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Morphological and physiological differences between the cysts of *Alexandrium catenella* and *A. tamarense* (Dinophyceae) in the Seto Inland Sea, Japan

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Abstract: Size composition and germination temperature of the cysts of *Alexandrium catenella* and *Alexandrium tamarense* were examined from two different areas (Tokuyama and Hiroshima Bay), located in the western part of the Seto Inland Sea, Japan. Based on past bloom records, naturally occurring *Alexandrium* cysts in Tokuyama Bay are suggested to be dominated by the cysts of *A. catenella*, while those in Hiroshima Bay are dominated by those of *A. tamarense*. Bottom sediment samples were collected in 1997 from one station in each bay. For cyst size measurements, cysts in the surface sediment samples were stained with the fluorochrome primuline and images of the cysts were captured under an epifluorescence microscope. The length and width of the cysts were measured using an image analysis program on a personal computer. There was a significant difference in the mean length of cysts between Tokuyama Bay (49.1±4.6 μm) and Hiroshima Bay (54.2±4.5 μm) ($p<0.01$). Cyst germination experiments were conducted under eight different temperature regimes (7.5°C to 25°C). The temperature "window" for the cyst germination was different between the cyst samples from the two bays (Tokuyama: 7.5°C to 25°C, Hiroshima: 7.5°C to 20°C). Optimum germination temperature for the cysts from Tokuyama Bay and Hiroshima Bay were 17.5°C and 12.5°C, respectively. The present results suggest that the naturally occurring cysts of *A. catenella* (Tokuyama Bay cysts) and *A. tamarense* (Hiroshima Bay cysts) have different size compositions and have different temperature windows for germination.

Key words: *Alexandrium catenella, Alexandrium tamarense*, resting cysts, size composition, temperature window

Introduction

Paralytic shellfish poisoning (PSP) outbreaks are increasing in their intensity and geographic distribution in southwest Japan (Anraku 1984, Yamamoto & Yamasaki 1996, Yamaguchi et al. 1996, Itakura & Yamaguchi 2001). Particularly in the Seto Inland Sea, toxic blooms of *Alexandrium catenella* (Whedon et Kofoid) Balech and *Alexandrium tamarense* (Lebour) Balech have repeatedly occurred in recent years (e.g. Yoshimatsu 1992, Asakawa et al. 1993). Tokuyama Bay and Hiroshima Bay are adjacent embayments located in the western part of the Seto Inland Sea, and both bays have experienced recurrent PSP events caused by these toxic *Alexandrium* species.

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In Tokuyama Bay, blooms of *A. catenella* have frequently been observed in early summer, while *A. tamarense* has not been a conspicuous phytoplankton species in recent years. Red tides of *A. catenella* have been reported in 1979, 1981 and 1997 in Tokuyama Bay (Sakamoto et al. 1999, Baba et al. 2000), and our recent bottom sediment survey revealed that a large number of ellipsoidal *Alexandrium* resting cysts are deposited in the bottom sediments of the inner bay (Yamaguchi et al. 2002).

In Hiroshima Bay, PSP events from *A. tamarense* blooms have been observed almost every year in late spring since 1992 (e.g. Asakawa et al. 1993, Itakura & Yamaguchi 2001). We have conducted investigations on the vegetative and cyst populations of *A. tamarense* in Hiroshima Bay since 1994 (Itakura & Yamaguchi 2001, Itakura et al. 2002), and the results have shown that the dense seedbed of

Alexandrium in the northern part of the bay is mostly composed of the cysts of *A. tamarense*. Germination characteristics of naturally occurring cysts in Hiroshima Bay have already been investigated in our previous study (Itakura and Yamaguchi 2001).

From a morphological and genetic point of view, *A. catenella* and *A. tamarense* are closely related species as they can be defined as a suite of divergent lineages, such as strains or varieties (Scholin 1998). For example, morphological characteristics of the vegetative cells of *A. catenella* and *A. tamarense* are very similar except for the presence or absence of a ventral pore in the 1′ apical plate, the overall shape of the cells and their propensity to form chains (Taylor 1984, Fukuyo 1985). Moreover, the cysts of these two species have been suggested to be indistinguishable from each other in their appearance and size; i.e. the cysts are elongate, ellipsoidal with rounded ends and central body from 38 to 56 μm in length and 23 to 32 μm in width (Fukuyo 1985).

