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Autofluorescent bodies in the toxic dinoflagellate *Alexandrium tamarense*: A potential indicator of the physiological condition of the species

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Abstract — Autofluorescent bodies were examined in cultured and natural cells of *Alexandrium tamarense* (Lebour) Balech collected from Ofunato Bay, Japan. Three different autofluorescent bodies (pale blue, light blue, and white) and the autofluoresced chloroplast were observed under ultraviolet light excitation. We suggest that the pale-blue and light-blue bodies represent a novel type in dinoflagellates based on their fluorescent properties. We also found that cells with pale-blue and light-blue bodies appeared predominantly in the early and middle to late exponential phases, respectively. A similar pattern was observed in natural populations during a 2-year field survey. Cells with pale-blue bodies were dominant almost one week before the bloom, whereas those with light-blue bodies were mostly apparent during the blooming stage. These results indicate that the observation of autofluorescent bodies could be useful as a physiological indicator for predicting natural population blooms of *A. tamarense* in Ofunato Bay.

Key words: *Alexandrium tamarense*, autofluorescent body, ultraviolet light excitation, epifluorescence microscopy, growth stage

Introduction

An accumulation of paralytic shellfish poisoning (PSP) toxins in bivalves causes severe damages almost annually to the fishery industry as well as public health along the Pacific coast of northern Japan (Oshima et al. 1982). Since the PSP event in Ofunato Bay, Iwate Prefecture, in 1961 (Kawabata et al. 1962), the toxicity of shellfishes and the abundance of toxic dinoflagellates have been monitored (Ogata et al. 1982, Sekiguchi et al. 1989) along the coast of Iwate Prefecture. Our studies revealed the presence of several *Alexandrium* species in this area (Kaga et al. 2006); bivalve contamination with PSP toxins has been caused by *A. tamarense* and *A. catenella* (Kodama et al. 1982, Ogata et al. 1982, Sekiguchi et al. 2001).

Many studies have addressed countermeasures for the problems caused by PSP. Two approaches emerged for preventing or minimizing damage. One approach is to regulate marketing by monitoring shellfish toxicity. The other approach is to predict blooming events of causative dinoflagellates to reduce risk and to manage planned bivalve culture. Various methods of bloom prediction based on bloom dynamics have been proposed. One method is to study the timing of cyst germination, focusing on environmental factors such as cumulative water temperature or cyst morphology changes including the appearance of chlorophyll fluorescence (Yentsch et al. 1980, Anderson and Keafer 1985, Itakura and Yamaguchi 2001). However, this method is effective only for the prediction of bloom initiation. Many investigations study changes in environmental factors to predict bloom development (e.g., Sekiguchi et al. 1989, Yamasaki and Tomosada 2001), but applying these data for short-term prediction is difficult because measuring factors such as nutrient concentration is time-consuming. The data are usually used to understand bloom dynamics following a bloom event.

Organelles called accumulation (AC) bodies or periodic acid–Schiff (PAS) bodies have been confirmed in some species of dinoflagellates (e.g., Taylor 1968, Tomas and Cox 1973, Schmitter 1971, Schmitter and Jurkiewicz 1981, Yentsch et al. 1985). These organelles are thought to change in size or number during growth stages. For example, the AC bodies of some symbiotic dinoflagellates such as *Peridinium balticum* were shown to increase in size with cell senescence (Taylor 1968, Tomas and Cox 1973). Schmitter (1971) indicated that the occurrence of PAS bodies in *Gonyaulax polye-*

dra is probably related to factors such as cell age or growth conditions. Zhou and Fritz (1993, 1994) proposed that AC and PAS bodies in dinoflagellates might be lysosomes.

AC bodies in *A. tamarense* were also observed as a yellow autofluorescence under blue light excitation (Yentsch et al. 1985) and as a blue-white autofluorescence under ultraviolet (UV) light excitation (Kodama et al. 1996). These data suggest that the autofluorescent bodies in *A. tamarense* cells play some roles in response to environmental changes such as nutrient limitation. In this study, we observed autofluorescent bodies in cultured and natural cells of *A. tamarense*. We found that the species possesses a novel type of autofluorescent body other than lysosomes. We propose herein that autofluorescent bodies can be used as an indicator of the physiological condition of natural populations of *A. tamarense*.

