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Green Autofluorescence in Dinoflagellates, Diatoms, and Other Microalgae and Its Implications for Vital Staining and Morphological Studies▽†

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Green autofluorescence (GAF) has been described in the short flagellum of golden and brown algae, the stigma of *Euglenophyceae*, and cytoplasm of different life stages of dinoflagellates and is considered by some researchers a valuable taxonomic feature for dinoflagellates. In addition, green fluorescence staining has been widely proposed or adopted to measure cell viability (or physiological state) in areas such as apoptosis of phytoplankton, pollutant stresses on algae, metabolic activity of algae, and testing treatment technologies for ships' ballast water. This paper reports our epifluorescence microscopic observations and quantitative spectrometric measurements of GAF in a broad phylogenetic range of microalgae. Our results demonstrate GAF is a common feature of dinoflagellates, diatoms, green algae, cyanobacteria, and raphidophytes, occurs in the cytoplasm and particularly in eyespots, accumulation bodies, spines, and aerotopes, and is caused by molecules other than chlorophyll. GAF intensity increased with time after cell death or fixation and with excitation by blue or UV light and was affected by pH. GAF of microalgae may be only of limited value in taxonomy. It can be strong enough to interfere with the results of green fluorescence staining, particularly when stained samples are observed microscopically. GAF is useful, however, for microscopic study of algal morphology, especially to visualize cellular components such as eyespots, nucleus, aerotopes, spines, and chloroplasts. Furthermore, GAF can be used to visualize and enumerate dinoflagellate cysts in marine and estuarine sediments in the context of anticipating and monitoring harmful algal blooms and in tracking potentially harmful dinoflagellates transported in ships' ballast tanks.

Green autofluorescence (GAF) has been described in the short flagellum of *Phaeophyceae*, *Synurophyceae*, *Chrysophyceae*, and *Prymnesiophyceae* (8, 17), the stigma of *Euglenophyceae* (8), and the cytoplasm of different life stages of dinoflagellates (6, 11, 20, 27). Among dinoflagellates, the presence of GAF was first considered to be specific to heterotrophic species (20) or unarmored taxa (27) but later was found to be a common feature (6, 11). For instance, Carpenter et al. (6) reported GAF from a number of dinoflagellates (both heterotrophic and autotrophic). Elbrächter (11) observed GAF in vegetative cells and cysts of dinoflagellates, although about half of the obligate heterotrophic dinoflagellates examined showed no GAF at all. Therefore, GAF, once considered to be a "phylogenetic enigma" (8) and a valuable taxonomic feature (11, 27), is now known to be widespread, but perhaps not ubiquitous, among dinoflagellates. In addition, GAF has been used to visualize some parasitic dinoflagellates in phytoplankton (7, 24, 26, 34).

A variety of stains that fluoresce green under excitation by light has been widely adopted or proposed to measure the viability, physiological state, or the presence of microalgae, including studies of phytoplankton apoptosis (12, 31), population dynamics (14, 29, 30), enumeration of dinoflagellate cysts in marine sediment (1, 2, 3, 18, 22, 23, 33), microzooplankton grazing (15, 16, 21), pollutant stresses on algal cells (4, 19), metabolic activity of algae (10), and efficacy testing of ballast water treatment technologies.

There are reports of GAF by microalgae interfering with green-fluorescing stains (10, 21, 31). Solutions have included either application of arbitrary criteria (31) or determining signal intensity by difference before and after staining (10). Some researchers, however, seem not to have been aware of possible interference from GAF with the desired green staining and instead were concerned only with interference from chlorophyll red autofluorescence (RAF) (16, 25). Of relevance, GAF can be compounded by interactions with chemicals, such as copper ions (19) and glutaraldehyde (20, 27).

In reviewing these studies of GAF in microalgae, we noted that a quantitative characterization of GAF and comprehensive observations across multiple taxa and varied physiological conditions had not been performed. These deficits make it difficult to assess and address potential limitations of using green-fluorescing stains to measure the viability of microalgal cells. Because we needed to judge the staining efficiency of widely used green-fluorescing stains (e.g., fluorescein diacetate, 5-chloromethylfluorescein diacetate [CMFDA]) using epifluorescence microscopy, we conducted a series of observations and measurements of GAF under blue light excitation in different taxa of microalgae (mainly dinoflagellates and diatoms) encompassing both marine and freshwater forms. Our study focused, although not exclusively, on GAF rather than the red, yellow, and orange autofluorescence elicited by blue light excitation, because of the popular use of green fluorescence staining.

MATERIALS AND METHODS

Cultures and field samples of microalgae. The dinoflagellate *Gymnodinium catenatum* strains GCDE08 and GCHU11 were obtained from the CSIRO Col-

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lection of Living Microalgae (Hobart, Tasmania, Australia), the diatom *Corethron hystrix* CCMP307 was from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Maine), the dinoflagellate *Takayama acrochocha* was kindly provided by M. J. Holmes (National University of Singapore), the raphidophyte *Chattonella globosa* was provided by M. A. Doblin (University of Technology, Sydney, Australia), and the dinoflagellates *Akashiwo sanguinea* and *Scrippsiella trochoidea* were isolated from the Elizabeth and Lafayette Rivers, Norfolk, Va., on 9 September and 14 July 2005, respectively. The cultures were maintained in GSe (9) or f/10 (for *C. hystrix* and *T. acrochocha*) medium in 18 or 21°C lighted incubators provided with cool fluorescent light (35 μM photons m^{-2} s^{-1}) operated on a 12 h-12 h light-dark photoperiod. Cysts of *G. catenatum* were produced in GSe-N-P medium by crossing GCDE08 and GCHU11 (9). Field samples of other algal species (including the dinoflagellates *Prorocentrum micans*, *Protoperidinium* sp., *Gyrodinium uncatenum*, *Ceratium furca*, diatoms *Asterionella*, *Skeletonema*, and two unidentified species, freshwater cyanobacteria *Woronichinia*, *Coelosphaerium*, and *Merismopedia*, and freshwater green algae *Staurastrum*, *Pediastrum*, *Desmodesmus*, and *Selenastrum*) were taken from the brackish Elizabeth River or a freshwater pond during 9 to 14 September 2005 (both near Old Dominion University) with a 20-μm-mesh-size nylon net. The unidentified cysts were concentrated from a ballast tank sediment sample using the method described by Bolch (5) after storage at 4°C for 4 years.