Benthic resting cysts play an important role in the initiation of blooms and subsequent dispersal and survival during interbloom periods (e.g. Wall 1971, Anderson 1984). Cyst surveys are essential for the ecological study of cyst-forming phytoplankton. For example, the distribution, abundance and species composition of cysts in the bottom sediments gives useful information on the past occurrence of vegetative populations in the area. If there is some detectable difference between the resting cysts of *A. catenella* and *A. tamarense*, it would greatly assist elucidation of the ecology, bloom dynamics and monitoring countermeasures for these two closely related species.

The purpose of this study was to examine any morphological and/or physiological differences in the cysts between *A. catenella* and *A. tamarense*. As stated above, the species composition of *Alexandrium* cysts in Tokuyama Bay and Hiroshima Bay based on bloom events are thought to be different, so we tested if there are any morphological and/or physiological differences between naturally occurring *Alexandrium* cysts in these two bays. For the examination of morphological difference of the cysts, size composition of *Alexandrium* cysts were determined for Tokuyama Bay and for Hiroshima Bay. To examine physiological differences of the cysts, the germination temperature range of the cysts from Tokuyama Bay and laboratory produced cysts from a strain of *A. tamarense* isolated from Hiroshima Bay were examined.

Materials and Methods

Sediment samples were collected with a gravity core-sampler (inner diameter 4 cm) in July 1997 from Station 1 in Tokuyama Bay and Station 11 in Hiroshima Bay (Fig. 1). Our recent bottom sediment survey at the area revealed that a high abundance of the cysts of *Alexandrium* spp. (*A. catenella* and/or *A. tamarense*) are deposited at these two stations (Yamaguchi et al. 1995a, Yamaguchi et al. 2002). The top 1 cm of the sediment samples were sliced off for each of five cores, placed in a plastic container and stored in the dark at 10°C.

Size analysis of the cysts was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). The length and width of the ellipsoidal *Alexandrium* cysts were measured using the NIH Image program. In order to obtain clear cyst images under the microscope, the primuline-staining method (Yamaguchi et al. 1995b) was used for the analysis. Five to 20 g (wet weight) aliquots of the sediment samples were suspended in distilled water, sonicated and sieved through plankton netting to obtain the size fraction between 20 and 150 μm. The material remaining on the 20 μm netting was then washed, fixed and stained with primuline. Microscopic observations were done using an epifluorescence inverted microscope (IX-70 with IX-FLA, Olympus, Tokyo, Japan) under blue light excitation. Then more than a hundred images of the cysts from Tokuyama Bay and Hiroshima Bay were captured using a CCD camera (DXC-108, Sony, Tokyo, Japan) and stored on a Macintosh computer.

The germination temperature range of the cysts was examined for naturally occurring *Alexandrium* cysts in Tokuyama Bay (Station 1), and also examined for laboratory produced *A. tamarense* cysts of a strain originally isolated from Hiroshima Bay. For the purpose of *A. tamarense* cyst production, field vegetative populations were collected at Hiroshima Bay in April 1997, when a bloom of *A. tamarense* occurred near Station 11 (Fig. 1). Then, based on the method described by Anderson et al. (1995) cysts of *A. tamarense* were produced under laboratory conditions;

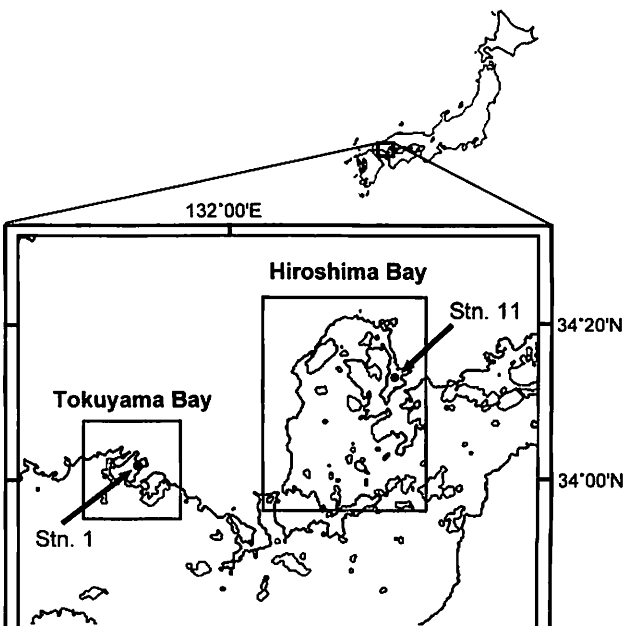


Fig. 1. Location of the bottom sediment sampling stations.