Materials and Methods

Culture experiment

Three strains (F-1–C-6, F-2–A-6, and F-2–B-4) of *A. tamarense* that were isolated from Ofunato Bay during a bloom event in 2008 were used in this study. Stock cultures were routinely maintained at 15°C and 33 psu salinity in a modified-enriched f/2 medium (Guillard and Ryther 1962, Guillard 1975) without silicate. Light was provided by cool-white fluorescent lamps at a light intensity of 80 μmol m^{-2} s^{-1} with a 12-h light (8:00–20:00)/12-h dark (20:00–8:00) cycle.

For all culture experiments, cells in the middle exponential phase were inoculated into 300 ml of fresh medium with an initial cell density of about 500 cells ml^{-1}. The cells of 3 strains were cultured under the same conditions for 40 days and examined for autofluorescent bodies. During the growth process, 1-ml aliquots of each culture were pipetted into 1.5-ml Eppendorf tubes every day during the early-light period (10:00–12:00) and centrifuged at 1,200×***g*** for 10 min at room temperature (17–25°C). A subsample of 20–242 cells was randomly selected from the concentrated cell suspension and observed for autofluorescent bodies. For strain F-2–B-4, the size of autofluorescent bodies was also measured using photographs and the micrometer part of image processor software. In addition, a 1-ml subsample was collected daily and fixed with 10% formalin (1% final concentration), and cell number was counted under a light microscope to estimate cell density.

Field experiment

Field studies were conducted at Shizu station (39°02′N, 141°43′E; approximate depth of 23 m) in Ofunato Bay from May 13 to July 10, 2008 and from April 21 to July 7, 2009. Scallops are cultured at the station at an approximate depth of 10 m, where *A. tamarense* has been reported to occur most densely (Ogata et al. 1982). Therefore, water samples were collected from 10-, 12-, and 14-m depths (3 layers) using a Van Dorn sampler (Rigo, Tokyo, Japan). To estimate average cell density, a 500-ml sample from each layer was concentrated to 5 ml by filtering through an 8-μm membrane filter and fixed with 10% formalin (1% final concentration). The thecal plate morphology of *A. tamarense* cells was examined using epifluorescence microscopy under UV light excitation after calcofluor staining (Fritz and Triemer 1985). *A. tamarense* was identified according to the morphological characteristics described by Yoshida (2000).

To observe the autofluorescent bodies, a 5-l water sample collected from the middle layer (12-m depth) was filtered successively through 100- and 20-μm-mesh sieves on the boat. Samples retained on the 20-μm-mesh sieve were recovered into a 15-ml polypropylene centrifuge tube and carried to the laboratory to prevent temperature increase.

Observation of autofluorescent bodies

Autofluorescent bodies in *A. tamarense* were observed under different conditions to compare the effect of excitation wavelength. Observation under UV light excitation was conducted using an Olympus epifluorescence microscope (Olympus BX60, Olympus, Tokyo, Japan) with a BP 330- to 385-nm excitation filter, DM400 dichroic mirror, and BA 420-nm barrier filter (U-MWU filter set, Olympus, Tokyo, Japan) equipped with a 100-W mercury lamp (USH-102D, USHIO, Tokyo, Japan). For the other light excitations, filter settings included a DM455 dichroic mirror and BA 475-nm barrier filter (U-MWBV filter set, Olympus, Tokyo, Japan) for BP 400- to 440-nm (blue-violet light) excitation and a DM500 dichroic mirror and BA 515-nm barrier filter (U-MWB2 filter set, Olympus, Tokyo, Japan) for BP 450- to 480-nm (blue light) excitation.

Twenty microliters of live cell suspension was mounted on glass slides under coverslips, and the autofluorescent bodies of *A. tamarense* were observed at 400× total magnification using a 40× fluorite objective lens (UPLFLN 40×, Olympus, Tokyo, Japan). The fine adjustment knob was used to observe the cells vertically. To prevent the fluorescence from fading, exposure to UV light excitation was controlled using a 25% neutral-density filter.

To capture digital images, samples were observed at 1,000× total magnification using a 100× Apochromat objective lens (UPlan Apo 100×, Olympus, Tokyo, Japan) and were photographed under a cooled charge-coupled-device camera (DP50, Olympus, Tokyo, Japan) with 1.5% neutral-density filter.