Sample preparation for microscopic observation. Algal cells were fixed with formaldehyde (1% [vol/vol] final concentration) for different times (as specified in Table 1, below), rather than glutaraldehyde, which can cause GAF of cells (reference 32 and personal observation). Other cells were killed with heat (80°C water bath for 15 min). To test the effect of pH on GAF intensity, the pH of medium containing *G. catenatum* or *C. hystrix* was adjusted with 1 N HCl or 1 N NaOH, and GAF intensity was measured within 3 h. Measurement of each sample took about 30 min. The brackish and freshwater field samples and the cysts from the ship's ballast tank sediment were observed and measured immediately after sampling or after fixation (see Table 1, below).

For comparison, live and fixed cells of *G. catenatum* and *C. hystrix* were stained with the vital stain CMFDA (5.0 μM final concentration, 18° or 21°C in the lighted incubator for 5 h) or the mortal stain SYTOX green (1.0 μM final concentration, 21°C in darkness for 0.5 to 2 h) (stains from Molecular Probes Inc., Oregon). To examine the possible contribution of chlorophyll to GAF, reagent-grade methanol was used to extract chlorophyll (4°C for 48 h in darkness) from vegetative cells and cysts of *Scrippsiella trochoidea* previously fixed with 2% (vol/vol) formaldehyde; after washing, the cells were examined for the presence of GAF.

Light microscope observations. Cells were observed with an Olympus BX50 light microscope (Olympus Optical Co., Tokyo, Japan) using bright field and epifluorescence illumination. For epifluorescence, two kinds of filter cubes were used: U-MNB (exciter BP470-490, dichroic DM500, and emitter LP515; Olympus Optical Co.) for GAF and chlorophyll-induced RAF, as well as for yellow and orange autofluorescence; U-N31001 (exciter D480/30X, dichroic 505DCLP, and emitter D535/40m; ChromaTechnology Corp., Vermont) for green fluorescence only (the emitter of the U-N31001 cube allows a relatively even transmission efficiency of 81 to 87% over the wavelength window from 520 to 554 nm). Images were captured with a DP70 digital camera (Olympus Optical Co.) and processed with DPController/Manager software (Olympus Optical Co.). Most observations and measurements were conducted at a total magnification of ×500 (10 × 40 × 1.25).

Spectrometric measurements of fluorescence intensity of individual cells. An S2000 miniature fiber optic spectrometer (Ocean Optics, Inc., Florida), connected to the microscope's viewing port, was used to measure the fluorescence intensity of individual cells; data were collected with OOIBase v1.5 software (Ocean Optics, Inc.). Filter cube U-N31001 was used when measuring green fluorescence only (all green fluorescence data in Table 1 and Fig. 2 and 4, below), while filter cube U-MNB was used when measuring GAF and RAF (data in Table 1 and Fig. 3, below). Filter cube U-MWU (exciter BP330-385, dichroic DM400, and emitter LP420; Olympus Optical Co.) was used only to expose cells to UV light; their subsequent GAF was measured after passing through filter cube U-N31001. All measurements were conducted at the same magnification (×500), field area (controlled with a shutter), and positioning of the fiber optics cable. The integration time (during which the accumulated irradiance was counted) was adjusted from sample to sample according to the signal intensity of fluorescence (1 to 10 seconds for GAF and 50 milliseconds for RAF), but within any one sample a constant integration time was used for all cells. The number of individual cells measured for each sample (Table 1) ranged from 5 to 40 (median, 21), reflecting both cell density and the need to minimize cells' exposure to a warm microscope slide. All comparisons between samples and treatments were based on relative fluorescence intensity, defined and calculated as 1/1,000 of the irradiance counts, integrated over 10 seconds, at the peak wavelength of the fluorescence spectrum.

Tests of significance for differences among treatments were conducted using a one-way analysis of variance (ANOVA) with Microsoft Excel 2002 and SPSS 10.1.0. Data were tested for homogeneity of variance (Fmax test), and if necessary to meet assumptions of ANOVA, were $\log_{10}$ transformed. Following ANOVA, Tukey's honestly significant difference test was calculated to compare all means pairwise. When homogeneity of variance was not achieved, a posteriori pairwise comparisons were made with the Games-Howell method. Unless noted otherwise, a critical P value of 0.05 was used.

RESULTS

GAF in different taxa of microalgae. GAF was exhibited in all species examined, covering 5 phyla: dinoflagellates, diatoms, cyanobacteria, green algae, and raphidophytes (Fig. 1; see more micrographs in the supplemental material). Among these were the dinoflagellates *G. catenatum* (vegetative cells and cysts) (Fig. 1a to g and m), *A. sanguinea* (Fig. 1n, o, and p), *T. acrochocha* (Fig. 1l), and *P. micans* (Fig. 1u); the diatom *C. hystrix* (Fig. 1h to k); green algae *Desmodesmus* (Fig. 1s) and *Pediastrum* (Fig. 1r); and the cyanobacterium *Woronichinia* (Fig. 1q). Other species in which GAF was observed included dinoflagellates *G. uncatenum*, *C. furca*, *Peridinium*, and *Protoperidinium*, diatoms *Cyclotella*, *Asterionella*, and *Skeletonema*, green algae *Staurastrum* and *Selenastrum*, cyanobacteria *Coelosphaerium* and *Merismopedia*, and raphidophyte *C. globosa* (images of these other species are available in the supplemental material). GAF also was observed in various unidentified cysts from a ballast tank sediment sample (Fig. 1t; see also the supplemental material). A possible endosymbiont species fluoresced brightly within a cell of *C. hystrix* (Fig. 1i to k). GAF was exhibited in both live and dead (heat-treated or formaldehyde-fixed) cells and cysts, although the intensity of GAF differed among different species and physiological states (see below). Therefore, GAF appeared to be a common feature in all phyla examined, and its presence was independent of the cells' physiological status.