i.e. the collected plankton sample was sieved through plankton netting to obtain the size fraction between 20 and 150 μm. The plankton remaining on the 20 μm netting were then rinsed and suspended in 500 ml of autoclaved sea water. The suspension was kept in the dark at 15°C (ambient bottom water temperature) for one month. The cysts so obtained were stored in the dark at 20°C for ca. six months in autoclaved sea water with 40 g granular activated charcoal (Wako, Osaka, Japan). The annual bottom water temperature range of Hiroshima Bay is ca. 10 to 25°C. Therefore, the cyst germination experiments were performed under eight different temperature conditions (7.5, 10, 12.5, 15, 17.5, 20, 22.5 and 25°C) at 50 μmol photons $m^{-2}s^{-1}$ of cool fluorescent light with a light : dark cycle of 12 hL : 12 hD. For each temperature condition, over fifty cysts isolated from the sediment or laboratory produced cysts were placed into cell culture clusters (48 well, Costar, MA, USA). Each well of the culture cluster was filled with 0.5 ml of autoclaved filtered sea water and generally one cyst was inoculated per well. Germination success was checked every day for individual resting cysts under an inverted epifluorescence microscope until about a month after the start of the incubation. So, the number of days required for the germination at each temperature was also observed in the cyst germination experiment.

Results

Size composition of the cysts

Figure 2 shows the length frequency distributions for the naturally occurring *Alexandrium* cysts isolated from Tokuyama Bay and Hiroshima Bays. The length of the cysts from Tokuyama Bay ranged from 33.8 to 68.5 μm with an average of 49.1 μm (±4.6 μm), and that of the cysts from Hiroshima Bay ranged from 42.6 to 62.9 μm with an average of 54.2 μm (±4.5 μm) (Fig. 2). There was a significant difference (t-test; $p<0.01$) in the mean cyst length between Tokuyama Bay and Hiroshima Bay.

Figure 3 shows the width frequency distributions for the naturally occurring *Alexandrium* cysts isolated from Tokuyama Bay and Hiroshima Bays. The width of the cysts from Tokuyama Bay ranged from 28.2 to 38.9 μm with an average of 32.4 μm (±1.9 μm), and that of the cysts from Hiroshima Bay ranged from 27.0 to 37.2 μm with an average of 32.4 μm (±1.7 μm) (Fig. 3). There was no significant difference in the mean cyst width between Tokuyama Bay and Hiroshima Bay.

Temperature range for cyst germination

Figure 4 shows the relationship between the incubation temperature and germination success (at a month after the start of incubation) of the naturally occurring cysts from Tokuyama Bay and laboratory produced *A. tamarense* cysts of a strain originally isolated from Hiroshima Bay. Naturally occurring cysts in Tokuyama Bay could germinate

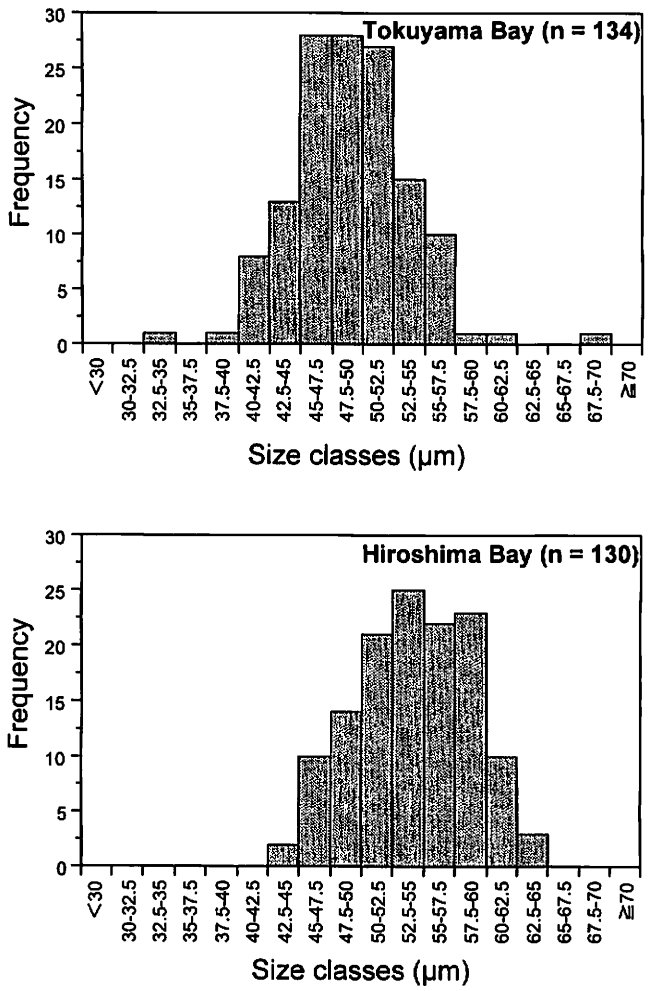


Fig. 2. Cyst length-frequency distributions of the naturally occurring *Alexandrium* cysts from Tokuyama Bay (upper) and Hiroshima Bay (lower).

