Promotion of cyst formation in the toxic dinoflagellate *Alexandrium* (Dinophyceae) by natural bacterial assemblages from Hiroshima Bay, Japan

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ABSTRACT: The relationship between the abundance of the toxic marine dinoflagellate *Alexandrium tamarense* (Lebour) Balech and *Alexandrium*-cyst-formation-promoting bacteria (Alex-CFPB) was investigated in the water column of Hiroshima Bay (Japan) from 1997 to 1998. Cell density of *A. tamarense* increased gradually from February to the middle of April, then peaked at the end of April and blooms declined rapidly in the beginning of May in both years. All seawater fractions collected from 5 m depth, where the density of *A. tamarense* cells was highest and which also contained the bulk of planktonic bacteria, promoted cyst formation of *A. catenella* (Whedon and Kofoid) Balech. This promotion was not caused by effects from nutrient limitation. The number of Alex-CFPB in seawater samples, analyzed by means of the most probable number (MPN) method, increased from the beginning of the *Alexandrium* bloom and reached 3.60×10^3 and 1.00×10^3 cells ml^{-1} at the peak bloom period at the end of April in 1997 and 1998, respectively. As the blooms declined, the number of Alex-CFPB decreased rapidly to less than 10 cells ml^{-1}. *Alexandrium*-cyst-formation-inhibiting bacteria (Alex-CFIB) were not detected. These results show a clear positive correlation between the abundance of *A. tamarense* and Alex-CFPB during blooms, which suggests that Alex-CFPB play a significant role in the process of encystment and bloom dynamics of *Alexandrium* in the field.

KEY WORDS: *Alexandrium* · Dinoflagellate · Bacteria · Cyst

INTRODUCTION

The toxic dinoflagellates *Alexandrium catenella* and *A. tamarense* commonly occur in temperate coastal waters throughout the world, resulting in recurrent outbreaks of Paralytic Shellfish Poisoning (PSP) (Hallegraeff 1993, Shumway 1993). Hiroshima Bay, a highly eutrophic, semi-enclosed embayment and a major shellfish culture area, is characterized by seasonal blooms of *A. tamarense* and shellfish toxicity is a recurrent annual problem.

The life cycles of these organisms are extremely important for their ability to form blooms. The dominant mode of reproduction is by simple asexual fission, but a transition to dioecious sexual reproduction can occur whereby motile mating types, plus and minus, which fuse to produce a motile diploid zygote (planozygote) are formed (Turpin et al. 1978, Yoshimatsu 1981). Planozygotes swim for several days before losing motility and becoming thick-walled resting cysts (hypnozygotes) (Pfiester & Anderson 1987). When favorable growth conditions return, cysts germinate and reinoculate the water with vegetative swimming cells (Steidinger 1975, Dale 1977, Anderson & Wall 1978). The roles suggested for these cysts include 'seed populations' for bloom initiation, short- and long-term survival through adverse conditions, species dispersal and preservation of genetic variation (Pfiester & Anderson 1987).

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In laboratory cultures, sexuality in dinoflagellates has been reported to occur in response to stress following nitrogen and/or phosphorus depletion (Pfiester & Anderson 1987). Environmental factors such as day length, temperature, light intensity, and dissolved gases have also been suggested as possible causes of sexuality (Pfiester & Anderson 1987). Anderson et al. (1984) examined the total cyst yield of *Alexandrium tamarense* in response to variations in temperature, light and initial nutrient availability in batch cultures, with nitrogen (nitrate/nitrite and ammonium) and/or phosphorus depletion being found to enhance encystment. Life cycle stages and the sizes of intracellular and extracellular pools of limiting nutrients (phosphorus) during *A. tamarense* encystment in batch culture were also investigated, which clarified that sexuality was induced as cellular phosphorus decreased to a subsistence cell quota (Anderson & Lindquist 1985). These laboratory culture experiments strongly suggest that sexuality of *A. tamarense* is induced by nutrient depletion.

There exist, however, field reports of encystment under seemingly favorable nutrient conditions for growth. Anderson & Morel (1979), Anderson et al. (1983) and Perez et al. (1998) reported cyst formation for natural populations of *Alexandrium tamarense* in the presence of relatively high levels of nitrate and phosphate which were able to support vegetative growth. Despite the emphasis on nutrient depletion in experimental studies, the precise set of environmental cues that trigger/stimulate encystment in the field has not been clearly defined. Considering the importance of life cycle phenomena, the principal objective of this study was to examine the factors inducing/promoting sexuality and encystment in natural toxic *Alexandrium* populations.

Although several studies on factors controlling encystment of *Alexandrium* have considered physical (water temperature and light intensity) (Anderson et al. 1984) and/or chemical aspects (inorganic nutrients or micro nutrients; e.g. iron) (Anderson et al. 1984, Anderson & Lindquist 1985, Doucette et al. 1989), no information regarding biological factors has been reported to date. Recently, close relationships between microalgae and bacteria have been observed under laboratory conditions, which suggest that bacterial assemblages play significant roles in the succession of marine phytoplankton communities, especially in the development and disintegration of blooms (Riquelme et al. 1987, Fukami et al. 1991, Yoshinaga et al. 1995). In the present study, we have undertaken an investigation of bacterial effects on cyst formation in natural toxic *Alexandrium* populations in Hiroshima Bay (Japan) during 1997 to 1998. Relationships between the population dynamics of toxic *Alexandrium* and the abundance of the bacteria that affect encystment are also examined.

MATERIALS AND METHODS

Organisms and culture methods. All the experiments were conducted with clonal toxic isolate 6ax (tentatively mating type plus) and TNY7 (mating type minus) of *Alexandrium catenella*, which were originally established by R. Kondo in 1995 from Uchiumi, Naruto, Japan and by Y. Sako in 1987 from Tanabe Bay, Wakayama, Japan, respectively. We re-isolated these isolates axenically using the micropipette washing and dilution method described by Sako et al. (1990). Sterility of each culture was periodically determined by culture tests using a liquid peptone medium, as well as by direct bacteria observations with DAPI (4'6-diamidino-2-phenylindole) staining and epifluorescence microscopy as described by Imai & Yamaguchi (1994). These cultures were grown in f/2 with no added silicate (f/2–Si) using 10^{-5} M Fe-EDTA as an iron source and chelator (Guillard & Ryther 1962, Anderson et al. 1984). Media preparation included precautions to avoid precipitation or chemical contamination. All glassware was routinely washed with detergent and rinsed thoroughly, then soaked for several days in 2N HCl and rinsed again with deionized, distilled water. Cultures were grown at 20°C with a 14:10 h light:dark cycle at 80 µmol photons m^{-2} s^{-1}, which was measured by Light Meter Model LI-250 (Li-Cor).