Results

Autofluorescent bodies in cultured *Alexandrium tamarense*

Epifluorescence micrographs of representative cells under UV excitation are shown in Fig. 1A–C. In addition to the red fluorescent chloroplast, 3 kinds of spherical autofluorescent bodies were observed according to their fluorescent color: pale-blue body (PBb), light-blue body (LBb), and white body (Wb). PBb distributes contiguously to chloroplasts. The margin of this body is not clear enough to compare with other two. LBb occurs between chloroplasts in many cases. Wb is larger than both PBb and LBb and locates predominantly in the center of the cell. These 3 kinds of bodies were not observed constantly, and the major body type varied from cell to cell. Wb was found in most of the cells over the course of the culture; PBb and LBb could not be detected in all of the cells. In some cells, all 3 of the body types were found. In addition, the number and size of these bodies varied from cell to cell. These significant variations suggest the difficulty in clearly categorizing all of the cells based on their fluorescent bodies. Therefore, 3 typical cell types that were easily distinguishable from each other were used in this study. A cell with PBb and Wb was designated as type 1 (Fig. 1A), a cell with LBb and Wb was designated as type 2 (Fig. 1B), and a cell with Wb only was designated as type 3 (Fig. 1C). The composition of these 3 cell types was examined in culture and field experiments.

Figure 1D shows an optical micrograph of a cultured cell of *A. tamarense*. Epifluorescence micrographs of the same cell under different excitation lights are shown in Fig. 1E–G (Fig. 1E, UV; Fig. 1F, blue-violet; Fig. 1G, blue). Wb was observed under all excitation lights tested. LBb and PBb could not be observed under blue light excitation but were observed under UV and blue-violet lights. All of the body types could be observed under blue-violet light, but distinguishing them was difficult due to the similarity of their fluorescent color. These results indicate that observation under UV light excitation is appropriate for studying the change of each autofluorescent body in an *A. tamarense* cell.

Changes in autofluorescent bodies in cultured *Alexandrium tamarense* cells

A sigmoidal growth curve was observed for 3 clonal cultures cultivated under the previously mentioned condition (Fig. 2). The number of each cell type mentioned above was counted over the growth process. The total number of the 3 cell types examined and cell type compositions were summarized by growth stages for each strain (Table 1). Results indicate a similar change in cell type appearance among the 3 strains. Type 1 occurred predominantly in the early exponential phase, and its percentage decreased significantly when the cultures entered the middle to late exponential phase. The percentage of type 2 increased in the middle to late exponential phase and decreased to less than 3% in the stationary phase. Although the percentage of type 3 remained high (more than 65%) throughout all of the growth stages, it tended to increase in the stationary phase.

