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Effect of nutrients and temperature on encystment of the toxic dinoflagellate *Alexandrium tamarense* (Dinophyceae) isolated from Hiroshima Bay, Japan

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Abstract: A sexual crossing experiment was carried out using 30 clonal strains of the toxic dinoflagellate *Alexandrium tamarense* isolated from Hiroshima Bay, Japan. Encystment through sexual reproduction was observed in 161 pairs (34.6%) out of a total of 465 pairs, which included 30 self-crossings. No planozygote formation or encystment were confirmed in any of the self-crossings. Encystment was observed in all the ten different N and P levels reduced from 1/1 to 1/10 of f/2 levels. Cyst yields at low nutrient enrichments of ≦1/5 level (NO_3^-, 176 μM; PO_4^{3-}, 7.2 μM) tended to be much higher than those at high nutrient enrichments of >1/4 level (NO_3^-, 220 μM; PO_4^{3-}, 9 μM) and there was a significant difference (ANOVA; F=172.29, p<0.001). Encystment was also observed at all the ten different metal enrichment levels reduced from 1/1 to 1/10 of f/2 levels. Cyst yields at high metal enrichments of >1/7 levels were significantly higher (ANOVA; F=40.99, p<0.001) than those at low metal enrichments of ≦1/7 levels.
In the mixed culture of two compatible strains for mating, vegetative growth was observed at temperatures ranging from 5°C to 24°C. The range of growth rates between 10–24°C was 0.62–0.74 divisions day^{-1}. Encystment was observed at temperatures ranging from 5°C to 24°C. The cyst yields at 5, 8 and 24°C were remarkably lower than at the other temperatures. The cyst yield at 14°C was significantly higher than those at other temperatures between 10–22°C (ANOVA; F=19.29, p<0.001). Cyst formation (cyst yield/maximum yield of motile cells×100 (%)) at 14°C was also significantly higher than those at other temperatures between 10–22°C (ANOVA; F=20.50, p<0.001). Therefore, the optimal temperature for encystment of *A. tamarense*, Hiroshima Bay-strains is considered to be around 14°C.

Key words: *Alexandrium tamarense*, cyst, dinoflagellate, encystment, planozygote

Introduction

In Japanese coastal waters, the toxic dinoflagellate *Alexandrium tamarense* (Lebour) Balech had been reported mostly from along the northern Pacific coast, such as in the Hokkaido and Tohoku regions until the 1980s (Uchida et al. 1980; Fukuyo 1982, 1985; Sekiguchi et al. 1986). In recent years, however, *A. tamarense* has become a conspicuous species in southwestern Japan (Yamamoto & Yamasaki 1996; Kotani et al. 1998), especially in Hiroshima Bay (Asakawa et al. 1995; Yamaguchi et al. 1995; Itakura et al. 2002; Yamaguchi et al. 2002).

A. tamarense is well known to have a dormant, sexually produced benthic stage that is called a 'resting cyst' or 'cyst' (Dale 1977; Turpin et al. 1978; Anderson 1980) and this species shows heterothallism in sexuality (Yoshimatsu 1985). The addition of actively growing cells to a nitrogen-deprived medium results in zygotic fusion and cyst formation (Turpin et al. 1978). Encystment was observed at temperatures ranging from 12 to 24°C by using a compatible pair isolated from Orleans, MA, and showed a sharp optimum near 21°C (Anderson et al. 1984). The abundance of cysts and germination characteristics of *A. tamarense* cysts derived from sediments of Hiroshima Bay have been well

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documented by Yamaguchi et al. (1995, 2002) and Itakura & Yamaguchi (2001). However, there have been no reports on cyst formation of *A. tamarense* in Hiroshima Bay up to now.

A. tamarense blooms usually disappear when the water temperature exceeds 15°C from April to May in Hiroshima Bay, and the water temperature, therefore, has been suggested to be the main controlling factor restricting the annual occurrence of vegetative cells in the Bay (Itakura et al. 2002). A high degree of synchronization of planozygote formation and the sedimentation of the clumping hypnozygotes have occasionally been observed at the end of *A. tamarense* blooms in Hiroshima Bay (Nagai unpublished data). Accordingly, investigation of the environmental factors controling encystment in *A. tamarense* in Hiroshima Bay is essential to understand the relationship between the decline of blooms and cyst formation. In this study, we succeeded in determining compatible strains for sexual reproduction and encystment, and demonstrated the effect of nutrient and essential metal concentrations and temperature on the encystment of the strains isolated from Hiroshima Bay in batch culture.