Because of the wide variation in algal cell size and shape, it is difficult to compare quantitatively the intensity of GAF among different taxa. For example, the dinoflagellate *G. catenatum* exhibited much higher intensity of GAF than the diatom *C. hystrix*, presumably because of its larger cell size and greater cellular content; the same result emerged for chlorophyll RAF and green fluorescence due to SYTOX green staining (Table 1). Even the smallest dinoflagellate in this study, *T. acrochocha* (in terms of its ventral area, only one-eighth that of the largest dinoflagellate, *A. sanguinea*), displayed brighter GAF than *C. hystrix* (Table 1; Fig. 1l). In general, live cells of diatoms and the raphidophyte exhibited weaker GAF than did dinoflagellates, cyanobacteria, and green algae, and of these, dinoflagellates had the strongest signal.

Distribution of GAF within cells. In all species, GAF generally was distributed evenly in the cytoplasm and mostly found in association with chloroplasts (Fig. 1; see also the micrographs in the supplemental material). It also occurred, however, in organelles lacking chlorophyll, such as accumulation bodies of vegetative cells and cysts (Fig. 1f, g, and l) and cell spines of green algae (Fig. 1r and s) but not spines of dinoflagellates (Fig. 1u), aerotopes (gas vacuoles) of cyanobacteria (Fig. 1q), cysts which had completely or partly lost chlorophyll (Fig. 1e, f, and t), and in particular, dinoflagellate stigma (eyespots), where the GAF was strongest in *G. catena-*

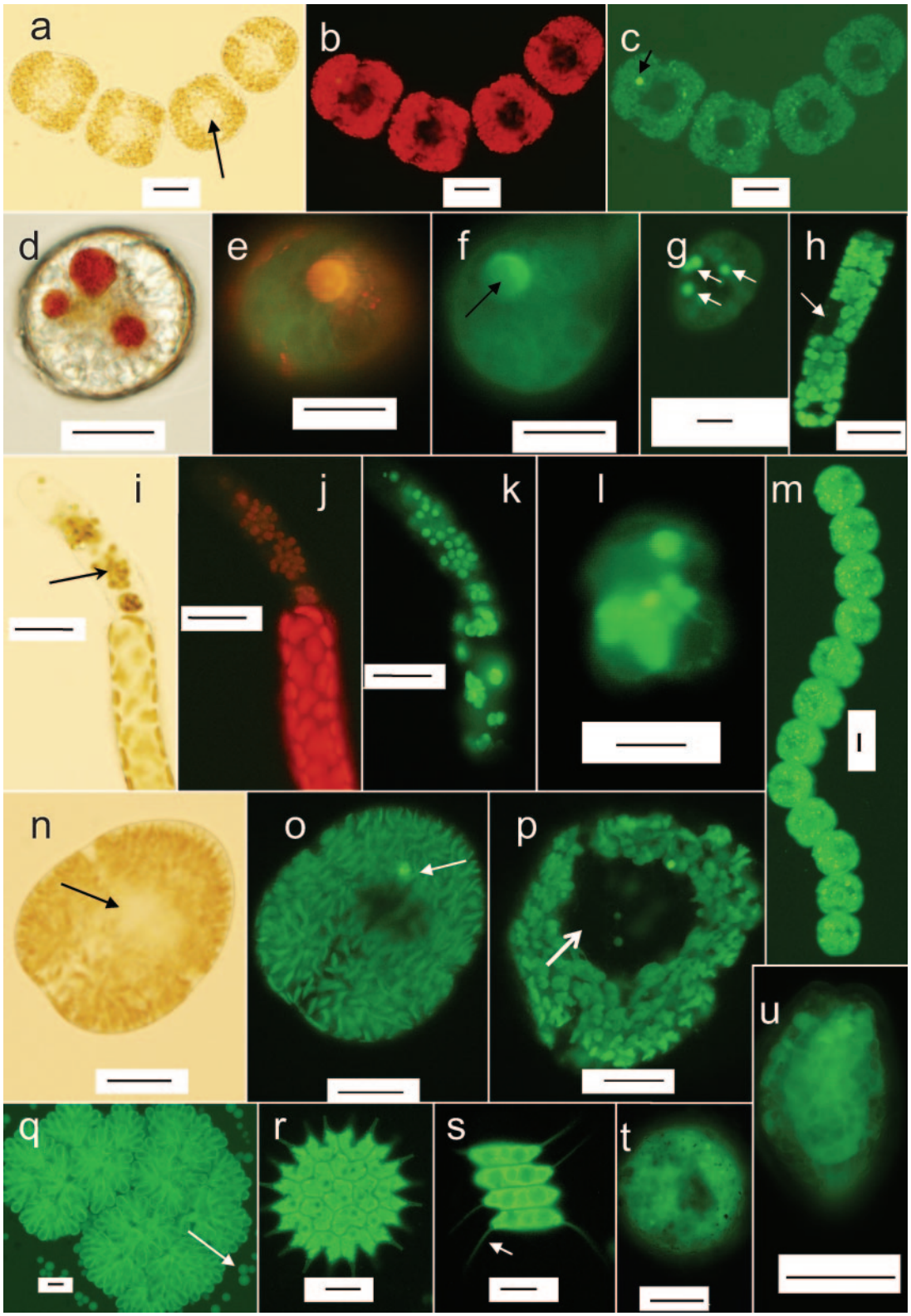

FIG. 1. Micrographs of GAF observed in microalgae, obtained using an epifluorescence microscope installed with filter cubes U-MNB (for chlorophyll RAF and green to orange autofluorescence) and U-N31001 (for GAF only). Unless noted otherwise, all images are of live cells. (a to g, m) *Gymnodinium catenatum* vegetative cells under bright field (a; arrow indicates nucleus), the same cells exhibiting RAF (b), the same cells exhibiting GAF (c; the arrow indicates eyespot), cyst under bright field (d), the same cyst observed with U-MNB filter (e), GAF of the cyst (f; the arrow indicates the lipid accumulation body), a vegetative cell indicating stronger GAF in eyespot and accumulation bodies (g; arrows), GAF of vegetative cells in a long chain after fixation with formaldehyde (m). (h to k) *Corethron hystrix* GAF (h; note the absence of GAF in the nucleus,

tum, *A. sanguinea*, and *T. acrochocha* (Fig. 1c, g, o, and p). GAF was not observed in the nucleus (Fig. 1a to c and n to p) or thecal plates of armored dinoflagellates (Fig. 1u). In chloroplasts or cytoplasm, GAF appeared to emanate from discrete bodies (Fig. 1c, h, m, and o). The brightness of GAF was not correlated with the presence or intensity of chlorophyll RAF, and cells fluoresced green even more strongly after their RAF was diminished following cell death or fixation by formaldehyde (see Fig. 2 and 3, below). Therefore, GAF seems not to be induced by chlorophyll, although these observations alone did not exclude the possibility GAF is related to the degradation products of chlorophyll.