Size of Wb

Wb occurred in most of the cells throughout all of the

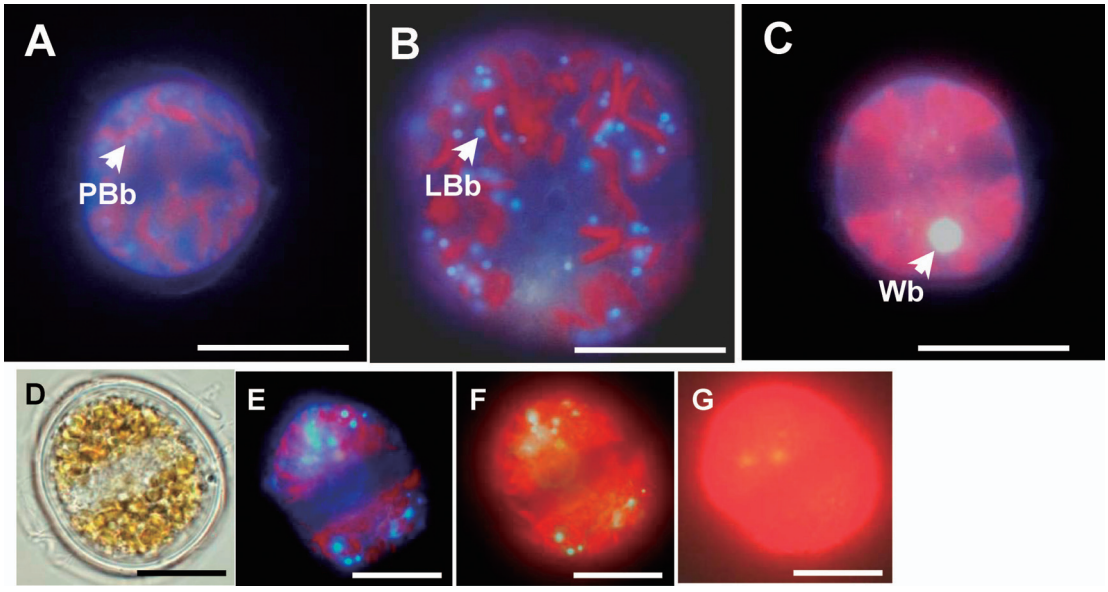

Fig. 1. Optical or epifluorescence micrographs of clonal cultured cells of *A. tamarense*. Fig. 1A–C. Typical cell type images under UV light excitation over the course of cultivation. Fig. 1A. Cell (type 1) with pale-blue fluorescent body (PBb). Fig. 1B. Cell (type 2) with light-blue body (LBb) and white body (Wb). Fig. 1C. Cell (type 3) with Wb only. Fig. 1D–G. Same cell observed under light microscopy (Fig. 1D) and epifluorescent microscopy with different excitation lights (Fig. 1E, UV; Fig. 1F, blue-violet; Fig. 1G, blue). Scale bars=20 μm.

Table 1. Total number of the 3 cell types examined and cell type compositions (mean±S.D. %) summarized by growth stages for each strain.

Strains growth stage (days)	Specimens (cells)	Type 1	Type 2	Type 3
F-1–C-6				
Early exponential phase (4–11)	462	11.6±8.0	1.1±1.6	87.3±7.7
Middle to late exponential phase (12–21)	606	1.4±1.9	7.0±6.0	91.6±6.7
Stationary phase (22–40)	1135	0.0±0.0	2.3±4.4	97.7±4.4
F-2–A-6				
Early exponential phase (3–11)	501	31.3±13.2	4.0±6.6	64.7±11.4
Middle to late exponential phase (12–26)	982	2.0±2.6	12.2±9.5	85.7±10.5
Stationary phase (27–40)	1644	2.1±2.7	1.8±3.1	96.1±3.2
F-2–B-4				
Early exponential phase (3–11)	524	6.1±7.6	5.2±9.0	88.7±8.5
Middle to late exponential phase (12–25)	967	0.1±0.4	21.1±16.2	78.8±16.1
Stationary phase (26–40)	1042	0.1±0.3	0.9±2.2	99.1±2.5

mean±S.D.=mean±Standard Deviation

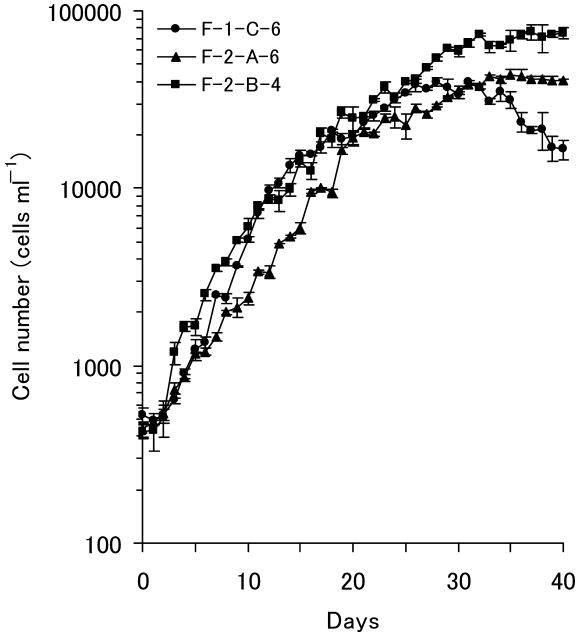

Fig. 2. Growth of 3 different strains of *A. tamarense*. Values are means±SD.

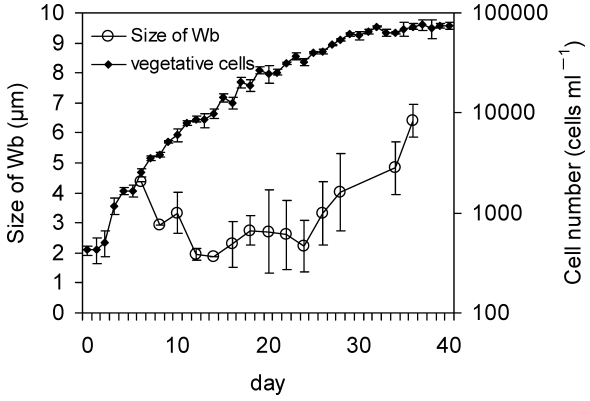

Fig. 3. Changes in Wb size and cell density in F-2–B-4 strain over the course of cultivation.

growth stages (Table1). The diameters of Wb were measured for strain F-2–B-4 every 2 days during the culture experiments. As shown in Fig. 3, the size of Wb in the exponential phase was estimated to be 2–11(6.4±2.4) μm and increased to 5–18(11.5±3.6) μm in the stationary phase ($p<0.01$, *t*-test). Thus, the size of Wb was confirmed to be larger in the stationary or senescent phase than in the exponential phase.