Materials and Methods

Maintenance of *A. tamarense* cultures

Thirty non-axenic and clonal strains of *A. tamarense* were isolated from an *A. tamarense* bloom in Hiroshima Bay (34°16′21″N; 132°16′9″E) in April 2001 by micropipetting single vegetative cells. f/2 medium (Guillard 1975; Guillard & Ryther 1962) was modified by addition of 10 μM of selenious acid (H_2SeO_3) and without copper sulfate hydrate in the stock solution of the metal mixture. Also no silicate was added. These clonal strains were maintained in 5 ml of f/2 medium based on enrichment of natural seawater collected from Hiroshima Bay (DIN, 0.9 μM; DIP, 0.17 μM; salinity adjusted to 30 psu) in glass test tubes at a temperature of 18°C under an irradiance of 100–150 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps with a 12 : 12 h L : D cycle.

Sexual crossing experiment

The crossing experiment was carried out in 1.9 ml of the modified f/2 medium in Iwaki 24 well microplates (Chiba, Japan) under the same conditions as the maintenance cultures. The culture medium was made up with 1/6 nitrate and phosphate, 1/3 metals and 1/10 vitamins. For self-crossing, 200 μl (ca. 1000 cells) of culture of each strain was transferred to single wells. For intercrossing, 100 μl of each strain (ca. 500 cells) was inoculated and mixed in wells for all possible pair combinations. The microplates were incubated for one month at 18°C under an irradiance of 100–150 μmol m^{-2} s^{-1} with a 12 : 12 h L : D cycle. Observations of sexuality and encystment were carried out through the base of the microplates using a Nikon TE-300 (Tokyo, Japan) inverted microscope. The number of cysts was also counted in each well and shown in the crossing matrix only for wells from which more than 200 cysts were obtained.

Effect of nutrients on encystment

The effect of nitrate and phosphate enrichment levels on encystment of this species was investigated at ten different NP levels (sets of N and P) reduced from 1/1 to 1/10 of f/2 levels (N, 882.4–88.2 μM; P, 36–3.6 μM). Metals and vitamins were added at the same levels as the crossing experiment. Encystment experiments were also carried out at 10 different metal levels reduced from 1/1 to 1/10 of f/2 levels ($Na_2EDTA \cdot 2H_2O$ & $FeCl_3 \cdot 6H_2O$, 1.2–11.7 nM; $CoCl_2 \cdot 6H_2O$, 5–50 nM; $ZnSO_4 \cdot 7H_2O$, 8–80 nM; $MnCl_2 \cdot 4H_2O$, 90–900 nM; $Na_2MoO_4 \cdot 2H_2O$, 3–30 nM; H_2SeO_3, 1–10 nM). NP and vitamins were added at the same levels as in the crossing experiment. From the result of the sexual crossing experiment, two compatible strains of opposite mating types (strain AT0104H15 and AT0104H26) were chosen and used in this experiment (see Fig. 1). Both the strains were grown separately for the NP experiment (made up with 1/10 NP, 1/3 metals and 1/10 vitamins) and for the metal experiment (made up with 1/6 NP, 1/10 metals and 1/10 vitamins), respectively for 2 weeks as the pre-incubation period. After the pre-incubation, 50 μl of each strain (ca. 250 cells) were inoculated, mixed, and added to each of 6 wells of a microplate for each nutrient mix. First 3 wells and the other 3 wells were used for counting vegetative cells/planozygotes and cysts, respectively as detailed below. The microplates were incubated for one month under the same temperature and light conditions as for the crossing experiment. In order to estimate the maximum yield of motile cells (vegetative cells and planozygotes), 100 μl of the *A. tamarense* culture from the wells of the microplates were sampled from each nutrient mix every 3 days and the number of cells was counted using a microscope. After the incubation, cysts were gently rubbed away from the bottom of the wells using a pipette and counted. Maximum yields of the cells and cyst yields were calculated. Percentage of cyst formation was also calculated by dividing the cyst yield by the maximum yield of motile cells.