Intensity of GAF and its change with storage time and pH. For *G. catenatum*, the mean relative intensity of GAF in live vegetative cells was 0.6% that of chlorophyll-induced RAF (3.4 versus 584.4), 0.25% that exhibited by formaldehyde-fixed cells stained with SYTOX green (3.4 versus 1,352.8), and equal to the GAF of live cysts (3.4 versus 3.4) (Table 1; Fig. 2A). GAF intensity exhibited by live vegetative cells stained with the vital dye CMFDA was only slightly higher, although statistically significant ($P < 0.05$), than that of unstained live cells and cysts (4.8 versus 3.4) (Table 1; Fig. 2A). GAF intensity in formaldehyde-fixed cells was greater than that of live cells ($P < 0.05$) and increased with time after fixation ($P < 0.05$) by up to 4.5 to 5.3 times after 3 days of fixation, following which intensity decreased ($P < 0.05$) through 168 h (Table 1; Fig. 2A). The significantly greater GAF after 3 weeks of fixation (Fig. 2A) is thought to be related to diffusion of the GAF-causing molecule(s), as discussed below.

For *C. hystrix*, the mean relative intensity of GAF in live cells was 8.8% that exhibited by formaldehyde-fixed and SYTOX green-stained cells (1.0 versus 11.1) and 64% the intensity of live cells stained with CMFDA (1.0 versus 1.5; $P < 0.01$) (Table 1; Fig. 2B). As with *G. catenatum*, GAF intensity increased following formaldehyde fixation and after 40 h was significantly higher than that of live cells ($P < 0.05$). The increase continued for 130 h and then faded somewhat by 180 h (1.9 versus 1.5; $P = 0.08$) (Table 1; Fig. 2B). Significantly increased GAF intensity over time following formaldehyde fixation ($P < 0.001$) was also observed in *A. sanguinea* and *P. micans* (Table 1).

The average GAF intensity of various unidentified cysts ($n = 40$) from ballast tank sediment stored at 4°C for 4 years was eight times that observed in live cysts of *G. catenatum* (27.0 versus 3.4) and also higher than that of fixed *G. catenatum* vegetative cells stored for 3 weeks (17.7) (Table 1).

The GAF intensity of *G. catenatum* and *C. hystrix* was significantly affected by pH ($P < 0.001$) over the range 4.0 to 9.5 for *G. catenatum* and 4.5 to 8.8 for *C. hystrix*, but with no discernible common pattern (Table 1). For both species, loss of cellular contents occurred at pH values of <5.

Temporal dynamics of GAF and RAF elicited by blue and UV light excitation. By exposing algal cells continuously to blue light and measuring periodically the intensity of GAF, RAF, and yellow-orange autofluorescence using filter cube U-MNB, we observed that the intensity of RAF decayed monotonically to extinction in a few minutes. On the other hand, the intensities of GAF and yellow-orange autofluorescence (510 to 620 nm), first increased significantly and then decayed for both *G. catenatum* and *C. hystrix* (Fig. 3A and B).

Using filter cube U-MWU to expose cells of *G. catenatum* and *C. hystrix* to UV light for 5-second intervals before measuring GAF and RAF, a similar pattern was observed, i.e., RAF decayed quickly and GAF increased up to fivefold after 45 seconds of exposure and then decreased (Fig. 4). The intensity after 160 seconds of UV exposure, however, remained more than twice that at the start.

Observations of GAF after chlorophyll extraction. There was no noticeable reduction in GAF when chlorophyll was extracted from formaldehyde-fixed vegetative cells and cysts of *S. trochoidea*. In fact, the GAF of vegetative cells tended to increase. These observations, especially in light of previous measurements on cysts of *G. catenatum* and *S. trochoidea* (28), suggested chlorophyll is not responsible for GAF.

DISCUSSION

Chemical nature of GAF. The broad phylogenetic distribution over which GAF manifests suggests it is caused by a compound(s) commonly found in microalgal cells. Although some GAF appeared to be colocated with chloroplasts, GAF and chlorophyll RAF were not always colocated within cells (see Fig. 1c, e to g, l, o to s, and u; see also the supplemental material). Indeed, GAF was also observed in cellular components where chlorophyll was absent, such as the eyespots of dinoflagellates, spines of green algae, and the aerotopes of cyanobacteria. While we do not know the compound(s) responsible for GAF, we presume it is not chlorophyll, based on the sometimes differing intracellular locations of GAF and chlorophyll RAF and the strong GAF in *S. trochoidea* cells and cysts after chlorophyll extraction.

The wavelengths of GAF maxima, at least in *G. catenatum*, shifted upward from 530 nm or less for live cells to 542 to 549 nm for fixed cells (Table 1). The shift suggests multiple compounds or derivatives of a single compound are stimulated to produce the GAF. Furthermore, the same group of molecules causing GAF may also be responsible for other autofluorescence, because algal cells fluoresced yellow and orange (Fig. 3), with a strength equivalent to GAF, under blue light (470 to 490 nm) excitation. While Kawai (17) suggested GAF was caused by a flavin-like molecule, Dorsey et al. (10) considered luciferin compounds to be responsible for GAF in natural

indicated by the arrow), a cell observed under bright field (i; the arrow indicates an unidentified endosymbiont microalga in the *C. hystrix* cell), RAF (j), or GAF (k). (l) GAF of *Takayama acrochocha*. (n to p) *Akashiwo sanguinea* cell observed under bright field (n; the arrow indicates the nucleus), GAF (o; the arrow indicates the eyespot), or GAF of the same cell after breakage (p; the arrow indicates the absence of GAF in the nucleus). (q) GAF of a colony of *Woronichinia* sp. (the arrow indicates the aerotopes). (r) GAF of *Pediastrum* sp. (s) GAF of *Desmodesmus* sp. (the arrow indicates the spines). (t) GAF of a cyst from ballast tank sediment stored at 4°C for about 4 years. (u) GAF of *Prorocentrum micans* (note the absence of GAF in the spine and plates). Bars: 20 μm (a to k, m to p, t, and u) or 10 μm (l and q to s).