Changes in autofluorescent bodies in natural populations

A typical micrograph, taken under UV excitation, of a concentrated plankton sample collected from Ofunato Bay is shown in Fig. 4. All of the live *Alexandrium* cells contained some of the autofluorescent bodies (PBb, LBb, and Wb). *Alexandrium* observed during the field survey was identified as *A. tamarense* based on morphological characteristics (Yoshida 2000).

The variation in cell density and cell type composition in natural populations of *A. tamarense* at the Ofunato Bay station in 2008 and 2009 are shown in Fig. 5. Cell density is represented by the average cell number per liter observed at 3 layers (10, 12, and 14 m).

In 2008, a small population of *A. tamarense* already was present on May 13 when the survey started. The abundance of *A. tamarense* increased gradually to June 10, subsequently showed a sharp increase, and reached a maximum (approximately 7,000 cells l^{-1}) on June 17. After the peak, the bloom disappeared in 2 weeks. In 2009, the bloom appeared almost 1 month earlier than in 2008 and with a much lower maximum density (approximately 600 cells l^{-1}).

As shown in Fig. 5, cell type composition varied during the blooming process in both years. Type 2 was predominant 3 weeks before the start of a remarkable bloom (May 13,

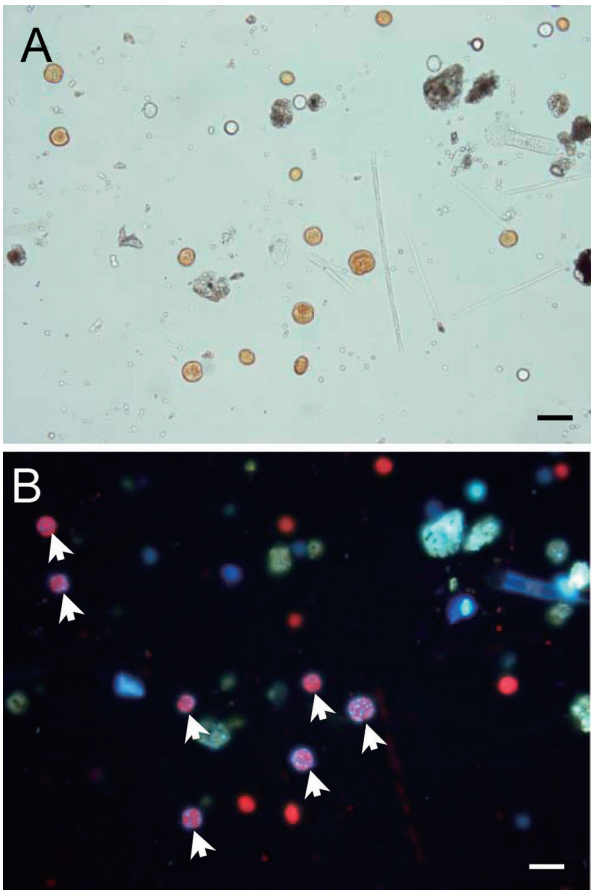

Fig. 4. Optical and epifluorescence micrographs, taken under UV excitation, of concentrated plankton sample collected from Ofunato Bay on May 26, 2009. (A) optical micrograph; (B) epifluorescence micrograph. White arrows indicate *A. tamarense* cells. Scale bar=50 μm.