Field measurements and sampling. Sampling was conducted from January 1997 to June 1998 once a month during non-bloom periods or once a week during bloom periods, at Stn 11, a shallow coastal site in Hiroshima Bay, western Seto Inland Sea, Japan (Fig. 1). Water samples for *Alexandrium tamarense* cell counts were taken with a Niskin sampling bottle (General Oceanics Inc.) from 0, 2, 5, 10, 20, and 1 m above the bottom at Stn 11 (depth ca 22 m). Water temperature and salinity were measured using a temperature-salinity bridge (Model 602, Yeo-Kal), at the same depth and time water samples were collected. The number of vegetative cells of *A. tamarense* in 1 ml of each sample concentrated from 500 to 10 ml were counted under a light microscope on the day of sampling.

One liter of subsamples collected from the 5 m depth layer at Stn 11 was poured into acid-washed, autoclaved glass bottles and kept at 4°C in the dark during transit to the laboratory for analysis of any bacterial effect on encystment. Nutrient samples were filtered through glass-fiber filters (GF/C) as well as 0.2 µm cellulose acetate filters DISMIC-25cs (Advantec Inc.) and poured into 100 ml acid-washed vials in the field and
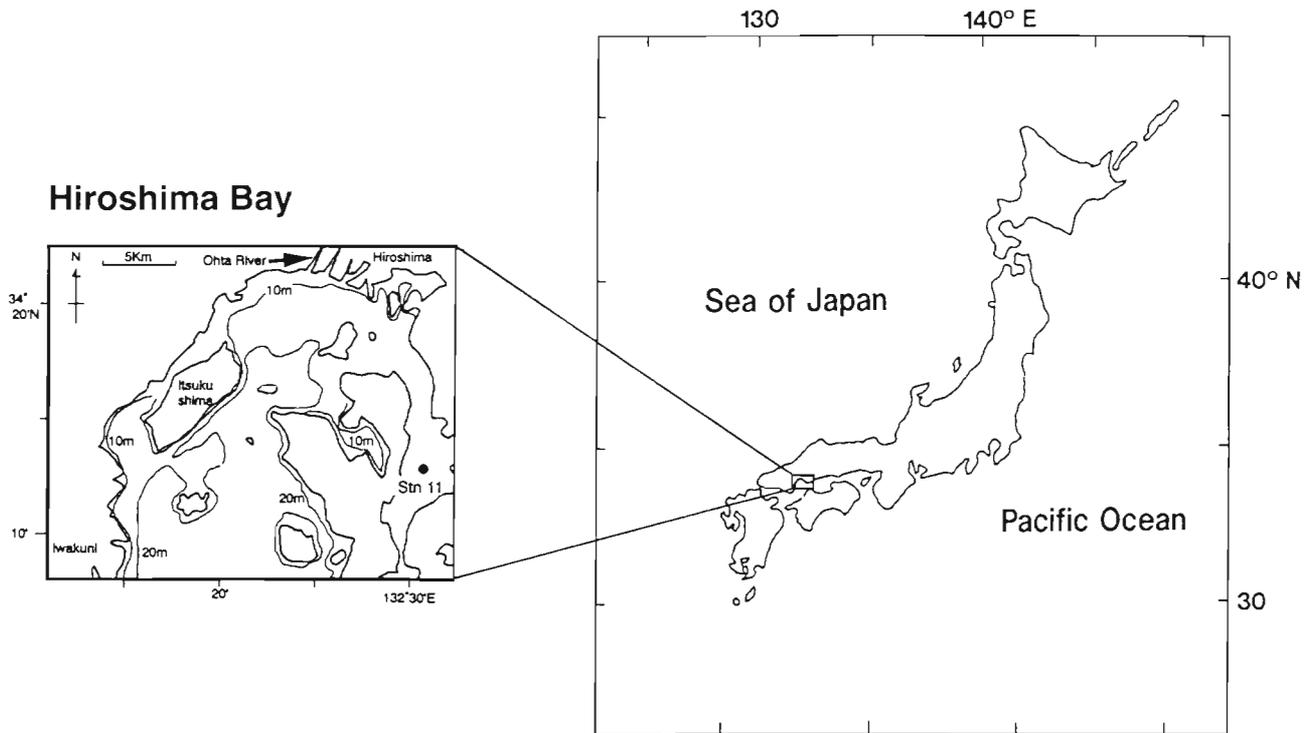
Fig. 1. Location of sampling station (Stn 11) in Hiroshima Bay, Japan

kept at 4°C until they could be frozen at –20°C. Reactive phosphate, nitrate/nitrite and ammonium concentration of collected seawater samples were determined by means of a TRAACS-800™ autoanalyzer (Bran Luebbe) according to the recommendations of the manufacturer. One hundred ml subsamples for total bacterial counts were fixed in the field with neutralized formaldehyde (final conc. 2%). The total bacterial number in seawater samples was determined by direct microscopic observations with DAPI staining (Porter & Feig 1980).

Small-scale bioassay. Fig. 2 shows the procedure of the small-scale bioassay for the detection and tentative enumeration of bacteria in seawater samples collected from the 5 m depth layer at Stn 11 from 1997 to 1998 promoting or inhibiting cyst formation in *Alexandrium*. Since isolates of *A. tamarense* from Hiroshima Bay did not form cysts when they were mated with each other, we used strains of *A. catenella* for bioassays, which readily form more cysts, have a close phylogenetic relationship with *A. tamarense* (Adachi et al. 1996), and form few cysts when mated with *A. tamarense* (Sako et al. 1990). Each seawater sample was first filtered through a glass-fiber filter (GF/F) and then through a 0.8 μm Millipore or 0.2 μm Nuclepore filter. The 0.8 μm filtrate was considered as the fraction containing the bulk of the planktonic bacteria (BF) and the 0.2 μm filtrate as the 'bacteria-free' fraction (BFF). The