TABLE 1. Relative intensities of GAF exhibited by individual cells of various microalgae[a]

Sample description	Avg relative intensity (AU)	SD	Maximum	Minimum	Sample size (cells)	Wavelength of peak signal (nm)
G. catenatum live cells	3.4	1.6	8.9	2.0	25	530
G. catenatum live cells stained w/CMFDA	4.8	2.0	9.8	1.9	20	526
G. catenatum fixed and stained w/SYTOX green	1,352.8	1,214.6	3,957.3	22.7	22	526–527
G. catenatum live cells, red fluorescence	584.4	140.4	795.5	327.4	10	681
G. catenatum live cysts	3.4	1.1	6.3	2.0	12	543–547
G. catenatum fixed 8 h	6.8	2.5	13.2	2.6	20	547–548
G. catenatum fixed 24 h	9.9	3.0	19.8	6.1	24	547–548
G. catenatum fixed 48 h	14.2	5.7	28.9	6.9	25	547–548
G. catenatum fixed 72 h	15.4	4.4	24.4	7.9	23	547
G. catenatum fixed 96 h	10.1	2.9	18.2	3.7	25	547
G. catenatum fixed 168 h	9.5	2.7	15.4	4.4	25	546
G. catenatum fixed 3 wks	17.7	7.6	39.7	5.1	25	544–546
G. catenatum live cells, pH 4.0	3.7	1.3	7.2	1.9	22	546–547
G. catenatum live cells, pH 5.1	2.9	1.2	7.3	1.4	21	530
G. catenatum live cells, pH 7.0	2.5	0.7	4.2	1.3	20	530
G. catenatum live cells, pH 9.0	4.8	3.6	16.9	1.8	21	527–530
G. catenatum live cells, pH 9.5	5.3	3.7	16.8	2.4	21	530
G. catenatum live cysts, pH 4.0	3.7	0.9	4.9	2.4	6	529–530
Corethron hystrix live cells	1.0	0.4	2.1	0.6	30	540
C. hystrix live cells stained w/CMFDA	1.5	0.8	3.2	0.8	21	525
C. hystrix fixed cells stained w/SYTOX green	11.1	3.4	17.9	5.8	21	526–527
C. hystrix fixed 12 h	1.0	0.3	1.6	0.7	27	542
C. hystrix fixed 40 h	1.3	0.3	1.6	0.8	20	542
C. hystrix fixed 105 h	1.9	0.7	3.8	1.1	30	542
C. hystrix fixed 130 h	1.9	0.7	3.3	1.0	30	542
C. hystrix fixed 180 h	1.5	0.5	2.7	0.8	35	542
C. hystrix live cells, pH 4.5	1.1	0.6	3.2	0.6	25	542
C. hystrix live cells, pH 6.3	0.9	0.3	1.7	0.6	25	542
C. hystrix live cells, pH 7.4	0.8	0.2	1.8	0.6	25	540
C. hystrix live cells, pH 7.8	0.9	0.5	2.7	0.6	25	540
C. hystrix live cells, pH 8.8	0.6	0.04	0.8	0.5	25	540
Akashiwo sanguinea live cells	2.4	0.7	3.4	1.2	12	530
A. sanguinea fixed 20 h	5.6	1.4	7.9	3.6	11	546.5
A. sanguinea fixed 168 h	8.1	3.9	18.2	3.8	10	546
Prorocentrum micans live cells	1.1	0.2	1.4	0.9	10	530
P. micans fixed 20 h	1.8	0.3	2.2	1.4	9	541–543
Peridinium sp. live cells	1.4	0.7	2.5	0.7	5	530–542
Protoperidinium sp. fixed 20 h	3.1	1.0	4.5	1.6	10	543
Takayama acrochocha live cells	1.1	0.3	1.7	0.8	10	526
Sediment cysts (stored at 4°C for 4 yrs)	27.0	15.1	55.6	6.9	40	542
Actinocyclus-like diatom fixed 168 h	2.1	1.0	4.6	0.8	11	543

[a] Calculated as 1/1,000 of the total irradiance counts integrated over 10 seconds (expressed in arbitrary units [AU]). With an emphasis on *G. catenatum* and *C. hystrix*, relative intensities are shown for live, stained, or formaldehyde-fixed cells (and cysts). Data also are shown for observations in which pH was adjusted. The wavelength at which peak values were observed was determined using filter cube U-N31001. Statistical comparisons are presented in the text.

populations of microalgae. Recently, Fujita et al. (13) isolated a flavoprotein that has a flavin mononucleotide as a chromophore from the green-fluorescing flagellum of the brown alga *Scytosiphon lomentaria*.

G. catenatum and *C. hystrix* exhibited a similar temporal pattern in GAF intensity following fixation (Fig. 2A and B). The pattern suggests at least two hypotheses. First, a GAF-causing molecule(s) was produced and either decomposed or leaked from cells over 168 h, and another such molecule(s) emerged between 168 h and 3 weeks. Second, and more simply, the pattern results from the interplay of production and leakage. Evidence for this latter thought comes from the history of the 3-week sample. For each alga, all previous samples (8 to 168 h) were taken from a single tube, which was well mixed before each sampling. The 3-week sample comprised its own tube, however, and was not mixed except once before the sample was taken. Therefore, the temporal pattern in GAF is consistent with the idea that mixing hastened the leakage of the GAF-causing molecule(s) from the cells. By extension, the nonlinear temporal pattern in GAF intensity caused by UV exposure (Fig. 4) is tentatively proposed to reflect simultaneous, short-term production and decomposition of the molecule(s) responsible for GAF, and after 60 seconds of exposure, decomposition exceeded production.