within the temperature range from 7.5°C to 25°C. High germination rates (>75%) were observed between 17.5°C to 20°C and the maximum germination success (93.9%) was obtained at 17.5°C (Fig. 4). Germination of the *A. tamarense* cysts occurred within the temperature range from 7.5°C to 20°C and no cyst germination was observed at 22.5°C and 25°C. High germination rates (>70%) were observed between 7.5°C to 15°C and the maximum germination success (95.9%) was obtained at 12.5°C (Fig. 4). The temperature "window" of the cysts (germination temperature range of the cysts) from Tokuyama Bay was 5°C higher than that of the cysts from Hiroshima Bay (*A. tamarense*).

Figure 5 shows the relationship between the incubation temperature and time to germination (days from the onset of the incubation experiment to the day of germination) observed in the cyst germination experiments. Germination time of the naturally occurring cysts in Tokuyama Bay was relatively short (ca. 5 days) at 25°C, while it was prolonged by about three times (ca. 17 days) at 7.5°C (Fig. 5). On the

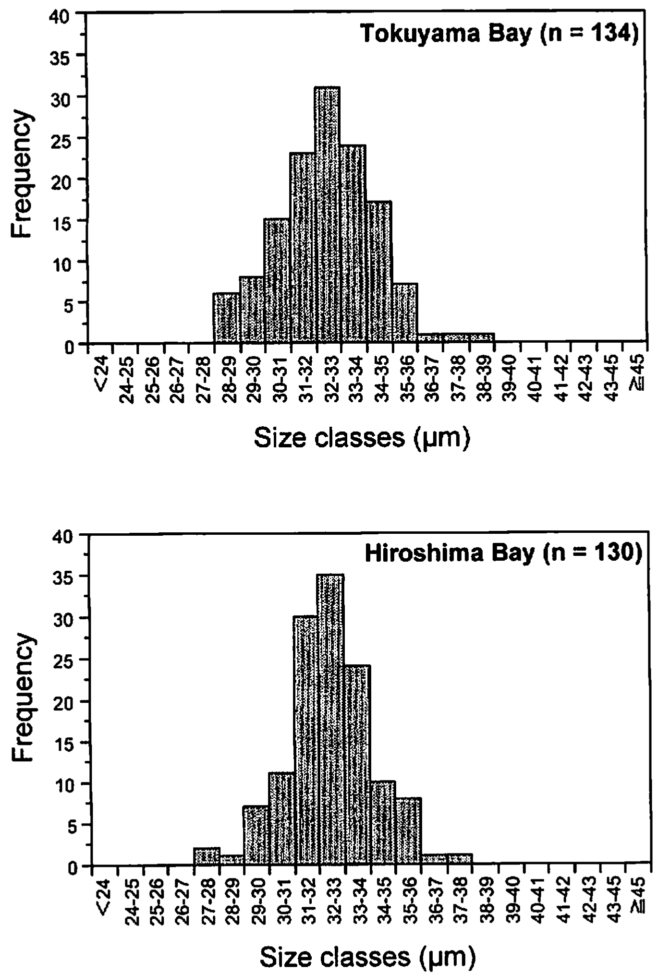


Fig. 3. Cyst width-frequency distributions of the naturally occurring *Alexandrium* cysts from Tokuyama Bay (upper) and Hiroshima Bay (lower).