BF filtrate was diluted serially from 10^{-1} to 10^{-5} with sterilized BFF. BFF, BF, serially diluted BF and f/2–Si medium (0.5 ml) were each inoculated into 16 wells of 48-well disposable sterilized tissue culture microplates (Iwaki Co.), respectively. In parallel, cultures of 6ax and TNY7 in the mid-exponential growth phase were adjusted to a concentration of 5.0×10^3 cells ml^{-1} with sterile f/2–Si medium, respectively and mixed together. One ml of the mixed cell suspension was inoculated into each of the 48 microwells and was mixed gently and incubated at 20°C with a 14:10 h light:dark cycle at 80 μmol photons m^{-2} s^{-1}. The inoculated microplates were tightly sealed with Parafilm to prevent evaporation. Two wk or 1 mo after the start of the incubation, the number of whole cysts formed in each well were counted under an inverted microscope IX-FLA (Olympus). Cyst yields in these fractions from April 21, 1997, were statistically compared by 1-way ANOVA (Sokal & Rohlf 1995). The wells in which the cyst number was more than 3 times and less than one-third of the mean cyst number formed in the bacteria-free wells (n = 18) were regarded tentatively as *Alexandrium*-cyst-formation-promoting and -cyst-formation-inhibiting bacteria (Alex-CFPB and Alex-CFIB) positive, respectively. Most probable number (MPN) values of Alex-CFPB and Alex-CFIB in the seawater samples were calculated from the numbers of Alex-CFPB- and Alex-CFIB- positive wells using the
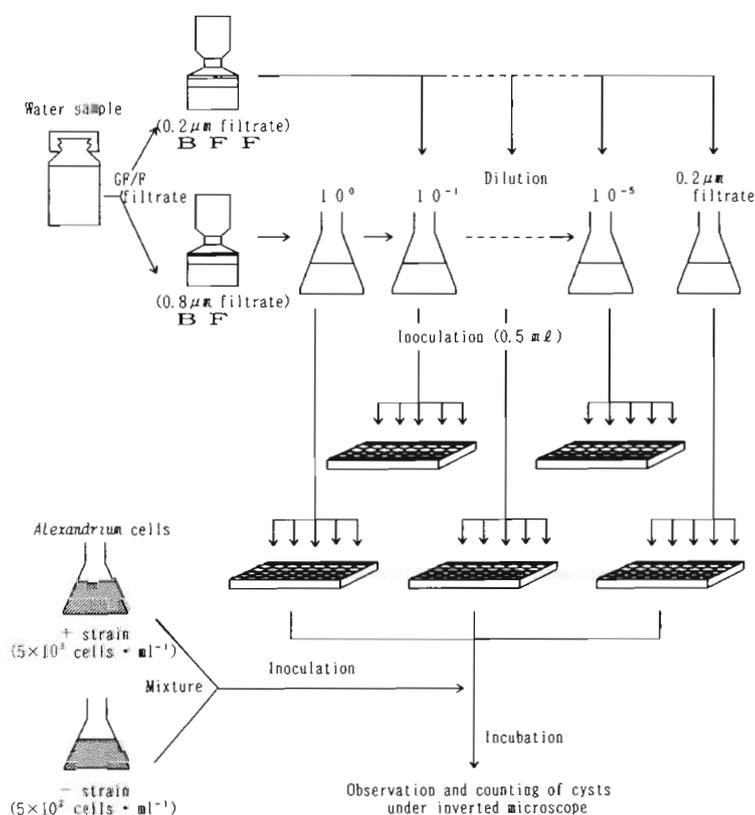
Fig. 2. Experimental procedure of the small-scale bioassay for detecting *Alexandrium*-cyst-formation-promoting and -cyst-formation-inhibiting bacteria

method of Imai et al. (1998). For the determination of excystment, cysts formed in the MPN wells were counted, stored at 5°C in the dark for 1 mo and then incubated at 20°C for 1 mo according to the cyst maturation method described by Sako et al. (1992). After incubation, cysts were recounted. Excystment efficiency was obtained as the ratio of cyst number before cold treatment:the germinated cyst number 1 mo after the start of the incubation. At least 600 cysts formed in 3 wells (total 1800 cysts) were counted for excystment efficiency.

Large-scale bioassay. A multi-flask approach was used to obtain more detailed information on the time course of encystment using field samples taken from February 9 to May 21, 1998. Twenty ml of BF or BFF from each seawater sample was poured into 15 sterile flasks of 100 ml for each fraction. Twenty ml of each culture of *Alexandrium catenella* 6ax and TNY7 at the mid-exponential growth phase, adjusted to a concentration of 5.0×10^3 cells ml^{-1} with sterile f/2–Si medium, was then added to the 30 flasks, mixed gently, and incubated for 2 wk or 1 mo under the conditions described before. During the incubation period, the

growth of *Alexandrium* cells and total bacteria in triplicate flasks of each fraction were monitored once in 3 d by the method described before. After incubation for 0, 4, 7, 14 and 30 d, external phosphate, nitrate/nitrite and ammonium concentrations in 3 separate flasks for each incubation were determined. After 2 wk or 1 mo, the bottoms of the triplicate flasks were agitated with a rubber scraper. After scraping, 12 ml aliquots of each culture were poured into 3 plastic disposable centrifugation tubes (15 ml, Iwaki Co.), respectively, and then centrifuged at $1000 \times g$ for 10 min. After 7 ml of supernatant was removed, the residue was sonicated with Urtrasonic Disruptor (Model UR-200P, Tomy Seiko Co.) at the maximum output (output 11) for 1 min to destroy most vegetative cells and centrifuged again at $1000 \times g$ for 10 min. After 4 ml of supernatant was removed, cysts were resuspended and transferred into a well of a 48-well tissue culture plate. All the cysts in a well were counted under an inverted microscope as described before. Counts with and without sonication indicated that no cysts were destroyed by this process. Encystment efficiency was obtained as the cyst:peak cell concentration ratio (Anderson et al. 1984).