Time course of the encystment process

In order to observe the sexuality and the encystment process, time-course measurements were carried out at temperatures of 16°C and 18°C under the same conditions as in the crossing experiment. The two compatible strains (strain AT0104H15 & AT0104H26) were pre-incubated at each temperature for 3 weeks. Strains so pre-cultured were mixed and added to each of six wells of a microplate for each temperature setting and incubated for one month. Three wells of each were used for counting motile cells and cysts, respectively. A portion of the *Alexandrium* culture

Mating type	Strain	5	1	15	18	30	9	10	6	21	27	3	17	20	23	26	28	8	25	13	19	22	29	7	11	24	12	16	4	2	14
+	AT0104H05	N	N	N	N	N	N	N	N	N	N	N	N	C	C	C	390	C	C	C	C	C	C	C	C	C	C	C	C	C	C
+	1	N	N	N	N	N	N	N	N	N	N	N	N	C	C	210	C	C	P	C	C	C	C	C	C	C	C	C	C	N	C
+	15	N	N	N	N	N	N	N	CCR	N	N	N	N	C	1840	730	C	C	C	C	N	350	C	C	210	N	C	C	1270	C	N
+	9	N	N	N	N	N	N	N	N	N	N	N	N	C	C	C	C	C	C	C	C	C	C	N	N	C	C	C	N	C	C
+	30	N	N	N	N	N	N	N	N	N	N	N	N	C	710	C	C	C	C	C	C	C	C	C	N	500	N	N	C	C	C
+	10	N	N	N	CCR	N	N	N	N	N	N	N	N	C	C	600	C	C	C	C	N	C	C	N	C	N	C	C	N	C	C
+	18	N	N	N	N	N	N	CCR	N	CCR	N	N	N	C	440	1850	480	N	C	N	C	C	C	C	700	C	C	C	C	N	N
+	27	N	N	N	N	N	N	N	N	N	N	N	N	C	C	C	C	C	C	C	C	N	C	C	N	C	N	N	C	N	N
+	3	N	N	N	N	N	N	N	N	N	N	N	N	C	C	C	C	C	C	C	C	C	N	P	C	N	N	N	C	N	N
+	6	N	N	CCR	N	N	N	N	N	N	N	N	N	C	390	C	C	C	C	N	C	C	C	C	N	C	N	N	N	N	N
+	21	N	N	N	CCR	N	N	N	N	N	N	N	N	C	C	340	430	N	C	C	C	N	N	C	C	C	C	N	N	N	N
+	17	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	C	N	N	N	N	N	N	C	N	N	C	N	N	N
-	26	C	210	730	1850	C	C	600	C	340	C	C	N	N	N	N	CCR	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	23	C	C	1840	440	710	C	C	390	C	C	C	N	N	N	N	N	N	CCR	N	N	N	N	N	N	N	N	N	N	N	N
-	20	C	C	C	C	C	C	C	C	C	C	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	28	390	C	C	480	C	C	C	C	430	C	C	N	N	N	CCR	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	8	C	C	C	N	C	C	C	C	N	C	C	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	25	C	P	C	C	C	C	C	C	C	C	C	N	N	CCR	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	13	C	C	C	N	C	C	C	N	C	C	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	19	C	C	N	C	C	C	N	C	C	C	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	22	C	C	350	C	C	C	C	C	N	N	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	29	C	C	C	C	C	C	C	C	N	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	7	C	C	C	C	C	N	N	C	C	C	P	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	11	C	C	210	700	N	C	N	C	N	C	C	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	CCR	N	N	N
-	24	C	C	N	C	500	C	N	C	C	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	4	C	C	1270	C	C	N	N	N	N	C	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	12	C	C	C	C	N	C	C	N	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	16	C	C	C	C	N	C	C	N	N	N	N	C	N	N	N	N	N	N	N	N	N	N	N	CCR	N	N	N	N	N	N
-	2	C	N	C	N	C	C	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
-	14	C	C	N	N	C	C	C	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

C, cyst formation; N, no cyst formation; P, planozygote formation but no cyst formation; CCR, cyst formation but contradictory result; The number of cysts is shown only in wells from which greater than 200 cysts were obtained.

Fig. 1. Crossing matrix of *Alexandrium tamarense* by use of 30 clonal strains isolated from Hiroshima Bay, Japan.

(100 μl) was sampled from each temperature incubation every 3 days and the number of motile cells counted using the microscope. Microplates with pre-drawn grids were used for enumeration of cysts by microscope.

Encystment experiment under different temperatures

Effect of temperature on encystment was investigated at eleven different temperatures of 5, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26°C under the same conditions as in the crossing experiment except for the temperature. The two compatible strains (strain AT0104H15 & AT0104H26) were pre-incubated at each temperature, except for 26°C, for 3 weeks. The strains were pre-incubated at 24°C instead of 26°C because less growth of the strains was observed at 26°C. After the pre-incubation, both strains were mixed in and added to each of ten wells of a microplate for each temperature setting and incubated for at least one month. Five wells of each were used for counting motile cells and cysts, respectively, and the maximum yields of motile cells, cyst yields and percentage of cyst formation were calculated as in the nutrient experiments.

