; Meseck et al., 2009; Nicholson et al., 2006). Spectrofluorometry and flow cytometry can determine intensity (Duhamel et al., 2010; González-Gil et al., 1998; Nedoma et al., 2003), but results need to be normalized for biomass. For our method we normalized to 450 nm, an application unique to spectrofluorometry since epifluorescent microscopes and flow cytometers typically cannot scan the visible light spectrum. Our normalization to 450 nm does not interfere with the ELF-97 emission (maximum 525 nm), and is more accurate than normalizing to chlorophyll-a (data not shown). We quantified detectable differences for cultures with as low as 2% labeling (data not shown), and suggest that modern instruments can be used for routine quantification of APA, in contrast to results from previous studies (González-Gil et al., 1998; Nedoma et al., 2003).

Fig. 1. *Amphidinium carterae* labeled with ELF-97 precipitate (green) at the sites of alkaline phosphatase activity, visualized using epifluorescent microscopy as described in the text. Red is autofluorescence from endogenous pigments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

By using a serial dilution of *A. carterae* we were able to set a repeatable upper limit for ELF-APA intensity (99.3 +/− 0.01% of the cells were labeled with 3 or more AP sites, n = 17) and for the baseline (<0.001 +/− 0.001%, n = 13) allowing us to determine relative intensity of natural samples. For multiple standard curves (separate cultures) the slopes (but not the intercepts) were not significantly different (ANCOVA, $p<0.05$, n = 5); it is therefore necessary to adjust for the baseline (intercept) fluorescence from run to run. The close correspondence with microscopic enumeration suggests that, despite the potential variability introduced with a biological standard, our proposed method is robust.

Microscopy cannot statistically quantify APA if a low quantity of labeled cells are present (Nedoma et al., 2003; Nicholson et al., 2006). In contrast, spectrofluorometric measurement account for changes in labeling-intensity due to both the proportion of cells labeled and the number of sites per cell. Our P-replete cultures (n = 5) exhibited fluorescence intensity 26.1 +/− 3.7 A.U. lower than samples grown under P-deplete conditions (n = 5). From field samples (n = 8) three were negative for APA (0% labeling by microscopy) and the others range from 11 to 34% labeled cells (Table 1). Field sample percent-labeled intensity standardized using *A. carterae* ranged from 0.8% to 34.0% (Table 1). Both dinoflagellates and diatoms exhibited positive APA (Table 2). There was excellent correspondence between microscopic and spectrofluorometric measurements, both with field samples and with cultures (Tables 1 and 3).

While epifluorescent microscopy can only determine APA in phytoplankton >2 µm (Li et al., 1998), flow cytometry has a typical upper limit of <70 µm (Dignum et al., 2004; Duhamel et al., 2010). From our field samples, diatoms (Table 2) and the *A. sanguinea* culture (~90 µm) were unable to be counted by flow cytometry due to clogging despite the use of a 150 µm nozzle. While the flow cytometer may be more quantitative for determining APA-intensity than spectrofluorometry, its cell size capabilities limit its use and may better represent prokaryote contributions (Duhamel et al., 2010; Grégori et al., 2011).

Our *A. cartarae* P-deplete culture (87.4% labeling by flow cytometry, 92.4% labeling by microscopy) exhibited a 12.9% stronger 525 nm fluorescence signal than P-replete media cells (3.4% labeling; Fig. 2; Table 3) as measured by flow cytometry. The two populations were significantly different ($p<0.01$; X^2, Kolmogorov–Smirnov). ELF-labeled and un-labeled heterotrophic bacteria could not be distinguished for the P-replete sample; for the P-deplete sample small populations of ELF-labeled and non-ELF-labeled bacteria were present (Fig. 3).

Spectrofluorometry exhibits fast acquisition of data without compromises caused by low ELF-labeled cell abundance, but like flow cytometry, does not allow for direct determination of species using APA. We feel this to be an acceptable compromise given the speed, efficiency, and ability to quantify labeling intensity rather than just presence/absence of ELF-labeling. Furthermore, unlike flow cytometry

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Table 1
Field samples analyzed for APA by microscopy and spectrofluorometry. Ancillary data from the sample collection site are provided for reference.

Date	Percent of cells labeled counted by microscopy (SD)	Percent of cells labeled intensity counted by spectrofluorometry	N [μM]	P [μM]	Si [μM]	Chlorophyll-a [μg l^{-1}]	Urea [μM]	Temperature [4 °C]	NH_4 [μM]
7/1/08	11.4 (0.7)	11.7	6.0	0.7	8.7	5.8	0.6	13.4	n/a[a]
7/15/08	0.0 (0.0)	2.1	1.0	0.4	12.6	5.5	0.5	15.4	n/a
7/22/08	0.0 (0.0)	1.4	1.6	0.3	8.0	3.6	0.2	15.6	n/a
7/29/08	0.0 (0.0)	0.8	1.1	0.4	9.1	8.6	0.1	15.1	0.6
8/5/08	2.7 (1.6)	4.3	0.5	0.4	5.9	7.6	0.2	14.6	1.0
8/19/08	9.4 (2.2)	7.8	4.2	0.8	14.3	6.5	1.3	15.7	26.1
8/26/08	34.2 (2.6)	34.0	0.1	0.4	14.8	12.7	0.2	16.9	0.3
9/2/08	11.2 (1.9)	12.0	0.1	0.5	15.9	5.3	0.2	16.5	0.7

[a] n/a = sample not analyzed.