Bioassay for enumeration of Alex-GPB and Alex-GIB. The numbers of *Alexandrium*-growth-promoting bacteria (Alex-GPB) and -growth-inhibiting bacteria (Alex-GIB) were determined by the MPN bioassay reported by Yoshinaga et al. (1995) in order to clarify the relationships between fluctuation of Alex-CFPB and fluctuations of Alex-GPB and Alex-GIB. A total of 1.5 ml of BF (10^0 to 10^{-5}) or BFF from field seawater samples collected from March 24 to May 21, 1998, was poured into 5 sterile MPN tubes (13 mm) for each fraction. Three ml of *Alexandrium catenella* 6ax on the mid-exponential growth phase, adjusted to a concentration of 5.00×10^3 cells ml^{-1} with sterile f/2–Si medium, was then added to the 35 MPN tubes, mixed gently, and incubated for 2 wk under the conditions described before. During the incubation period, the growth of *Alexandrium* cells in 5 replicates of each fraction were monitored every 5 d by *in vivo* autofluorescence excited by blue-light, using a Turner Fluorometer Model 10-AU (Turner Designs Inc.). After 2 wk incubation, the MPN tubes in which the algal growth was less than one-third and more than triple of that in the BFF control cultures were regarded as 'Alex-GIB-positive' and 'Alex-GPB-positive', respectively. MPN values of Alex-GIB and Alex-GPB in the seawater samples were determined by a series of numbers for these 'positive' tubes by the method described by Yoshinaga et al. (1995).

RESULTS

Alexandrium tamarense blooms and environmental conditions. Seasonal trends in water temperature at around 5 m depth at Stn 11 each year were similar, with a continuous increase from ca 10°C in February to the annual maximum of 25 to 26°C in August and a steady decrease after September (Fig. 3C). *Alexandrium tamarense* were first observed at a seawater temperature of 10°C in February 1997 and 1998 and peaked around 15°C at the end of April (Fig. 3A). Maximum densities of 9.33 and 2.50×10^4 cells l^{-1} occurred at the 5 m depth layer on April 21, 1997, and April 23, 1998, respectively. Blooms rapidly declined in the beginning of May in both years. Since *A. tamarense* was densely distributed at around 5 m depth during the peak period of blooms (data not shown), samples for estimating bacterial effects were collected from 5 m depth. Salinity ranged between a high of >33 in late winter and a low of 28 in July 1997 (Fig. 3C). Nutrient concentrations remained relatively high during the bloom initiation periods (February to beginning of March 1997 and 1998), decreased during the early bloom development periods (beginning of April 1997 and end of March 1998), and then became constant at a low level until the beginning of summer (Fig. 3D, E, F). The total number of bacteria fluctuated little (1.06 to 2.86×10^6 cells ml^{-1}, mean $2.16 \times 10^6 \pm$ SD: 4.69×10^5 cells ml^{-1}) over the observed period (Fig. 3B).

Bacterial effects on cyst yield. Fig. 4 shows the results of the small-scale bioassay (Fig. 2) using field seawater sampled on April 21, 1997, the period of *Alexandrium tamarense* bloom. After 1 mo of incubation, 822 (SD = 187) cysts well^{-1} (n = 16) were formed in the 0.8 µm filtrate seawater (bacteria fractions [BF]) wells, whereas fewer cysts (139 [SD = 91.0] cysts well^{-1}, n = 18) were obtained in the bacteria-free fractions (BFF: 0.2 µm filtrates) wells (Fig. 4) and a significant difference in cyst yield was found (*t*-test; $t = 12.7$, $p < 0.001$). Cyst formation declined with decreased bacterial inoculum (BF 10^{-1} to 10^{-5}); the BF 10^{-5} concentration approximated that of the BFF medium (Fig. 4). The *F*-value of the 1-way ANOVA ($F_{6,117}$) was 55.6 (p < 0.001). Some cysts (95.8 [SD = 49.3] cysts well^{-1}, n = 18) were formed in the wells with f/2–Si media (Fig. 4). Most bacterial fractions of other seawater samples collected from 1997 to 1998 (data not shown) also promoted cyst formation of *A. tamarense* as shown in Fig. 4.
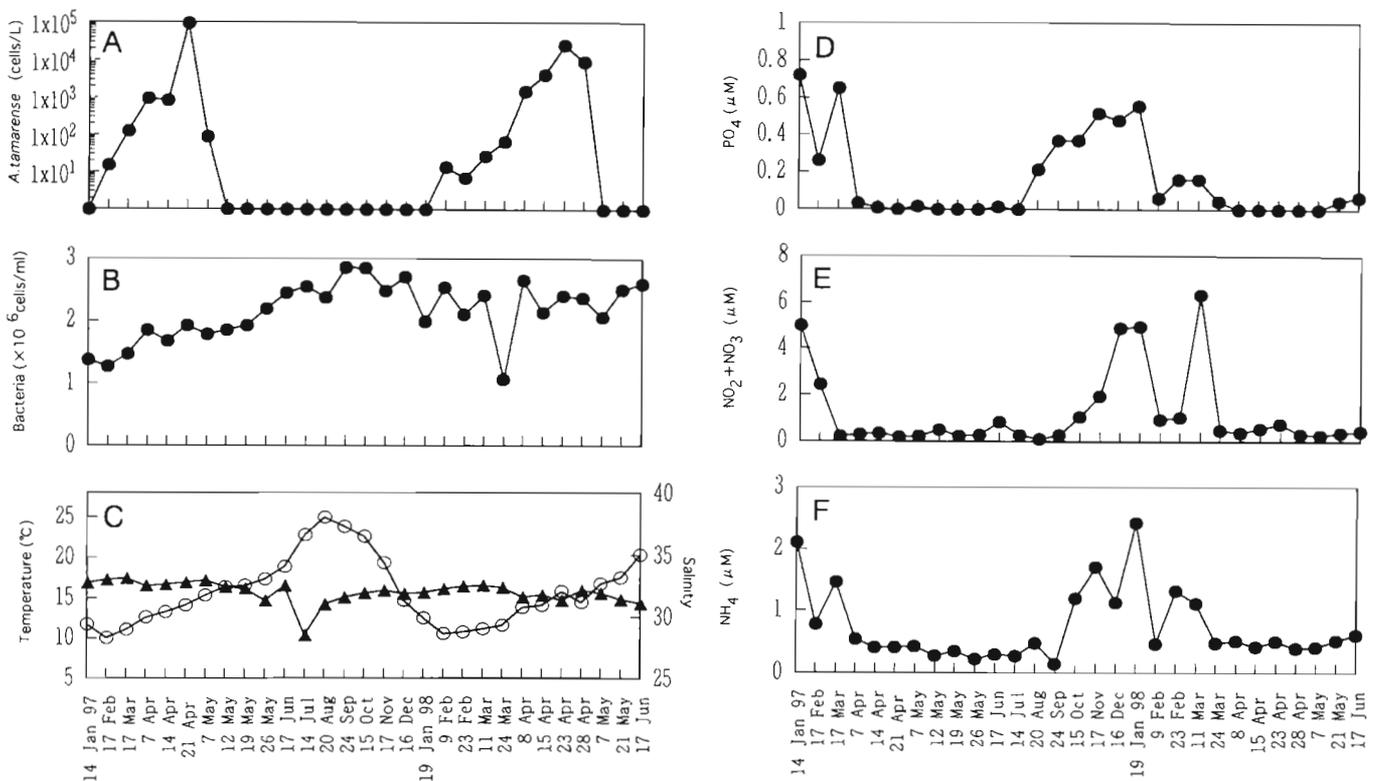