Results

Sexual crossing experiments

Encystment through sexual reproduction was observed in 161 pairs (34.6%) out of the total of 465 pairs, which included 30 self-crossings (Fig. 1). No planozygote formation or encystment were confirmed in any of the self-crossings. These results clearly show that *A. tamarense* is heterothallic, i.e. compatible strains of the opposite mating type are required for sexual reproduction. First of all, the mating type of strain AT0104H01 was arbitrarily designated mating type +. All other strains were subsequently identified as + or − mating type based on their pattern of sexual compatibility with strain AT0104H01. Contradictory results to the determined mating type were obtained in 6 of the strain pairs (3.7% of the compatible pairs), suggesting the existence of ambiguous mating types. Twelve strains were considered as mating type + and another eighteen strains were considered as mating type −. The percentage of encystment success within the possible +/− combinations was in the ranges of 10–60 (44.7±13.1, Mean±SD)% and 17–43 (30.4±7.6)%, respectively. In 2 of the strain pairs, planozygote formation was observed but cysts were not produced. Most of the pairs showed low cyst productivity (<100 cysts per well), but 17 pairs (3.7% of the total, 10.6% of the compatible pairs) showed high cyst productivity (210–1,850 cysts per well). The strains of AT0104H15 and AT0104H26 showed the highest cyst productivity as mating type + and −, respectively, and these strains, therefore, were used in the following encystment experiments under various nutrient concentrations and temperatures regimes.

Effect of nutrients on encystment

Encystment was observed at all ten different N and P levels reduced from 1/1 to 1/10 of f/2 levels (Fig 2A), ranging from 17±3 cysts ml^{-1} (Mean±SD) at the 1/1 N, P level (N, 882.4 μM; P, 36 μM) to 1,390±145 cysts ml^{-1} at the 1/9 N, P level (N, 98 μM; P, 4 μM). Cyst yields in the low nutrient enrichments of the ≦1/5 level (N, 176 μM; P, 7.2 μM) were significantly higher than those in the high nutrient enrichments of >1/4 level (N, 220 μM; P, 9 μM) (ANOVA; $F=172.29$, $p<0.001$). Cyst yields were not significantly different between the ≦1/5 N, P level incubations ($p>0.05$, t-test).

Maximum yields of motile cells (vegetative cells and planozygotes) of the mixed cultures ranged from 5,000±500 (Mean±SD) cells ml^{-1} to 75,833±5,774 cells ml^{-1} and these yields increased linearly with increasing N, P levels.

The percentage of cyst formation ranged from

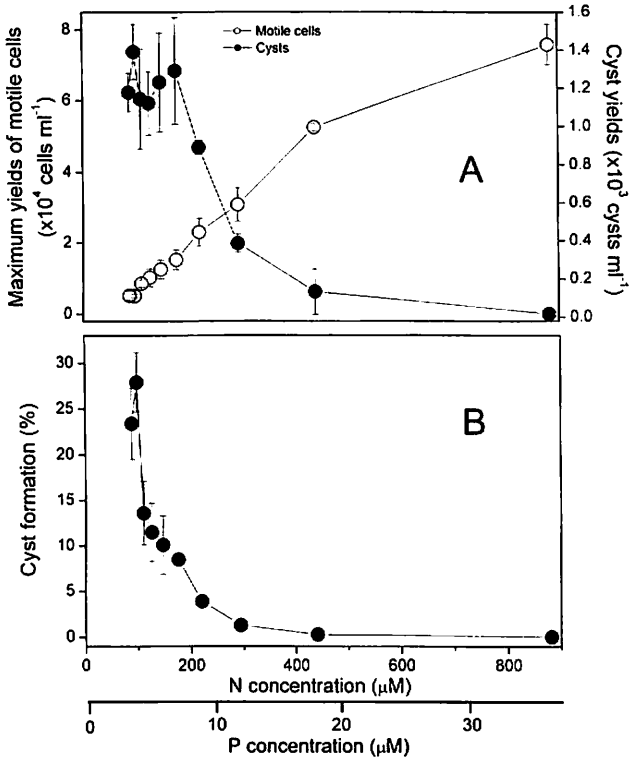

Fig. 2. Effect of the nitrogen and phosphate enrichment level of f/2 medium on encystment of *Alexandrium tamarense* batch culture. (A) Maximum yields of motile cells and cyst yields; (B) Cyst formation.