Taxonomic implications of GAF. The wide distribution of GAF we report here, together with its observation in the flagellum of golden and brown algae (8, 17), led us to conclude the utility of GAF's presence or absence as a taxonomic fea-

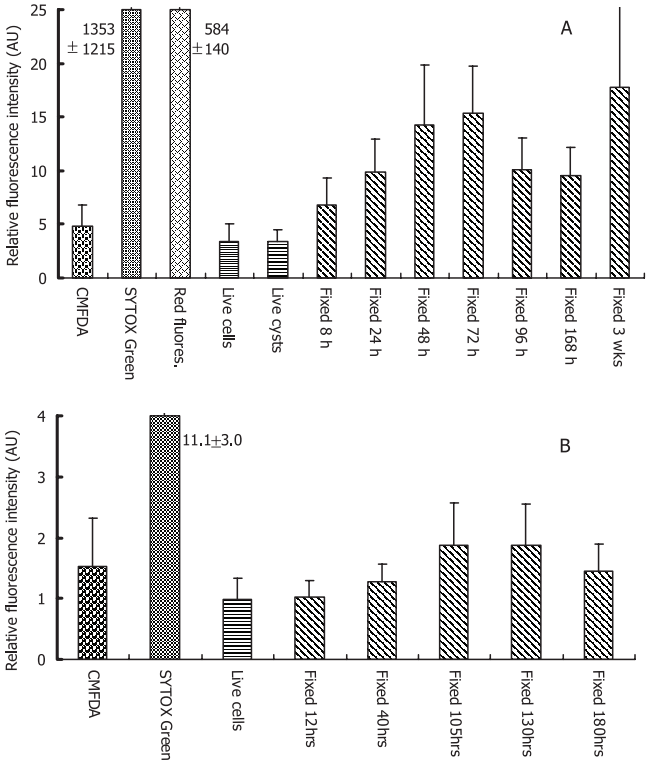

FIG. 2. Relative intensity of GAF in stained, live, or formaldehyde-fixed cells of (A) *Gymnodinium catenatum* and (B) *Corethron hystrix*. CMFDA and SYTOX green stains were applied to live and dead cells, respectively. The relative intensity of chlorophyll-induced RAF in live *G. catenatum* cells is shown for comparison. Bars represent means ± 1 standard deviation for $n = 10$ to 35 cells. Corresponding data are presented in Table 1. AU, arbitrary units.

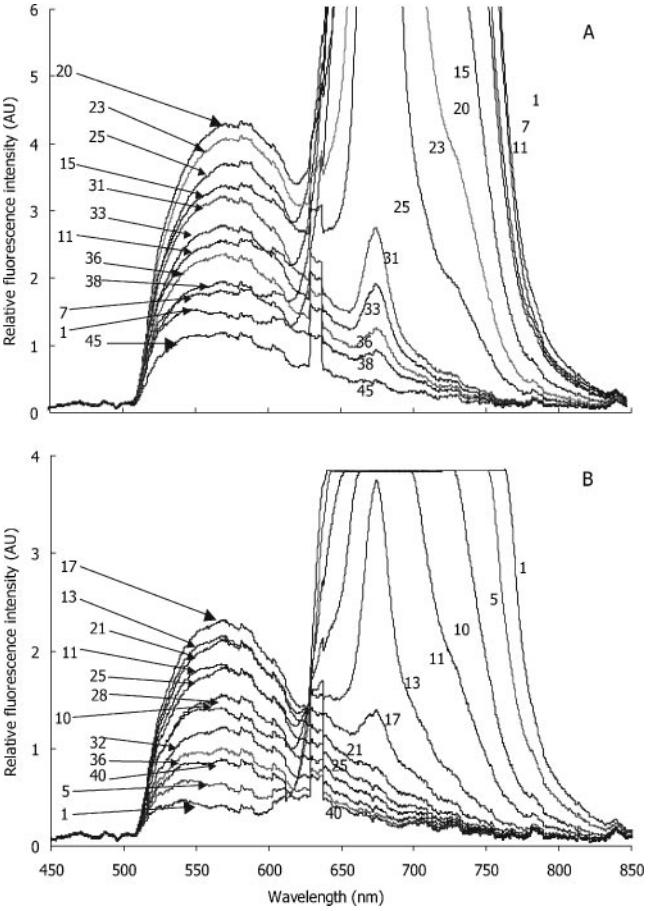

FIG. 3. Temporal dynamics in the relative intensity of GAF and chlorophyll-induced RAF in (A) *Gymnodinium catenatum* (vegetative cells) and (B) *Corethron hystrix*. Individual cells were exposed continuously to blue light (470 to 490 nm; filter cube U-MNB). From 40 (*C. hystrix*) or 45 (*G. catenatum*) spectra evenly spaced over 10 (*C. hystrix*) or 15 (*G. catenatum*) minutes, 12 for each are shown here in chronological order (numbers indicate the order of measurements). These spectra are representative of those observed emanating from other cells (for *G. catenatum*, $n = 5$ cells; for *C. hystrix*, $n = 4$ cells). AU, arbitrary units.

ture is limited. That GAF explicitly was not observed in cytoplasts of golden and brown algae (8, 17) or in some species of dinoflagellates (6, 11, 27) may be attributed to its relatively weak signal in live samples, which can be overwhelmed by the much stronger signal of chlorophyll RAF.