Fig. 3. Seasonal changes of (A) *Alexandrium tamarense* cell density, (B) total bacteria, (C) temperature (O) and salinity (▲), (D) inorganic nutrient phosphate, (E) nitrate/nitrite, and (F) ammonium at 5 m depth at Stn 11 from 1997 to 1998
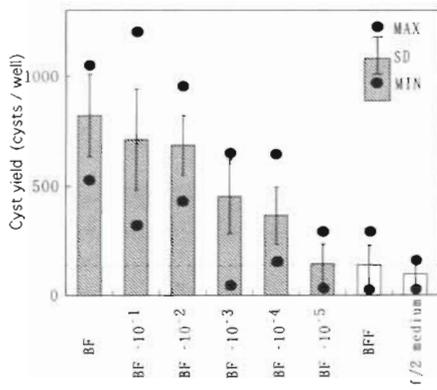
Fig. 4. Effects of bacterial assemblages in serially diluted seawater fractions collected from Hiroshima Bay on April 21, 1997, on cyst formation in *Alexandrium catenella* in BF (n = 16) and other fractions (n = 18) analyzed by the small-scale bioassay (see 'Materials and methods'). *F*-value of the 1-way ANOVA ($F_{6, 117}$) was 55.6 (p < 0.001). (●) Maximum cyst number formed in each fraction (upper) and minimum cyst number in each fraction (lower)

Bacterial effects on encystment efficiency.
When the large-scale bioassay was conducted using BF and BFF sampled during the period of bloom initiation (February 9) and during the bloom (April 28) in 1998, *Alexandrium catenella* cells grew almost similarly in flasks containing BF or BFF samples of these periods and the peak algal cell density of the BF/ BFF was 2.90 (SD = 0.345) × 10^4 cells ml^{-1}/2.92 (SD = 0.181) × 10^4 cells ml^{-1} (ratio: 99.3 % on February 9) and 3.28 (SD = 0.105) × 10^4 cells ml^{-1}/3.67 (SD = 0.346) × 10^4 cells ml^{-1} (ratio: 89.3 % on April 28), respectively (Fig. 5A,F, Table 1). In flasks with bacteria from February 9 and with bacteria from April 28, total bacteria increased from 5.40 × 10^5 to 5.62 (SD = 0.340) × 10^7 cells ml^{-1} and from 7.92 × 10^5 to 6.80 (SD = 0.568) × 10^7 cells ml^{-1} during the 1 mo incubation, respectively (Fig. 5B,G). The number was almost 30 times the mean total number of bacteria in the field seawater (2.16 × 10^6 cells ml^{-1}) (Fig. 3B). The phosphate concentration in flasks containing seawater of February 9 and of April 28, 1998 decreased similarly from 18.6 to 0.77 µM (BF)/0.95 µM (BFF) and from 18.1 to 0.68 µM (BF)/0.86 µM (BFF), respectively (Fig. 5C,H). Their nitrate/nitrite concentration similarly decreased from 5.37 × 10^2 µM to 3.94 µM (BF)/4.87 µM (BFF) and from 5.40 × 10^2 µM to 6.44 µM (BF)/6.93 µM (BFF), respectively (Fig. 5D,I). The concentration of ammonium in the flasks added with BF sampled on February 9, 1998 was less than that in the BFF flasks throughout the incubation period (Fig. 5E). In the case of seawater of
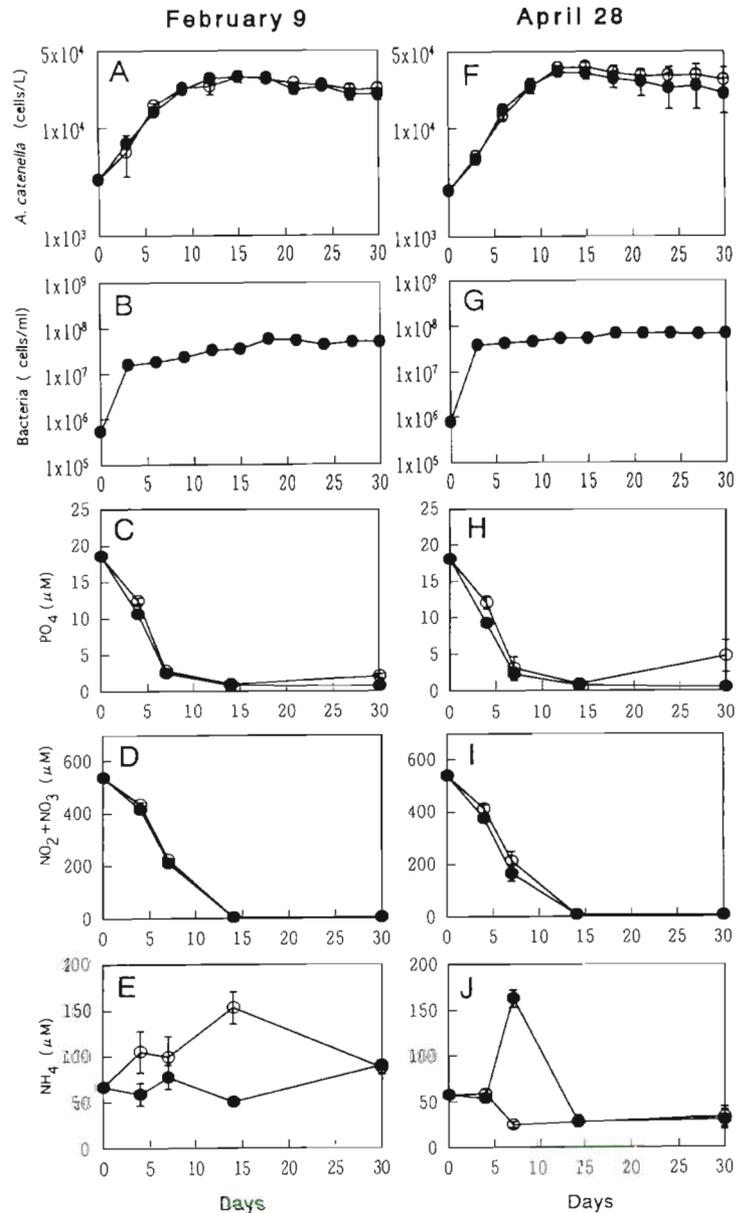