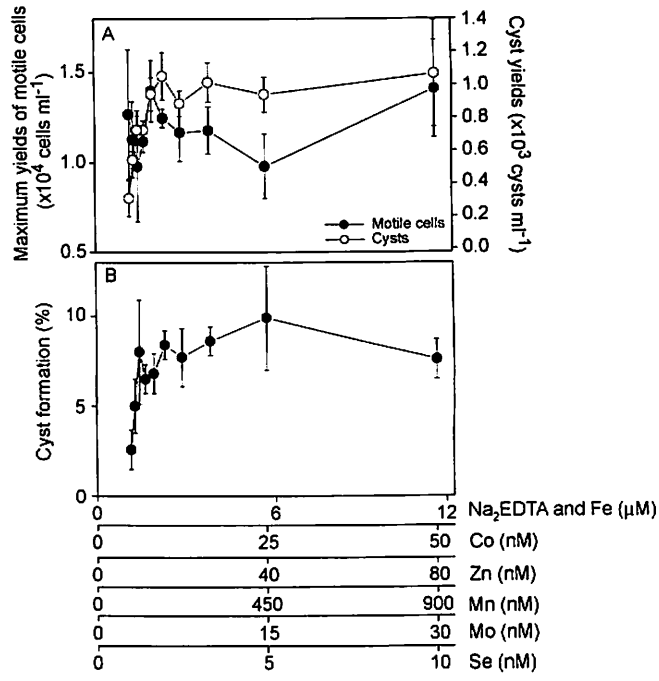

Fig. 3. Effect of the metal enrichment level of f/2 medium on encystment of *Alexandrium tamarense* batch culture. (A) Maximum yields of motile cells and cyst yields; (B) Cyst formation.

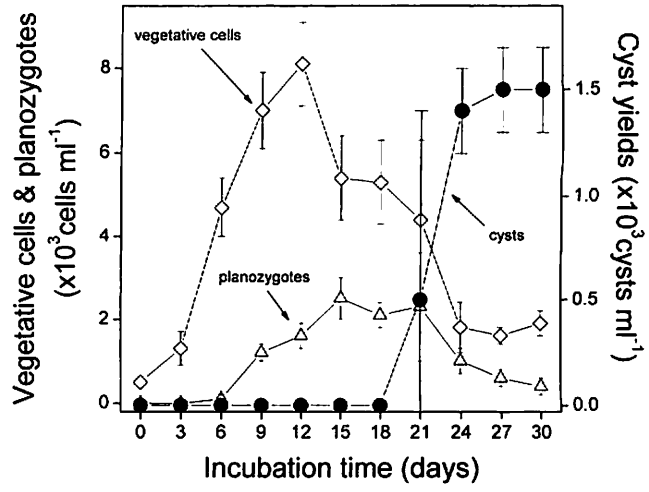

Fig. 4. Encystment process of *Alexandrium tamarense* batch culture at 16°C.

0.02±0.002% (Mean±SD) to 27.9±3.2% (Fig. 2B). Minimum and maximum percentages were obtained at 1/1 N, P and 1/9 N, P levels, respectively. The percentage tended to be high at low N, P levels and low at high N, P levels.

Encystment was observed in all the ten different metal enrichments reduced from 1/1 to 1/10 of f/2 levels (Fig. 3A) and cyst yields ranged from 310±111 cysts ml^{-1} (Mean±SD) at the 1/10 level to 1,067±325 cysts ml^{-1} at the 1/1 level. Cyst yields at high metal enrichments of >1/7 tended to be higher than those at low metal enrichments of ≦1/7 and the difference was significant (ANOVA; F=40.99, P<0.001). There was no significant difference in the cyst yields between ≧1/6 metal levels (p>0.05, t-test).

Maximum yields of motile cells in the mixed cultures ranged from 9,767±3,101 (Mean±SD) cells ml^{-1} to 14,067±2,695 cells ml^{-1} and no trend in maximum yields was detected between the different metal enrichment levels.

The range of percentage of cyst formation was 2.6±1.1 (Mean±SD)% and 9.6±2.9%. The minimum and maximum percentages were obtained at 1/10 and 1/2 metal enrichment levels, respectively. The percentages for the low metal enrichment levels (1/9 and 1/10) were lower than those for other levels, due to the low cyst productivity.