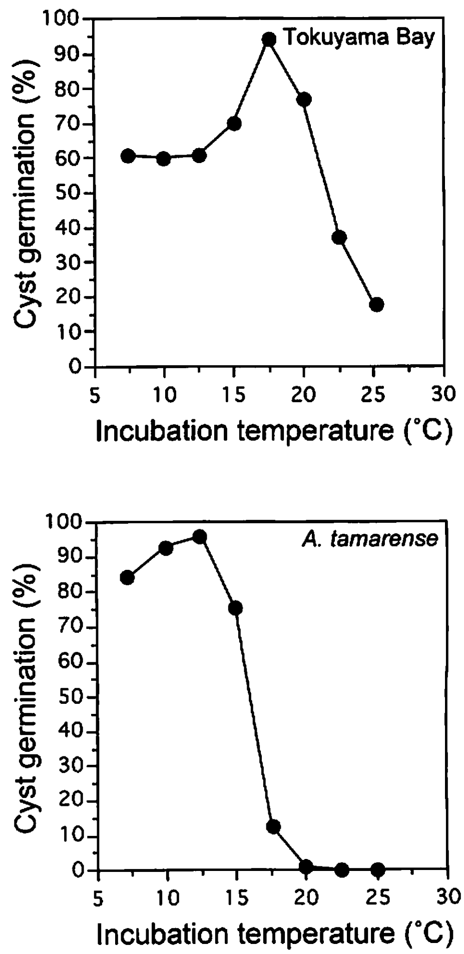


Fig. 4. Relationship between incubation temperature and germination success of naturally occurring *Alexandrium* cysts from Tokuyama Bay (upper) and laboratory produced *A. tamarense* cysts from Hiroshima Bay (lower).

other hand, *A. tamarense* cysts exhibited a nearly constant (ca. 10 days) germination time at a temperature range between 7.5°C to 20°C (Fig. 5).

Discussion

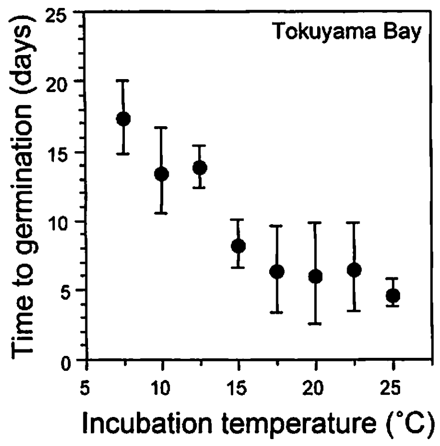
Size measurement of the cysts

In the present study, primuline-stained samples were used for size measurements of the cysts. This has several advantages as follows; primuline-staining allows ready location of cysts within sediment particles, and it also enables the whole cyst image to be observed even if the cyst is embedded in detritus (Yamaguchi et al. 1995b). Furthermore, the images of primuline-stained cysts under blue light excitation are suitable for image analysis on a computer, because this technique produces sufficient brightness and contrast. So, the primuline-staining method is useful not only for the enumeration of cysts, but also for the morphological analysis of cysts in bottom sediment.

The present data showed a difference in size composition of the *Alexandrium* cysts between Tokuyama Bay and Hiroshima Bay; i.e. the *Alexandrium* cyst population of Hiroshima Bay was composed of longer cells compared to the cyst population of Tokuyama Bay (Fig. 2). The observed difference in the length of the cysts indicate that there are some internal (genetic) or external (environmental) factors that separate these two *Alexandrium* cyst populations.

Germination temperature of naturally occurring cysts in Tokuyama Bay

Naturally occurring cysts in Tokuyama Bay could germinate at a temperature range between 7.5°C and 25°C, and the optimal germination temperature of the cysts was 17.5°C (Fig. 4). This optimal temperature for germination was 5°C higher than that of *A. tamarense* cysts (Fig. 4). Takeuchi (1994) and Hallegraeff et al. (1998) have shown the optimal temperature conditions for the germination of *A. catenella* cysts. Cyst germination of the Japanese *A.*



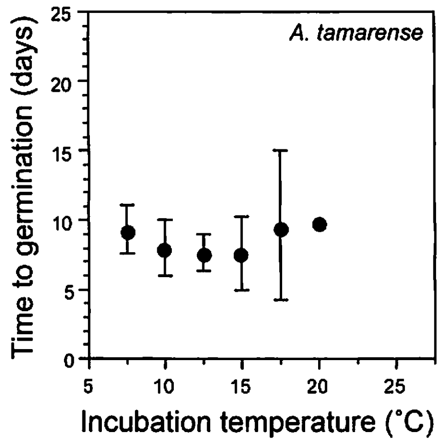


Fig. 5. Relationship between incubation temperature and germination time of naturally occurring *Alexandrium* cysts from Tokuyama Bay (upper) and laboratory produced *A. tamarense* cysts from Hiroshima Bay (lower).