(Duhamel et al., 2010), spectrofluorometry can be used in conjunction with epifluorescent microscopy (the same sample can be used for both instruments) for 48 h with no decrease in label efficiency or intensity (data not shown). Thus spectrofluorometry in combination with limited epifluorescent microscopy could provide both a quantitative assessment and community composition of APA expression and could be used to estimate the intensity of ELF-labeling of APA in natural assemblages. When combined with a biological standard (stressed culture material) spectrofluorometric estimates of relative APA intensity can be obtained both within a lab (experiment to experiment) and potentially across instruments.

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Table 2
List of genera positively labeled for APA using epifluorescent microscopy from samples collected at the Santa Cruz Municipal Wharf, Monterey Bay, between July 1 and September 2 2008.

Genera	Group	Date positive
Akashiwo	Dinoflagellate	July 1st, August 5th, 19th, 26th, and September 2nd, 2008
Protoperidinium	Dinoflagellate	July 1st, 2008
Ceratium	Dinoflagellate	July 1st, August 5th, 19th, 26th, and September 2nd, 2008
Dinophysis	Dinoflagellate	August 5th, August 19th, 2008
Gymnodinium	Dinoflagellate	August 19th, August 26th, September 2nd, 2008
Prorocentrum	Dinoflagellate	August 26th, September 2nd, 2008
Thecate dinoflagellate 1	Dinoflagellate	August 8th, 2008
Thecate dinoflagellate 2	Dinoflagellate	September 2nd, 2008
Pseudo-nitszchia	Diatom	July 1st, August 19th, 2008
Chaetoceros	Diatom	July 1st, 2008

Table 3
Summary results for the inter-comparison of microscopy, spectrofluorometry, and flow cytometry using the dinoflagellate *A. carterae* grown under varying levels of P-stress.

Percent labeled cells counted by microscopy (SD)	Percent labeled cells intensity counted by spectrofluorometry	Flow cytometry geometric mean	Percent labeled particles (>3 μm) counted by flow cytometry
0.0 (0.0)	0.2	205	3.2
17.4 (1.4)	19.1	n/a	n/a
35.4 (0.8)	38.1	n/a	n/a
92.4 (1.9)	92.4	218	87.4

n/a = sample not analyzed.

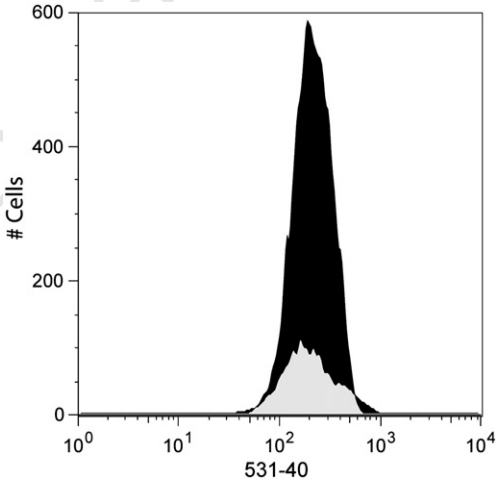

Fig. 2. Cytograms showing the number of events with 531 +/− 40 nm green fluorescence in batch cultures of *A. carterae* grown under P-replete conditions (gray; geometric mean = 205) and P-deplete conditions (black; geometric mean = 218).

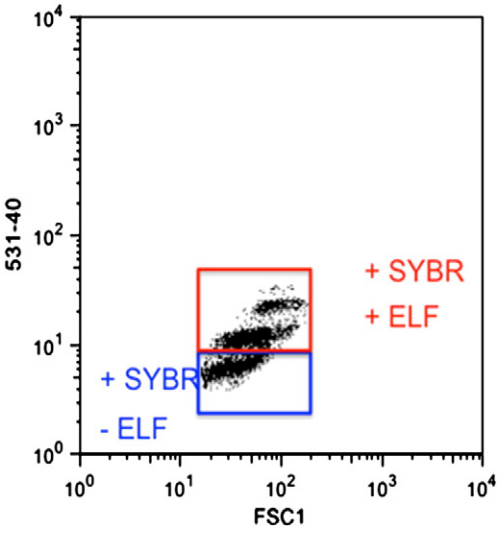

Fig. 3. Cytogram dot plot showing the number of events with 531 +/− 40 nm green fluorescence for heterotrophic bacteria in batch cultures of *A. carterae* grown under P-deplete conditions, labeled with ELF-97 and SYBR green I.

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