Implications of GAF to green fluorescence staining, morphological study, and visualization of dinoflagellate cysts. Green fluorescence staining has been widely adopted in a variety of applications (see the introduction). In applying such fluorescent stains, therefore, it is critical to have a well-defined demarcation between the introduced green fluorescence and GAF. While different species of microalgae exhibit GAF with various intensities at different physiological states, the signal is generally bright enough to visualize using epifluorescence microscopy. The signal is not stable over time, however, even if only one algal species is considered. In our study, for example, GAF of *G. catenatum* increased appreciably with time after fixation or following 5 seconds' excitation by UV light. Lage et al. (19) reported a three- to fourfold increase in GAF for the dinoflagellate *Amphidinium carterae* when cells were exposed to a toxic dose of copper. In these and other scenarios, therefore, GAF could confound green-fluorescing, vital-staining studies, especially in cases where the stained cells are observed and evaluated microscopically (see Fig. 1 and Table 1 for the comparison between CMFDA staining and cells stored for days or weeks after fixation). Even for cases where GAF can be quantitatively measured by flow cytometry, its intensity in fixed cells, at least for 3 weeks following fixation (Table 1), may be strong enough to smear the boundary of stained and unstained cells in cytograms. Widely used nucleic acids stains like SYTOX green (29) produce green fluorescence strong enough to overwhelm the GAF of microalgal cells; however, such stains are generally applicable for mortal staining only.

If present, GAF may provide a useful tool to visualize certain cellular components of microalgae, such as eyespots, accumulation bodies, nuclei, aerotopes, spines, and even chloroplasts. Furthermore, the intracellular absence of GAF may prove useful. For example, the location and shape of the nucleus, an important taxonomic feature of dinoflagellates, can be easily visualized by adjusting the focus (Fig. 1c and p), since the nucleus does not exhibit GAF. The absence of GAF in the plates of armored dinoflagellate species (Fig. 1u; see also the supplemental material) is also useful to visualize their cellular

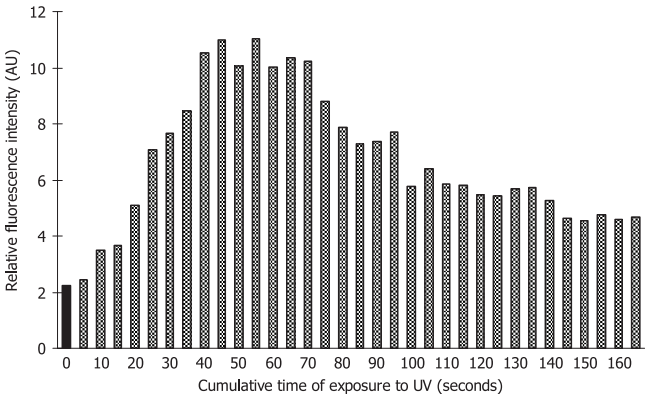

FIG. 4. For an individual cell of *G. catenatum*, the time course of additional GAF elicited by exposure to UV light (330 to 385 nm; filter cube U-MWU) is shown. Prior to all but the first measurement, the cell was exposed for 5 seconds to UV light and then to blue light (470 to 490 nm; filter cube U-N31001) while the intensity of GAF was determined. Within 1 to 2 min, the next exposure to UV light and subsequent measurement of GAF was begun. The first measurement, however, represents the relative intensity of GAF elicited by blue light alone. Compare these values with those shown in Fig. 3A. The time course shown here is representative of those seen in other cells of this species (*G. catenatum*; $n = 4$ cells) and another species (*C. hystrix*; $n = 4$ cells). AU, arbitrary units.

structures. An established use of GAF is to detect infection of phytoplankton by the parasitic dinoflagellate *Amoebophrya* sp. (7, 24, 26, 34); the parasite exhibits GAF but no chlorophyll RAF. Its use for quantifying parasites in the population, however, can be problematic, as GAF is typically weak in early infections, or even absent in some strains, or fades rapidly following fixation (24, 34).

More importantly, perhaps, GAF could be used in place of the primuline staining method (33) to visualize and enumerate dinoflagellate cysts in marine and estuarine sediments in the context of anticipating and monitoring harmful algal blooms and in tracking potentially harmful dinoflagellates transported in ships' ballast tanks (28). Since the primuline staining protocol is very time-consuming (at least 2 days) and requires chemicals, using GAF alone for the same purpose would reduce costs significantly.