catenella was most frequent at 20°C (Takeuchi 1994) while the optimal temperature for the Australian cyst germination was 17°C (Hallegraeff et al. 1998). Takeuchi (1994) also examined germination success and germination time of the *A. catenella* cysts at different incubation temperatures. The germination temperature window of the *A. catenella* cysts was 10°C to 25°C, and the germination time of the cysts was prolonged with a decrease in incubation temperature (Takeuchi 1994). All these germination characteristics of *A. catenella* cysts agree with those of the naturally occurring cysts in Tokuyama Bay (Figs. 4 and 5). In the present germination experiments, the morphological characteristics of 12 (germinated) vegetative cells were examined. All of the observed vegetative cells from Tokuyama Bay cysts were identical to *A. catenella*. So, it is most likely that the benthic cyst population in Tokuyama Bay is mostly composed of the cysts of *A. catenella*.

Germination temperature of laboratory produced *A. tamarense* cysts

In the present study, cyst germination of *A. tamarense* was observed within the temperature range between 7.5°C to 20°C, and the optimal germination temperature of the cysts was 12.5°C (Fig. 4). Anderson (1998) and Perez et al. (1998) have examined the germination temperature of *A. tamarense* cysts in Perch Pond (Cape Cod, MA USA) and the St. Lawrence estuary (Quebec, Canada). The germination temperature "window" for *A. tamarense* cysts in Perch Pond ranged from 5°C to 21°C, and it ranged from 4°C to 16°C in the St. Lawrence estuary, respectively. All these results agree well with our recent data on the germination temperature of naturally occurring *Alexandrium* cysts in Hiroshima Bay (Itakura and Yamaguchi 2001). Furthermore, in our previous study, the germination time of naturally occurring *Alexandrium* cysts in Hiroshima Bay was observed to be almost constant (ca. 10 days) regardless of the incubation temperature (Itakura and Yamaguchi 2001). Also this result is consistent with the present data on germination time of laboratory-produced *A. tamarense* cysts (Fig. 5), and it differs from the present data on the germination time of the cysts from Tokuyama Bay (Fig. 5). Furthermore, in our previous germination experiments on the naturally-occurring cysts from Hiroshima Bay, morphological characteristics of (germinated) vegetative cells were examined. All of the observed vegetative cells (28 germinated vegetative cells) from Hiroshima Bay cysts were identical to *A. tamarense*. Consequently, the greater part of the cysts in the bottom sediments of Hiroshima Bay are considered to be the cysts of *A. tamarense*.

Conclusion

The present results and the recent bloom records of *Alexandrium* species strongly suggest a difference in the species composition of the benthic cyst populations between Tokuyama Bay and Hiroshima Bay. Table 1 summarizes the size composition and germination characteristics of *Alexandrium* cysts from Tokuyama Bay and Hiroshima Bay, with reference to the cyst germination characteristics of *A. catenella* and *A. tamarense*. Germination characteristics of the naturally occurring cysts in Tokuyama Bay and Hiroshima Bay are in accordance with the germination characteristics of *A. catenella* and *A. tamarense*, respectively (Table 1). So, the observed difference in size composition of the cysts between the two bays indicates the possibility that the cysts of *A. catenella* and *A. tamarense* have different size (length) compositions. If this is the case, the cyst size composition would be important information for understanding the ecology and bloom dynamics of *A. catenella* and *A. tamarense*, and also for the analysis of past bloom events. However, further study is needed to clarify whether the different size compositions of the cysts is based on genetic or environmental factors.

Table 1. Size composition and germination characteristics of *Alexandrium* cysts from Tokuyama Bay and Hiroshima Bay, with reference to the cyst germination characteristics of *A. catenella* and *A. tamarense*.

	Tokuyama Bay	*Alexandrium catenella*	Hiroshima Bay	*Alexandrium tamarense*
Length of the cysts (Average)	33.8 to 68.5 μm* (49.1 μm)	—	42.6 to 62.9 μm* (54.2 μm)	—
Germination temperature window (Optimum)	7.5 to 25°C* (17.5°C)	10 to 25°C*** (20°C) (17°C)****	10 to 20°C** (15°C)	7.5 to 20°C* (12.5°C) 50 to 21°C***** 4 to 16°C******
Required time to germination	5 to 17 days* (temperature dependent)	4 to 27 days*** (temperature dependent)	*ca.* 10 days** (constant)	*ca.* 10 days* (constant)

* Data from the present study
** Itakura and Yamaguchi (2001)
*** Takeuchi (1994)
**** Hallegraeff et al. (1998)
***** Anderson (1998)
****** Pelez et al. (1998)

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