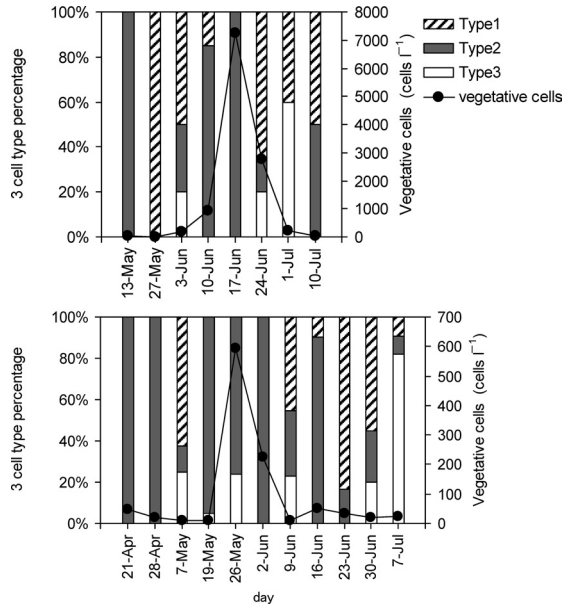

Fig. 5. Changes in cell density of vegetative cell and cell type percentages in natural populations of *A. tamarense* at the Ofunato Bay station in 2008 (A) and 2009 (B); right axis, average cell density in water column (10, 12, and 14 m).

2008 and April 28, 2009). Cells with a high percentage of type 1 appeared almost 1 week before bloom initiation (May 27, 2008 and May 7, 2009). The major cell type changed from type 1 to type 2 when significant proliferation began. Type 2 remained dominant (more than 75% in both years) until the bloom reached a peak. As the bloom declined after the peak, cell type fluctuated widely and composition changed. A relatively high percentage of type 3 was observed during these periods.

Discussion

Three autofluorescent body types were found in both cultured and natural populations of *A. tamarense* cells in this study. Of these types, Wb emits white or yellow fluorescence under UV or blue-light excitation, respectively. Wb size tended to increase in the stationary or senescent phase compared to the exponential phase. These properties coincide with those of the body referred to as an AC body (Yentsch et al. 1985) or PAS body (Schmitter and Jurkiewicz 1981). Yentsch et al. (1985) reported that the AC body in *A. tamarense* appears larger because of cell aging. Zhou and Fritz (1994) referred to the body as a dinoflagellate lysosome. According to their findings, Wb in this study might function in the degradation and reuse of cell constituents for survival under nutrient-limited conditions.

The fluorescent properties of the other autofluorescent body types, PBb and LBb, are clearly different from those of Wb. In contrast to Wb, PBb and LBb could not be detected under blue-light excitation and disappeared when the culture entered the stationary phase. As far as we know, there are no reports describing this kind of body in dinoflagellate cells. As mentioned above, these bodies are undetectable under blue-light excitation, which may be a reason why they were not previously discovered.

PBb and LBb showed similar characteristics, but we tentatively categorized them on the basis of fluorescence intensity and clearness of the body margin. They emit a similar fluorescent color and locate between chloroplasts. In addition, LBb tended to appear after PBb disappeared during the middle to late exponential phase in culture. These findings imply that the LBb and PBb are essentially the same, and that their fluorescent color is altered from pale to light blue during active cell growth.

As described above, a relatively high percentage of type 1 and type 2 appeared in the early exponential phase and middle to late exponential phase, respectively, in culture experiments. Type 1 could not be detected in the stationary or senescent phase. The percentage of type 3 increased during those growth stages. These results indicate that cell type composition based on morphology of the autofluorescent body could be applicable as an indicator of *A. tamarense*

growth stage or physiological condition. Although measurement is time-consuming, a change in body size of Wb also could be used as a supplementary indicator of the stationary or senescent phase.

In both the years (2008 and 2009), natural populations of *A. tamarense* exhibited a similar pattern of changes in the cell type composition during the process from the pre-blooming stage to the peak of the bloom. A high percentage of type 2 appeared during preblooming and growing periods. Type 1 dominated 1 week before bloom initiation. Although some discrepancies were noted between natural and cultured cells, i.e., the percentage of type 3 was much lower in natural cells than in cultured cells, the pattern of change in type 1 and type 2 was similar in both environments. Based on the results of this study, the following observations could be used to predict an *A. tamarense* bloom, at least in Ofunato Bay:

1. Type 1 appears in a high percentage almost 1 week before a bloom initiation.
2. The dominant type changes from type 1 to type 2 during the growing stage of a population.

Our results suggest that autofluorescent bodies of *A. tamarense* may provide real-time information on physiological condition. Observation can be completed in a short time and does not require a high degree of skill or experience. Thus, this method easily could be introduced at the site of an *A. tamarense* outbreak. To establish a more precise prediction method, further field investigations, as well as investigations into the function of autofluorescent bodies, are needed.

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