Fig. 5. Time-course measurements of (A,F) *Alexandrium catenella* density, (B,G) total bacteria, and (C,H) inorganic nutrient phosphate, (D,I) nitrate/nitrite and (E,J) ammonium in the large-scale bioassay cultures (see 'Material and methods') using BF and BFF of the field seawater of February 9 (bloom initiation period) (A to E) and of April 28 (bloom peak) (F to J), 1998. (O) BFF; (●) BF

April 28, ammonium concentration in BF was higher than that in BFF flasks after 1 wk of incubation (Fig. 5J). The cyst numbers counted after 2 wk and after 1 mo of incubation were almost the same (data not shown). The cyst yield in flasks containing BF and BFF from February 9, 1998, were 1.93 (SD = 1.05) cysts ml^{-1} (BF) and 2.30 (SD = 1.70 × 10^{-1}) cysts ml^{-1} (BFF),

Table 1. Peak number of cells, cyst yields and encystment efficiency in cultures of the large-scale bioassay using bacterial (BF) and bacteria-free fractions (BFF) of field seawater sampled on February 9 and April 28, 1998 (ns: not significant, ***p < 0.001)

Seawater	Fraction	Maximum cell yield ± SD (cells ml^{-1})	Cyst yield ±SD (cysts ml^{-1})	Encystment efficiency[a] (cysts cell^{-1})
February 9	BF	$2.90 \pm 0.345 \times 10^4$ ns	1.93 ± 1.05 ns	6.64×10^{-5}
	BFF	$2.92 \pm 0.181 \times 10^4$ ns	2.30 ± 0.170 ns	7.86×10^{-5}
	BF/BFF (%)	99.3	83.9	84.5
April 28	BF	$3.28 \pm 0.105 \times 10^4$ ns	15.7 ± 0.538***	4.79×10^{-4}
	BFF	$3.67 \pm 0.346 \times 10^4$ ns	2.59 ± 1.22***	7.05×10^{-5}
	BF/BFF (%)	89.3	606	679

[a]Encystment efficiency: cyst yield/maximum cell yield

respectively (Table 1). The encystment efficiency (the cyst yield: maximum cell ratio) in flasks containing BF and BFF from February 9, 1998, were 6.64×10^{-5} cysts cell^{-1} (BF) and 7.86×10^{-5} cysts cell^{-1} (BFF), respectively (Table 1). The cyst yield in flasks containing BF and BFF from April 28, 1998, were 15.7 (SD = 5.38 × 10^{-1}) cysts ml^{-1} (BF) and 2.59 (SD = 1.22) cysts ml^{-1} (BFF), respectively (Table 1). The encystment efficiency in flasks containing BF and BFF from April 28, 1998, were 4.79×10^{-4} cysts cell^{-1} (BF) and 7.05×10^{-5} cysts cell^{-1} (BFF), respectively (Table 1). These results show that in the seawater sampled in the bloom initiation period, the encystment efficiency under the BF conditions was almost the same as that under the BFF condition, whereas the encystment efficiency under the BF conditions was almost 7 times that under BFF conditions using seawater sampled during the blooming period (Table 1, Fig. 6B), although the nutrient level was not different between the 2 series of the large-scale experiments (Fig. 5). The ratio of encystment efficiency under the BF conditions and that under the BFF conditions increased during the bloom initiation period (Fig. 6B).

Relationships between abundance of *Alexandrium tamarense* and fluctuation of CFPB. The number of *Alexandrium*-cyst-formation-promoting bacteria (Alex-CFPB) in the seawater samples determined by the small-scale MPN bioassay increased from the beginning of the *A. tamarense* bloom on February 17, 1997, and reached 3.60×10^3 cells ml^{-1} at the peak bloom period on April 21, 1997 (Fig. 6A). As the bloom declined at the beginning of May, Alex-CFPB decreased rapidly and dropped to less than 1 cell ml^{-1} on May 12, 1997 (Fig. 6A). After the bloom in 1997, the next peak of Alex-CFPB was observed on June 17, 1997, when dinoflagellate cells were not detected, and then Alex-CFPB fluctuated from 1.56×10^0 to 1.58×10^3 cells ml^{-1}. Alex-CFPB dropped to less than 1 cell ml^{-1} by the initiation of the bloom on February 23, 1998, then increased

and reached 1.00×10^3 cells ml^{-1} by the end of April 1998 when the peak of *A. tamarense* was observed. As the bloom decayed, the number of Alex-CFPB decreased to about 10 cells ml^{-1} (Fig. 6A). These results show a clear positive correlation between number of cells of Alex-CFPB and the abundance of *A. tamarense* during the blooms in 1997 and 1998 ($Y = 24.6X - 2.65 \times 10^3$, $r^2 = 0.861$, $t = 7.863$, p < 0.001, X: Alex-CFPB, Y: *Alexandrium*). *Alexandrium*-cyst-formation-inhibiting bacteria (Alex-CFIB) were not detected by the MPN method during the observation period. These results also showed a clear positive correlation between the number of cells of Alex-CFPB (Fig. 6A) and the ratio of encystment efficiency under BF/BFF during the bloom in 1998 (Fig. 6B).

Relationships between abundance of *Alexandrium tamarense* and fluctuation of Alex-GPB and Alex-GIB. The number of *Alexandrium*-growth-promoting bacteria (Alex-GPB) determined by the MPN bioassay increased from the beginning of the *Alexandrium tamarense* bloom on March 24, 1998, and reached 4.67×10^2 cells ml^{-1} on April 15, 1998, just before the peak bloom period. After that, Alex-GPB decreased rapidly and dropped to less than 1 cell ml^{-1} in the period of bloom disintegration (Fig. 6C). The number of *Alexandrium*-growth-inhibiting bacteria (Alex-GIB) increased from April 8, 1998, reached 5.53×10^1 cells ml^{-1} at the peak bloom period on April 23, 1998, and then dropped to about 1.00×10^1 cells ml^{-1} (Fig. 6C).

