Germination Capability of Resting Cysts of *Alexandrium* spp. (Dinophyceae) Enclosed in the Fecal Pellets of Macrobenthic Organisms

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Abstract: A high abundance of *Alexandrium* spp. resting cysts occur in the fecal pellets of macrobenthic organisms in Hiroshima Bay. However, whether cysts enclosed within fecal pellets of macrobenthic organisms are able to germinate remains unclear. Therefore, cysts enclosed in fecal pellets were incubated for 29 days under two light intensities, sufficient light intensity for germination and near bottom light intensity at 24 m depth in Kure Bay, 50 μmol photons/m\(^2\)/s and 0.28 μmol photons/m\(^2\)/s, respectively. Subsequently, the germination frequency of cysts and the number of remaining cysts in the wells of microplates in each culture condition were compared. It was found that at 0.28 μmol photons/m\(^2\)/s the cysts remaining enclosed in the fecal pellets were mostly unable to germinate. For cysts extracted from the fecal pellets, 27% of the total cysts were not able to germinate at a light intensity of 50 μmol photons/m\(^2\)/s, and 75% at 0.28 μmol photons/m\(^2\)/s. Accordingly, at sites of similar depth to Kure Bay, it is suggested that resting cysts which are ingested and excreted in fecal pellets by macrobenthic organisms largely lose the ability to germinate.

Key words: *Alexandrium* spp., fecal pellets, macrobenthic organisms, resting cysts

INTRODUCTION

During the last decade, paralytic shellfish poisoning caused by the toxic dinoflagellate *Alexandrium tamarense* has occurred almost every spring in Hiroshima Bay, Seto Inland Sea, Japan (Yamamoto et al. 2002). A high density of resting cysts of *Alexandrium* spp. was observed on the bottom sediment of Hiroshima Bay (Yamaguchi et al. 1995b; Tsujino et al. 2001). The accumulated cysts of *Alexandrium* can lie dormant for a long time (Anderson 1984). The resting cysts have been considered to be important for the initiation and termination of blooms (Anderson 1978; Mggillinguddy et al., 2003). Persson (2000) reported the possibility of phytoplankton cysts predation by bivalves and copepods. In fact, the cysts of phytoplankton were observed within the fecal pellets of Copepoda (Reid & Boalch 1987). And Ichimi and Montani (2001) suggested that there were abundant cysts inside the fecal pellets of deposit feeder at some waters in Japan. There is a high density of macrobenthos in Hiroshima Bay, which is mainly composed of Poly-chaeta (Tsujino et al. 2001), and the resting cysts of *Alexandrium* spp. were observed within the fecal pellets of macrobenthic organisms (Tsujino et al. 2002). The resting cysts of *Alexandrium* spp. isolated from the fecal pellets had almost the same germination capability as those from the bottom sediment (Ichimi & Montani 2001; Tsujino et al. 2002). However, it has not been investigated whether the resting cysts of *Alexandrium* spp. can germinate in the condition enclosed in the fecal pellets of macrobenthic organisms in which light energy is reduced and excystment might be physically interrupted.

This study compared the number of vegetative cells germinated from the resting cysts enclosed inside and extracted from the fecal pellets under two light conditions to examine whether the resting cysts remaining enclosed in the fecal pellets of macrobenthos can germinate at sites of low light level such as the sampling site. At the end of the experiment, the number of cysts which did not germinate in each well of culture microplates under each culture condition was counted. The influence on germination of enclosure of the cysts within the fecal pellets of
macrobenthos is discussed.

**MATERIALS AND METHODS**

**Germination of Alexandrium spp. cysts remaining enclosed in the fecal pellets of macrobenthos at two different light levels**

Bottom sediment containing a high density of cysts of *Alexandrium* spp. was collected using a Smith-McIntyre grab sampler at one station (about 24 m depth) in Kure Bay of eastern Hiroshima Bay in April, 2000. The sediment collected was sieved through a 1 mm mesh to remove macrobenthos. The material obtained was placed in a plastic container and stored in the dark at 11°C until use.

The fecal pellets of macrobenthic organisms isolated from the bottom sediment and the cysts enclosed in them are shown in Fig. 1. In order to obtain the fecal pellets of macrobenthic organisms from the bottom sediment, 1 g wet weight (water percentage 81%) aliquots of well mixed sediment were sieved through a 100 μm mesh size 24 times. Half of the obtained fecal pellets were put into each well of two microplates (6 wells) with 6 mL filtered seawater (passed through a Whatman GF/C glass microfibre filter). The other half of the prepared fecal pellets were sonicated for 60 s with a sonicator (TAITEC, VP-60S) and sieved through a 20 μm mesh size to extract the cysts. The remaining cysts on the mesh were put into each well of two microplates (6 wells) with 6 mL of the filtered seawater. Pairs of plates, each pair with one plate containing cysts remaining in fecal pellets and the other plate with cysts extracted from the fecal pellets were incubated for 29 days under 50 μmol photons/m²/s or 0.28 μmol photons/m²/s. Temperature was regulated at 12.5°C, and photoperiod was set at 12-h light/dark.