Time course of encystment

Vegetative cells (Fig. 6A) in the mixed culture at 16°C grew exponentially and the growth reached a peak (8,100±1,000 cells ml^{-1}) on Day 12 with density thereafter decreasing due to the increase in the number of planozygotes (Fig. 4). Vegetative chains composed of 2–8 cells were dominant during the early and mid exponential growth phase (until Day 9) but after that single vegetative cells became dominant. Planozygote formation was first observed on Day 3 (Fig. 6B). Several clumps of single vegetative cells, this behavior being termed the "mating dance", were observed at the bottom of the wells around Days 6–12 (Fig. 6C) and active gamete fusions were seen (Fig. 6D, E). The density of planozygotes increased gradually until Day 15

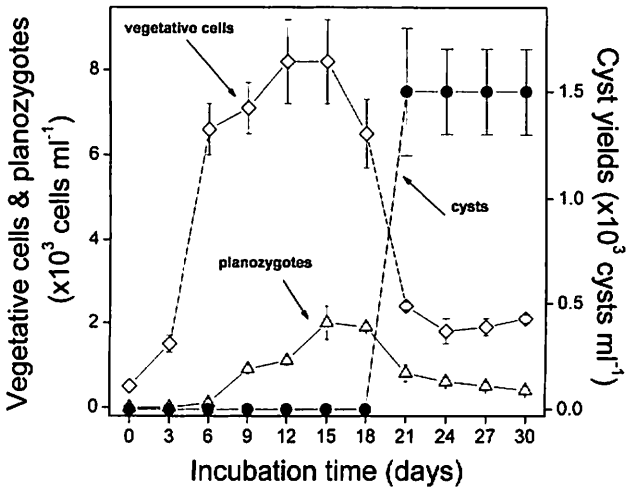

Fig. 5. Encystment process of *Alexandrium tamarense* batch culture at 18°C.

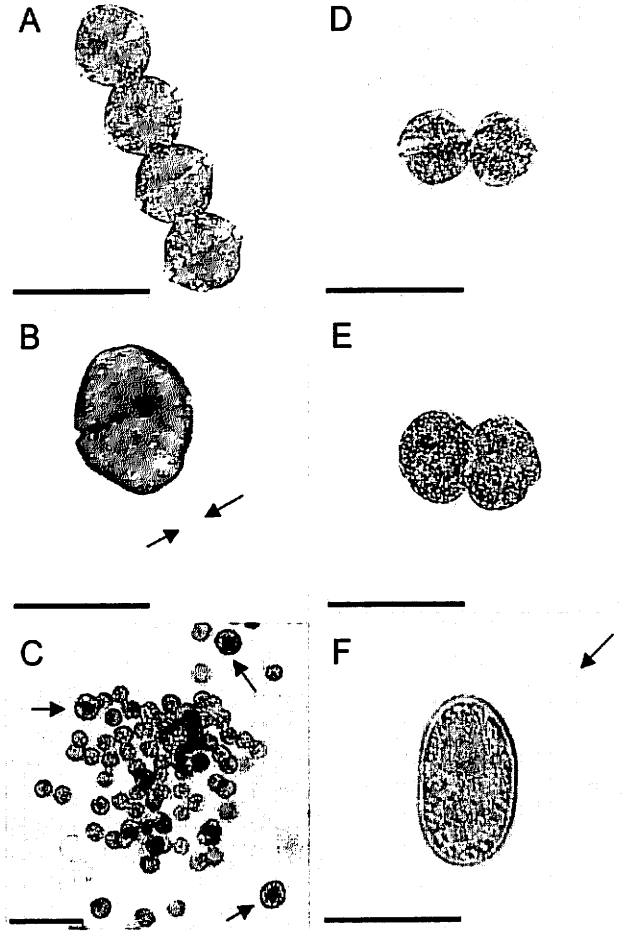

Fig. 6. Life cycle stages of *Alexandrium tamarense*. Scale bars=50 (A, B D, E and F), Scale bar=100 μm (C).
(A) Vegetative cells forming a 4 cell chain; (B) Planozygote with two longitudinal flagella indicated by arrows; (C) Clump of single vegetative cells, in the mating dance with planozygotes indicated by arrows; (D) Mating pair of isogametes (initial contact phase); (E) Conjugating gametes; (F) Resting cyst covered with transparent mucilaginous matter indicated by an arrow.