Forms of cysts and their germination. Forms of cysts obtained from BF and BFF were normal and similar to those found in the field bottom sediment. All of the cysts formed in BF had the developed clear-zones around the cells, whereas cysts formed in BFF had undeveloped and faint clear-zones. The probability of excystment ranged from 85.2 to 97.8 % (mean = 92.8%, SD = 6.71) when they were incubated at 20°C with a 14:10 h light:dark cycle after storage at 5°C in the dark for 1 mo.
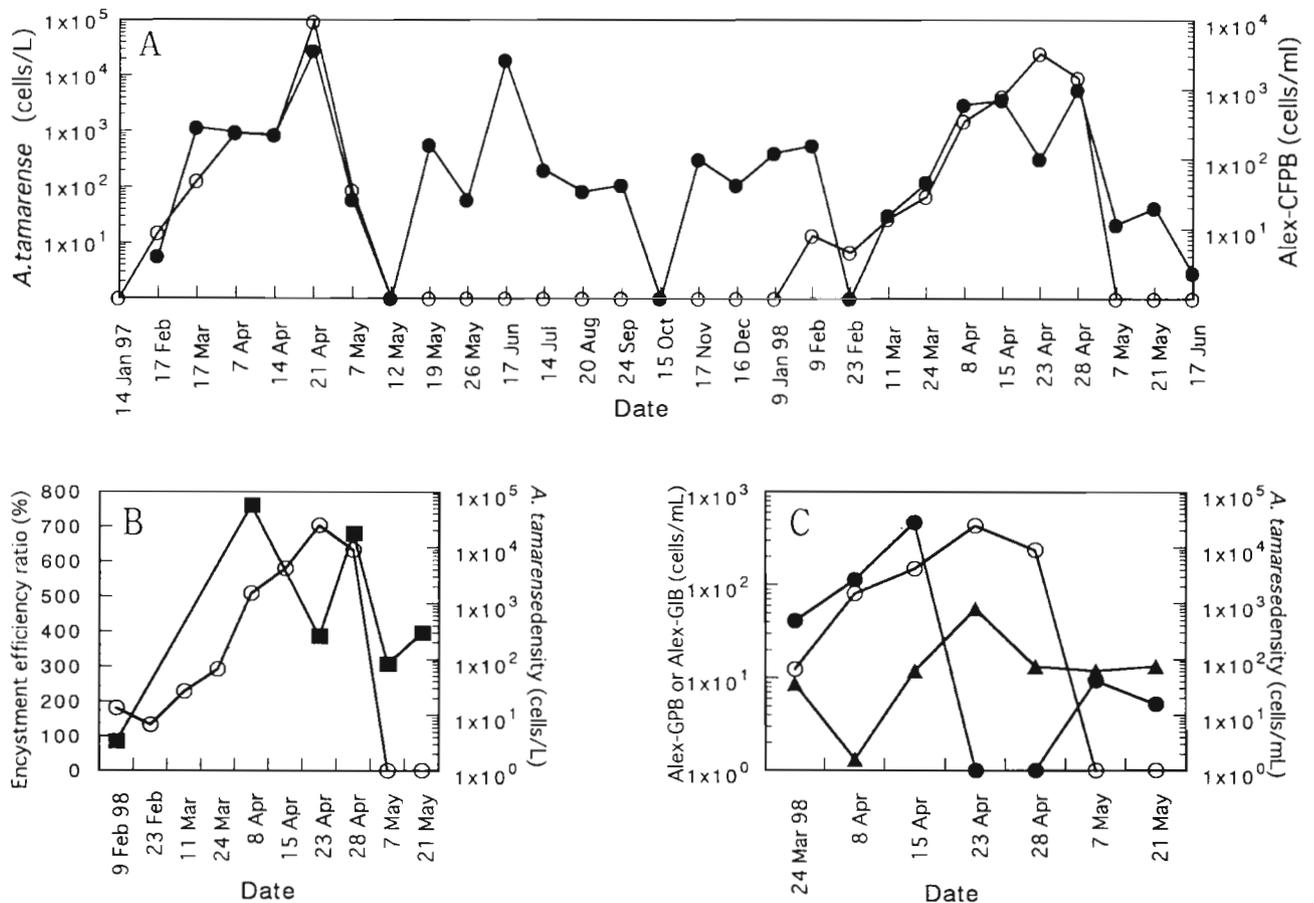
Fig. 6. Relationships between fluctuations of (A) the number of Alex-CFPB (●) determined by the small-scale bioassay, (B) the encytment efficiency ratio (■) of BF to BFF by the large-scale bioassay or (C) the number of Alex-GPB (●) and Alex-GIB (▲), and *Alexandrium tamarense* density (○) at Stn 11 in Hiroshima Bay

DISCUSSION

Promotion of encystment by natural bacterial assemblages. We focused on one of the possible biological factors, bacteria, as the factor that triggers/promotes encystment of the toxic dinoflagellate *Alexandrium* and conducted small- and large-scale bioassays using BF or BFF of natural seawater from Hiroshima Bay in 1997 to 1998 to examine the roles of natural bacterial assemblages on cyst formation of toxic *Alexandrium* in the field. Results suggest that the natural bacterial assemblages enhanced encystment of *Alexandrium*. The abundance or activity of Alex-CFPB increased as the bloom developed and reached the maximum concentration at the peak period of the bloom. After that, Alex-CFPB decreased rapidly as the bloom declined. The clear positive correlation between the abundance of *A. tamarense* and Alex-CFPB during the bloom periods suggests that the Alex-CFPB play a significant role in the process of encystment and bloom disintegration in the field. The existence of Alex-CFPB

during the non-blooming period suggests that Alex-CFPB exist throughout the year.

The MPN counting of Alex-CFPB. In each MPN microwell containing BF, not only Alex-CFPB but also Alex-CFIB, although the latter were not detected in this study, probably exist, so that a succession of these bacterial populations may occur in each well. Therefore, the MPN of Alex-CFPB in this study might be underestimated, since the stimulative effects on encystment may reflect the balance of effects of Alex-CFPB and Alex-CFIB. The number of Alex-CFPB and Alex-CFIB may also be affected by the coexisting Alex-GPB and Alex-GIB in the MPN wells, since the growth in the wells was not monitored in our small-scale bioassay. Results of the fluctuation of Alex-GPB and Alex-GIB in Hiroshima Bay showed that the Alex-GPB disappeared and many Alex-GIB were detected at around the peak bloom period of *Alexandrium tamarense* as well as Alex-CFPB during the blooming in 1998, which suggests that the number of Alex-CFPB at that time have perhaps been underestimated. Fur-

thermore, our estimates of bacteria may be underestimated by the filtration of field seawater with GF/F as well as 0.8 μm filter in some cases.