During the experiments, 5 mL aliquots of the culture medium were sampled on the 7th, 10th, 13th, 16th, 22nd, 26th, and 29th day from each well of incubating 4 microplates to count the excysted vegetative cells. The samples were fixed with 0.5 mL of 0.1% glutaraldehyde. After sampling, 5 mL of filtered seawater was added to each well. All vegetative cells in the samples were counted under an inverted microscope (OLYMPUS, IX70).

**Number of non-germinated cysts**

Under each culture condition, the number of cysts which did not germinate in each well of culture microplates was counted. Enumeration of cysts was carried out using the primuline-staining direct count method (Yamaguchi et al. 1995a). For counting all cysts of *Alexandrium* spp. in the fecal pellets before and after the experiment, the fecal pellets were sonicated for 60 s with the sonicator, then sieved through a 20 μm mesh. The cysts retained on the mesh and the cysts not germinated in each well of culture plates were poured into 15 mL polycarbonate centrifuge tubes of which the volume was made up to 10 mL with sterilized seawater. The suspension was fixed with glutaraldehyde for 60 min., and then centrifuged at 700 × g for 10 min. After decantation, 5 mL of cold methanol was added to the pellet, which was then placed in a refrigerator overnight. Then the methanol was replaced with 10 mL of distilled water and to each tube was added 1 mL of the primuline stock solution (2 mg/mL). Samples were left for 1 h in the dark. After staining, the supernatant was removed using centrifugation and the pellet was re-suspended in distilled water to remove traces of the primuline solution. After centrifugation, the supernatant was decanted and finally the pellet was suspended in 5 mL of distilled water. The living cysts of *Alexandrium* spp. in the samples were counted under an epifluorescence inverted microscope (OLYMPUS, IX70) with blue light excitation, using a 400–440 nm band pass filter and a DM455 dichroic splitting mirror.

**Fig. 1.** The fecal pellet isolated from the sediment in Kure Bay of eastern Hiroshima Bay and the cysts enclosed inside the fecal pellet. A: Fecal pellet, B: Broken fecal pellet and appeared cysts.
RESULTS

Fecal pellets in the sediment

The dry weight of the fecal pellets contained in the bottom sediment of Kure Bay is 165 mg±13/g dry sediment. The shape of the fecal pellets was ordinarily elliptical. The size of those was 366 μm±70×198 μm±43 (n=200).

Germination of Alexandrium spp. cysts remaining enclosed in the fecal pellets of macrobenthos at two different light levels

The number of vegetative cells of Alexandrium spp. germinated from the cysts, both extracted from and enclosed inside the fecal pellets, are shown in Fig. 2. Under high light intensity condition (50 μmol photons/m²/s), the fluctuation on the number of vegetative cells germinated from the cysts was not different between the cysts enclosed inside and extracted from the fecal pellets (ANOVA, p>0.05). The vegetative cells germinated from cysts were first observed on the 7th day after the start of the culture at both the cysts enclosed inside and extracted from the fecal pellets. Thereafter, the number of vegetative cells germinated from cysts enclosed inside the fecal pellets was the largest on the 13th day, and after decreased. Those of cysts extracted from the fecal pellets progressed 50–100 cells/well from 10th to 30th day. On the other hand, the number of vegetative cells produced from the cysts enclosed inside and extracted from the fecal pellets was below 10 cells/well throughout the experiment under the low light intensity condition of the sea bottom at the sampling station (0.28 μmol photons/m²/s).