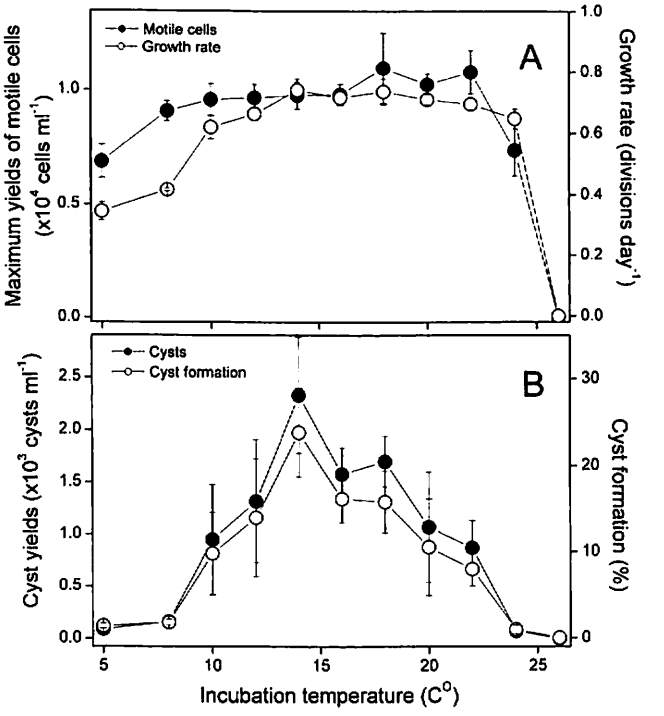

Fig. 7. Effect of temperature on growth and encystment of *Alexandrium tamarense* batch culture.
(A) Maximum yields of motile cells and growth rates; (B) Cyst yields and cyst formation.

(2,467±451 cells ml^{-1}) and then decreased on Day 24, due to transformation into cysts. Cyst formation occurred 15 days after the beginning of first incubation (Fig. 6F) and a massive increase in cyst density was seen between Days 18 and 24, with density slightly increasing until the end of the incubation (1,473±166 cysts ml^{-1}). However, not all planozygotes transformed into cysts.

At a temperature of 18°C vegetative cells grew exponentially and reached a peak of 8,200±985 cells ml^{-1} on Day 12, with density thereafter decreasing due to an increase in the number of planozygotes (Fig. 5). Planozygote formation was first observed to have occurred on Day 3 and the density increased gradually until Day 15 (2,000±361 cells ml^{-1}), thereafter decreasing until around Day 21 due to transformation into cysts. Encystment occurred 15 days after first incubation, as with the 16°C incubation, and a high degree of synchronization of cyst formation was observed on Day 21.

Effect of temperature on encystment

In the mixed culture, vegetative growth was observed at temperatures ranging from 5°C to 24°C and the maximum yields of motile cells ranged from 6,880±716 cells ml^{-1} at 5°C to 10,980±1,553 cells ml^{-1} at 18°C. No growth was seen at 26°C. The growth rate ranged from 0.34±0.03 divisions day^{-1} at 5°C to 0.74±0.04 divisions day^{-1} at 14°C and 18°C (Fig. 7A) and the rates at 5°C and 8°C were re-

markably lower than those at the other temperatures. The range of growth rates between 10–24°C was 0.62–0.74 divisions day^{-1}. Encystment was observed at temperatures ranging from 5°C to 24°C and cyst yields ranged from 68±34 cysts ml^{-1} at 24°C to 2,326±551 cysts ml^{-1} at 14°C (Fig. 7B). The cyst yields at 5, 8 and 24°C were remarkably lower than those at the other temperatures. The cyst yield at 14°C was significantly higher than those at other temperatures between 10–22°C (ANOVA; $F=19.29$, $p<0.001$). Cyst formation was in the range of 0.9±0.5% at 24°C to 23.8±5.1% at 14°C. Effect of temperature on cyst formation tended to be very similar to that for the cyst yield. Cyst formation at 14°C was also significantly higher than values at other temperatures between 10–22°C (ANOVA; $F=20.50$, $p<0.001$). Therefore, the optimal temperature for encystment of *A. tamarense* strains from Hiroshima Bay is concluded to be around 14°C.