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REFERENCES

1. **Anderson, D. M., Y. Fukuyo, and K. Matsuoka.** 2003. Cyst methodologies, p. 165–190. *In* G. M. Hallegraeff, D. M. Anderson, and A. D. Cembella. (ed.), Manual on harmful marine microalgae. Monographs on Oceanographic Methodology 11. UNESCO, Paris, France.
2. **Anderson, D. M., B. A. Keafer, D. J. McGillicuddy, M. J. Mickelson, K. E. Keay, P. Scott Libby, J. P. Manning, C. A. Mayo, D. K. Whittaker, J. Michael Hickey, R. Y. He, D. R. Lynch, and K. W. Smith.** 2005. Initial observations of the 2005 *Alexandrium fundyense* bloom in southern New England: general patterns and mechanisms. Deep-Sea Res. **52:**2856–2876.
3. **Anderson, D. M., C. A. Stock, B. A. Keafer, A. Bronzino Nelson, D. J. McGillicuddy, M. Keller, B. Thompson, P. A. Matrai, and J. Martin.** 2005. *Alexandrium fundyense* cyst dynamics in the Gulf of Maine. Deep-Sea Res. **52:**2522–2542.
4. **Berglund, D. L., and S. Eversman.** 1988. Flow cytometric measurement of pollutant stresses on algal cells. Cytometry **9:**150–155.
5. **Bolch, C. J. S.** 1997. The use of sodium polytungstate for the separation and concentration of living dinoflagellate cysts from marine sediments. Phycologia **36:**472–478.
6. **Carpenter, E. J., J. Chang, and L. P. Shapiro.** 1991. Green and blue fluorescing dinoflagellates in Bahamian waters. Mar. Biol. **108:**145–149.
7. **Coats, D. W., and K. R. Bockstahler.** 1994. Occurrence of the parasitic dinoflagellate *Amoebophrya ceratii* in Chesapeake Bay populations of *Gymnodinium sanguineum*. J. Eukaryot. Microbiol. **41:**586–593.
8. **Coleman, A. W.** 1988. The autofluorescent flagellum: a new phylogenetic enigma. J. Phycol. **24:**118–120.
9. **Doblin, M. A., S. I. Blackburn, and G. M. Hallegraeff.** 1999. Growth and biomass stimulation of the toxic dinoflagellate *Gymnodinium catenatum* (Graham) by dissolved organic substances. J. Exp. Mar. Biol. Ecol. **236:**33–47.
10. **Dorsey, J., C. M. Yentsch, S. Mayo, and C. McKenna.** 1989. Rapid analytical technique for the assessment of cell metabolic activity in marine microalgae. Cytometry **10:**622–628.
11. **Elbrächter, M.** 1994. Green autofluorescence: a new taxonomic feature for living dinoflagellate cysts and vegetative cells. Rev. Palaeobot. Palynol. **84:** 101–105.
12. **Franklin, D. J., and J. A. Berges.** 2004. Mortality in cultures of the dinoflagellate *Amphidinium carterae* during culture senescence and darkness. Proc. R. Soc. Lond. B **271:**2099–2107.
13. **Fujita, S., M. Iseki, S. Yoshikawa, Y. Makino, M. Watanabe, T. Motomura, H. Kawai, and A. Murakami.** 2005. Identification and characterization of a fluorescent flagellar protein from the brown alga *Scytosiphon lomentaria* (Scytosiphonales, Phaeophyceae): a flavoprotein homologous to Old Yellow enzyme. Eur. J. Phycol. **40:**159–167.
14. **Grégori, G., A. Colosimo, and M. Denis.** 2001. Phytoplankton group dynamics in the bay of Marseilles during a 2-year survey based on analytical flow cytometry. Cytometry **44:**247–256.
15. **Johnson, M. D., M. Rome, and D. K. Stoecker.** 2003. Microzooplankton grazing on *Prorocentrum minimum* and *Karlodinium micrum* in Chesapeake Bay. Limnol. Oceanogr. **48:**238–248.
16. **Kamiyama, T.** 2000. Application of a vital staining method to measure feeding rates of field ciliate assemblages on a harmful alga. Mar. Ecol. Prog. Ser. **197:**299–303.
17. **Kawai, H.** 1988. A flavin-like autofluorescent substance in the posterior flagellum of golden and brown algae. J. Phycol. **24:**114–117.
18. **Kirn, S. L., D. W. Townsend, and N. R. Pettigrew.** 2005. Suspended *Alexandrium* spp. hypnozygote cysts in the Gulf of Maine. Deep-Sea Res. **52:**2543–2559.
19. **Lage, O. M., F. Sansonetty, J. E. O'Connor, and A. M. Parente.** 2001. Flow cytometry analysis of chronic and acute toxicity of copper(II) on the marine dinoflagellate *Amphidinium carterae*. Cytometry **44:**226–235.
20. **Lessard, E. J., and E. Swift.** 1986. Dinoflagellates from the North Atlantic classified as phototrophic or heterotrophic by epifluorescence microscopy. J. Plankton Res. **8:**1209–1215.
21. **Li, A. S., D. K. Stoecker, D. W. Coats, and E. J. Adam.** 1996. Ingestion of fluorescently-labeled and phycoerythrin-containing prey by mixotrophic dinoflagellates. Aquat. Microb. Ecol. **10:**139–147.
22. **Matsuoka, K., and Y. Fukuyo.** 2000. Technical guide for modern dinoflagellate cyst study. WESTPAC-HAB/WESTPAC/IOC. WESTPAC-HAB, Tokyo, Japan.
23. **McGillicuddy, D. J., R. P. Signell, C. A. Stock, B. A. Keafer, M. D. Keller, R. D. Hetland, and D. M. Anderson.** 2003. A mechanism for offshore initiation of harmful algal blooms in the coastal Gulf of Maine. J. Plankton Res. **25:**1131–1138.
24. **Park, M. G., W. Yih, and D. W. Coats.** 2004. Parasites and phytoplankton, with special emphasis on dinoflagellate infections. J. Eukaryot. Microbiol. **51:**145–155.
25. **Pouneva, I.** 1997. Evaluation of algal culture viability and physiological state by fluorescent microscopic methods. Bulg. J. Plant Physiol. **23:**67–76.
26. **Salomon, P. S., S. Janson, and E. Granéli.** 2003. Parasitism of *Dinophysis norvegica* by *Amoebophrya* sp. in the Baltic Sea. Aquat. Microb. Ecol. **33:** 163–172.
27. **Shapiro, L. P., E. M. Haugen, and E. J. Carpenter.** 1989. Occurrence and abundance of green-fluorescing dinoflagellates in surface waters of the northwest Atlantic and northeast Pacific oceans. J. Phycol. **25:**189–191.
28. **Tang, Y. Z., and F. C. Dobbs.** 2007. Green autofluorescence of dinoflagellate cysts can be used instead of primuline staining for cyst visualization and enumeration in sediments. J. Phycol. **43:**65:–68.

29. **Veal, D. A., D. Deere, B. Ferrari, J. Piper, and P. V. Attfield.** 2000. Fluorescence staining and flow cytometry for monitoring microbial cells. J. Immunol. Methods **243:**191–210.
30. **Veldhuis, M. J. W., T. L. Cucci, and M. E. Sieracki.** 1997. Cellular DNA content of marine phytoplankton using two new fluorochromes: taxonomic and ecological implications. J. Phycol. **33:**527–541.
31. **Veldhuis, M. J. W., G. W. Kraay, and K. R. Timmermans.** 2001. Cell death in phytoplankton: correlation between changes in membrane permeability, photosynthetic activity, pigmentation and growth. Eur. J. Phycol. **36:**167–177.
32. **Vives-Rego, J., P. Lebaron, and G. Nebe-von Caron.** 2000. Current and future applications of flow cytometry in aquatic microbiology. FEMS Microbiol. Rev. **24:**429–448.
33. **Yamaguchi, M., S. Itakura, I. Imai, and Y. Ishida.** 1995. A rapid and precise technique for enumeration of resting cysts of *Alexandrium* spp. (Dinophyceae) in natural sediments. Phycologia **34:**207–214.
34. **Yih, W., and D. W. Coats.** 2000. Infection of *Gymnodinium sanguineum* by the dinoflagellate *Amoebophrya* sp.: effect of nutrient environment on parasite generation time, reproduction, and infectivity. J. Eukaryot. Microbiol. **47:**504–510.