Number of non-germinated cysts

The number of resting cysts that remained non-germinated at the end of the experiment in each well is shown in Fig. 3. The mean number of cysts at the beginning of the experiment was 986±315 cysts/well. Under the light level of 50 μmol photons/m²/s, the number of cysts that did not germinate during the incubation was 392±99 cysts/well for the cysts enclosed in fecal pellets and 272±62 cysts/well for those extracted from the fecal pel-

![Fig. 2. The number of vegetative cells of Alexandrium spp. germinated from the cysts extracted and enclosed in the fecal pellets of macrobenthic organisms incubated under two light intensities (dashed lines, 50 μmol photons/m²/s and continuous line, 0.28 μmol mol/m²/s).](image)

![Fig. 3. The number of cysts not germinated and remaining in the wells of the culture microplates incubated under two light intensities (50 μmol photons/m²/s and 0.28 μmol mol/m²/s) at the end of the experiment.](image)
lets. Under the low light level of 0.28 μmol photons/m²/s, the number of non-germinated cysts was 1,007±132 cysts/well for the cysts enclosed in the fecal pellets and 740±114 cysts/well for the cysts extracted from the fecal pellets. At either light levels, the number of non-germinated cysts was more for the cysts remaining enclosed within the fecal pellets than cysts extracted from the fecal pellets. No difference in the number of cysts was observed between the start and the end of the incubation for the cysts enclosed in the fecal pellets under the low light intensity (two-sample t-test, p>0.05). For cysts extracted from the fecal pellets 27% of the beginning cysts under the light intensity of 50 μmol photons/m²/s, and 75% under 0.28 μmol photons/m²/s did not germinate.

**DISCUSSION**

Polychaeta was the most dominant macrobenthos and mollusca was the second most dominant group in Kure Bay. The most dominant species of polychaeta was Lumbrineris longifolia (Scoletoma longifolia, Imajima (2001)). Parapriprionospio sp. form B was the second most abundant. Mollusca was mainly Theora fragilis (Tsujino et al. 2000). Parapriprionospio sp. form A excretes feces in a cylinder-like shape with a diameter approximately one-third of the body width, which is 1.37 mm in adults (Yokoyama 1988). The mean size of the fecal pellets of T. fragilis is 382 μm±44×215 μm±17 (n=18) (Tsujino & Uchida 2004). Therefore, it is considered that these cylinder-like pellets extracted from the bottom sediment are feces excreted by macrobenthic organisms occurring in the sediment of Kure Bay.

In this experiment, under high light intensity (50 μmol photons/m²/s), more than 50% of the cysts enclosed inside the fecal pellets germinated. The number of cysts not germinated lastly in the wells enclosed inside the fecal pellets was slightly larger than that in the wells of the cysts extracted from the fecal pellets. This difference was not observed in the fluctuation of the number of vegetative cells germinated between the cysts enclosed inside the fecal pellets and those extracted from them (ANOVA, p>0.05). Accordingly, under high light intensity, it is estimated that the germination is not disturbed by enclosing the cysts inside the fecal pellets.

On the other hand, under low light intensity (0.28 μmol photons/m²/s) which is the same light level as the sampling site of the cysts used in the present study, the cysts within the fecal pellets were largely unable to germinate, whereas ca. 25% of the total cysts extracted from the fecal pellets were able to germinate in this light condition. Since the cysts inside the fecal pellets could germinate when exposed to a relatively high light intensity (50 μmol photons/m²/s), physical stress of being enclosed by the fecal pellets may not suppress excystment of the resting cysts. The fecal pellets are considered to reduce the available light energy for the excystment of the resting cysts of *Alexandrium* spp. within the pellets. This may cause inactivation of excystment of resting cysts at the sea bottom where the light level is low.

In the estuarine station of Hiroshima Bay, the number of *Alexandrium* spp. cysts in the fecal pellets of macrobenthos accounted for about 30% of the total cysts on April (Tsujino et al. 2001). Blooms of *Alexandrium tamarense*, which is a major species of *Alexandrium* in Hiroshima Bay, cease toward the end of May (Yamamoto & Yamasaki 1996). It is considered that the resting cyst production of *Alexandrium* spp. hardly occurs after the cessation of the bloom. Thereafter the proportion of the cysts enclosed in the fecal pellets to the total cysts may increase because macrobenthic organisms continue to ingest the resting cysts. Most of the cysts inside the fecal pellets of macrobenthos are alive (Tsujino et al. 2001), however, enclosure of the cysts within the fecal pellets is considered to suppress the excystment by reducing the available light energy at the sea bed especially where the light energy level is critically low.

In the case of the fecal pellets decayed, the cysts enclosed in these pellets can catch the chance of germination. But, as the fecal pellets have been excreted by macrobenthos, it is thought that as many as the percentage of the cysts distributed in the fecal pellets are certainly suppressed, when the sea water temperature is suited for germination of cysts in spring and autumn. It can be emphasized that macrobenthos plays an important role in the regulation of blooms of the toxic dinoflagellate *Alexandrium* spp. by enclosing the resting cysts within the fecal pellets.

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**REFERENCES**


Germination capability of resting cysts