Discussions

No planozygote formation or encystment were confirmed in any of the self-crossings, clearly indicating the heterothallism of this species as reported by Turpin et al. (1978), Anderson (1980) and Yoshimatsu (1985). Yoshimatsu (1981) reported zygote formation in *Alexandrium catenella* (36–54% success; based on three replicate trials) and the percentages of conjugation success within the possible +/− combinations were in the range of 14–86%. Nagai et al. (2003) reported that encystment through sexual reproduction in *A. tamiyavanichii* isolated from the Seto Inland Sea was observed in 54 pairs (39.7%) out of the 136 pairs, which included 16 self-crossings. The percentage of conjugation success within the possible +/− combinations was in the range of 25–100%. Blackburn et al. (1989) also observed cyst formation through sexual reproduction in 12 out of 24 pairs of *Gymnodinium catenatum* Graham (50% success) and the percentages of conjugation success within the possible +/− combinations were in the range of 33–83%. Based on these results it appears that some strains readily conjugate and others do not, and that sexual compatibility is variable. Variation in sexual compatibility of *G. catenatum* within the possible +/− combinations may reflect the incompatibility of some crosses or may be due to some undetermined effect of culturing (Blackburn et al. 1989; Nagai et al. 2003).

Cyst yields at ≦1/5 N, P levels were significantly higher than those at the higher concentrations but no significant differences in yields were observed between ≦1/5 N, P levels (Fig. 2). Anderson et al. (1984) demonstrated that in phosphate-limited cultures of *A. tamarense* batch cultures, the cyst yield was optimal with the initial concentration near 3 μM (1/12 of f/2 medium). A relatively high valuefor cyst production (826 cysts ml^{-1}) in the batch culture with 88.3 μM of nitrate (1/10 N of f/2 medium) was also reported. This finding agrees with the results of the present study. In most studies, sexuality has been induced by nutrient starvation, often by re-suspending actively growing cells in a culture medium lacking one essential nutrient, usually nitrogen (e.g. Pfiester 1975; Turpin et al. 1978; Walker and Steidinger 1979). In an *A. tamarense* culture grown in nutrient depleted medium, the C/N ratio was relatively constant in the presence of excess nutrients, but sharply increased as the external nitrogen supply disappeared (Pfiester & Anderson 1987). Planozygotic cells were actively formed when a sharp increase in C/N ratio occurred (Pfiester & Anderson 1987). Thus, some kind of change in metabolism such as the C/N ratio, may have affected the synchronization of encystment in this species. On the other hand, judging from the results of the present study, where cyst yields were not significantly different between ≦1/5 N, P levels, it is assumed that nitrogenous nutrients at high concentrations simply inhibit encystment. In an *A. tamiyavanichii* batch culture, the cyst productivity at low nutrient levels (1/6–1/10 N, P) was significantly higher than that at high nutrient levels (1/1–1/2 N, P) (Nagai et al. unpublished data), the same result as for *A. tamarense*. However, the influence of initial cell densities on cyst production is remarkably large under low nutrient conditions (1/6 N, P levels) and ca. 250 cells ml^{-1} is the optimum initial density for cyst production, suggesting that there is some interaction between initial nutrient levels and cell densities to enhance conjugation (Nagai et al. unpublished data). Further quantitative work on the role of nutrient stimuli (nitrogen depletion) is needed to clarify the mechanism of cyst formation, perhaps using methods such as semi-continuous culture.

Data on the effect of metals on encystment in batch culture of dinoflagellate species have been rarely reported, perhaps because no easily recognizable in cyst productivity has been detected. However, our data showed significant differences in the cyst yield between >1/7 and ≦1/7 metal levels. *A. tamarense* seems to require metals in order to have sufficient growth and cyst productivity, but there is a dearth of data as to which metals are more effective in the induction of sexuality.

A. tamarense isolated from sediments of Mill Pond, Orleans, MA, USA did not grow below 7°C or above 26°C and the optimum range was between 11°C and 22°C under laboratory conditions (Anderson et al. 1984). Encystment was observed at temperatures ranging from 12 to 24°C, and cyst production was highly variable, with a sharp optimum near 21°C. On the other hand, *A. tamarense* isolated from Hiroshima Bay, Japan grew even at 5°C (Fig. 7A) and formed cysts at this temperature, although the cyst yield was much lower than at other temperatures. The cyst formation was relatively constant at 10–22°C (Fig. 7B).

Surface water temperatures in Hiroshima Bay increase from approximately 10°C in March to an annual maximum (25–28°C) in August and steadily decrease after September. Within the bloom period, the water temperature ranges from 10°C to 20°C and the abundance of *A. tamarense* usually starts to increase in March (10°C), with the blooms disap-

pearing when the water temperature exceeds 15°C in April–May (Itakura et al. 2002). The water temperature at which the blooms disappear is close to the optimum temperature of encystment. Thus, active encystment might contribute substantially to disappearance of the bloom.

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