Promotion of encystment not by nutrient depletion but by bacteria. In the large-scale bioassay, cyst numbers counted after 2 wk and after 1 mo incubation were almost the same, indicating that cyst formation mainly occurred within the first 2 wk. Considering that it takes about 1 wk to mature from fused cells (planozygotes) to resting cysts (hypnozygotes) (Anderson et al. 1983), fusion probably mainly occurred within the first week. During this fusion period, macronutrient (phosphate/nitrite/nitrate) concentrations in flasks containing BF and BFF sampled on February 9, 1998 decreased at almost the same rate as the nutrient concentration in flasks containing BF and BFF sampled on April 28, 1998, whereas the difference between the encystment efficiency in these samples at bloom initiation and bloom periods was detected. These results suggest that the difference was caused not by the effects from macronutrient limitation but by the effects of bacteria sampled at the bloom period.

Promotion effects of encystment by bacteria. Encystment efficiency under BF conditions using seawater containing 'bloom peak' bacteria in the large-scale bioassay was much higher than that under BF conditions using seawater containing 'bloom initiation' bacteria, though total bacteria grew almost similarly in flasks containing these 2 types of seawater. These results suggest that the florae of bacteria which were contained in these 2 types of seawater were quite different and the encystment promotion was not caused by 'general' bacteria but by some bacteria that increased at the bloom period.

Timing of encystment in the field. Anderson et al. (1983) reported that, in their study of dynamics of *Alexandrium tamarense* in Perch Pond, USA, planozygotes had already emerged during the bloom development period and reached a maximum at around the peak of the bloom and then decreased as the bloom declined. Although planozygotes were not counted in our work, our observations that Alex-CFPB increased during the bloom development, reached the maximum at the around bloom peak, and then decreased during the bloom disintegration period correspond to their observations on planozygote dynamics.

Trigger of encystment. In the laboratory cultures, some cysts were formed in wells containing BFF and nutrient-replete f/2–Si media, which suggests that neither the bacterial assemblages nor nutrient-depletion were the trigger for cyst formation in our experiments. The trigger of encystment remains obscure in our experiments. The possibility of an endogenous or 'clock' regulated sexuality might be considered (Anderson et al. 1995).

In the field, it is not clear that natural bacteria triggered the encystment, whereas they promoted the cyst formation. Yet this does not necessarily exclude the possibility that bacteria act as the trigger of encystment in the field. Considering the field environmental parameters, after nutrient concentration decreased rapidly at the beginning of April 1997 and at the end of March 1998, cells of *Alexandrium tamarense* were supposed to divide several times until the peak bloom period and to contain cellular nitrogen or phosphorus at critical or threshold levels before or at the peak bloom period. The putative nutrient depletion might have triggered the encystment during the blooms in 1997 and 1998.

Encystment efficiency. We estimated the effects of bacteria on the encystment efficiency calculated from the division of cyst yields by maximum cell yields according to the method described by Anderson et al. (1984). Considering that the cyst number counted 2 wk and 1 mo after the crossing was almost the same and that the maximum density was always obtained around 2 wk after the crossing, the efficiency may be reliable. More accurate estimates require continuous monitoring of cyst abundance as well as planozygote cells throughout each experiment, so that cells that successfully fuse to form planozygotes or cysts can be included in the motile cell totals (Watanabe et al. 1982).

Difference of cyst yield in small- and large-scale bioassays. Cyst yield produced in each well (1.5 ml) of disposable sterilized polystyrene tissue culture microplates used for small-scale bioassay was always higher than that obtained in each grass flask (100 ml) used for large-scale bioassay. However, the clear positive correlation between abundance of Alex-CFPB obtained using small-scale bioassay and the activity of Alex-CFPB obtained using large-scale bioassay suggests that the difference of cyst yields was not caused by an error in experiment but by the difference of material, size or shape between wells of microplates and flasks.

Nature of cysts formed in our experiments. Anderson et al. (1984) reported that some of the cysts produced under phosphate depletion were not of the typical shape. Forms of cysts obtained in this study were normal under BF and BFF treatments and similar to those found in field bottom sediments. All of the cysts formed in BF had developed clear-zones around the cells, whereas cysts formed in BFF had undeveloped and faint clear-zones. This suggests that the clear-zones may be induced by the existence of natural bacteria assemblages. The high excystment rate also suggests that the cysts formed in our experiments were normal.

Promotion and inhibition of phytoplankton growth by natural bacterial assemblages. Fukami et al. (1991) clarified that the activity of *Gymnodinium mikimotoi-*

growth-promoting-bacteria (Gm-GPB) by their bioassay increased as the bloom developed and reached maximum at the peak period of the bloom in Uranouchi Inlet, Japan. Yoshinaga et al. (1995) reported that the number of *Gymnodinium mikimotoi*-growth-inhibiting bacteria (Gm-GIB) determined by the MPN method increased during the peak bloom period in Tanabe Bay, Japan. Our results of fluctuation of Alex-GPB and Alex-GIB almost corresponded with their results of Gm-GPB and Gm-GIB, which suggests that natural bacterial assemblages may play an important role in the development and decay of algal blooms.

Natural bacteria affect bloom dynamics of marine phytoplankton. This is the first report on the ecological role of natural bacterial assemblages on sexual reproduction of marine phytoplankton species. Recently, some bacteria that affect sexuality or growth of various marine phytoplankton have been isolated from natural marine environments. A bacterium that promotes sperm formation of the diatom *Coscinodiscus wailesii* Gran was isolated from sediment from Harima-Nada (Japan) (Nagai et al. 1994). Sawayama et al. (1993) reported that a bacterium inhibited the mating reaction of *Alexandrium catenella*. These bacteria might affect sexual reproduction of natural phytoplankton species in the field, although these papers did not analyze their abundance or ecological roles in the field. Furthermore, some researchers have clarified that bacterial assemblages in natural seawater affect the growth of specific marine phytoplanktons as well as their succession in the field (Riquelme et al. 1987, Fukami et al. 1991, Yoshinaga et al. 1995). These findings suggest that natural bacterial assemblages play an important role in bloom dynamics of phytoplankton in marine ecosystems.

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