Comparative Study on the Toxic Effects of Red Tide Flagellates *Heterocapsa circularisquama* and *Chattonella marina* on the Short-Necked Clam (*Ruditapes philippinarum*)

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*Heterocapsa circularisquama* showed much higher toxic effects on short-necked clams than *Chattonella marina*. Clams exposed to *H. circularisquama* exhibited morphological changes concomitant with an accumulation of mucus-like substances in the gills, a profound reduction in filtration activity, and lysosomal destabilization in hemocytes. *Chattonella marina* was less effective than *H. circularisquama*, and *Heterocapsa triquetra* was almost harmless in all these criteria. These results suggest that *H. circularisquama* exerted its lethal effect on short-necked clams through gill tissue damage and subsequent induction of physiological stress.

Key words: red tide; phytoplankton; *Heterocapsa circularisquama*; *Chattonella marina*; short-necked clam

Harmful algal blooms or the so-called red tide frequently cause mass mortality of cultured fish, shellfish, and various wild marine living organisms. Various species of phytoplankton have been identified as causative organisms of red tide, and their toxic profiles are considered to be quite different depending on the species.1 Despite the long history of studies on the toxic or harmful effects of various red tide phytoplankton, the exact mechanism by which the phytoplankton can kill fish or shellfish remains to be clarified. The dinoflagellate, *Heterocapsa circularisquama*, is known to have caused mass mortality of bivalves in embayments of western Japan.2 Blooms of *H. circularisquama* can kill more than 12 bivalve species, but no harmful effects on wild fish populations, cultured fish, or on public health in general have so far been reported.3,4 The lethal effect of *H. circularisquama* on the pearl oyster (*Pinctada fucata*) has been confirmed under laboratory conditions.5 In contrast to the severe shellfish toxicity of *H. circularisquama*, no significant toxic effects of *Heterocapsa triquetra*, which is known as a morphologically analogous species to *H. circularisquama* and has worldwide distribution, on bivalves and other marine organisms have so far been observed.6 Matsuyama et al. have shown several lines of evidence supporting the idea that unstable toxic substances located on the surface of *H. circularisquama* cells may be responsible for its toxicity to bivalves.5 Since such other harmful dinoflagellates as *Gyrodinium aureolum* are known to kill shellfish, finfish and crustacean species, the shellfish-specific toxicity of *H. circularisquama* is a unique characteristic of this alga.7 We have found in our previous studies that *H. circularisquama* showed potent hemolysis toward rabbit erythrocytes through direct cell-to-cell contact, whereas *H. triquetra* showed no significant hemolytic activity.8,9 These findings suggested that *H. circularisquama* may exhibit its lethal effect on bivalves via a certain hemolytic toxin.

In contrast, *Chattonella* sp., a noxious raphidophyccean flagellate, is generally known to be highly toxic to fish, especially to the yellowtail, *Seriola quinqueteradiata*.10–13 It has been reported that a red tide of *Chattonella antiqua*, that occurred at Konagai (Nagasaki Prefecture, Japan) in August 2000 was associated with the mass mortality of cultured short-necked clams (*Ruditapes philippinarum*) as well as of other wild marine organisms including fish. This incidence suggests that *Chattonella* exerted harmful effects not only on fish, but also on shellfish. It was found in laboratory exposure experiments that *Chattonella marina* exhibited a lethal effect on short-necked clams through its direct detrimental action.14 Although details of the toxic mechanism of *Chattonella* on clams is still unclear, it has been speculated that gill tissue injury mediated by reactive oxygen species might be partly responsible for...
the toxicity as has been proposed as the fish-killing mechanism. It is generally considered that the sensitivity of clams to red tide phytoplankton can be influenced by various environmental factors, and the results obtained by laboratory exposure experiments have fluctuated according to the experimental conditions and physiological condition of the clams. There is a possibility that such environmental stress as depletion of dissolved oxygen might also be responsible for shellfish mortality by the incidence of red tide. Under these circumstances, we thought that a comparison between H. circularisquama and C. marina, in terms of their toxic effects on clams under the same experimental conditions, would be necessary to gain further insight into their toxic mechanisms towards shellfish. We therefore evaluated in this study the comparative effects of H. circularisquama and C. marina on short-necked clams. The effects of H. triqueta as a typical non-toxic flagellate were also examined.

H. circularisquama and H. triqueta were respectively isolated in Ago Bay and Hiroshima Bay. C. marina, which had been isolated in Kagoshima in 1985, was generously provided by Kagoshima Prefectural Fisheries Experimental Station of Japan. Clonal cultures of all these flagellate strains were obtained by repeated washing using capillary pipettes. The algae were cultured at 26 °C in a sterilized Erd-Schreiber modified (ESM) medium at pH 8.2 under illumination from a fluorescent lamp (30 mE/m²/S) with a cycle of 12 h light and 12 h dark. Flagellates in the exponential growing phase were used throughout the experiments, and all cultivation was conducted by using sterilized instruments. Cells were counted with a hemocytometer.

The culture supernatant was obtained from 200 mL of the exponential growth culture (3.0–4.0 × 10⁴ cells/mL) of each flagellate by centrifugation at 1000 × g for 10 min at 4 °C. A disrupted cell suspension was obtained by sonicating 200 mL of the exponential growth culture for 60 s at 20 °C in bath-type sonicator. Microscopic observation confirmed that all the cells had been ruptured by this treatment. Mortality tests were conducted at 23–24 °C in the dark on 10 individual short-necked clams (Ruditapes philippinarum) as described previously. In brief, short-necked clams with shell size from 25 to 40 mm (a shell length of 37 ± 4 mm) were exposed to each flagellate cell suspension (3.0–4.0 × 10⁴ cells/mL) in filtered seawater with mild aeration. The condition of each clam was observed at 12-h intervals. The clams were judged to be moribund by their lack of response to physical stimulation. The ESM medium alone was also tested under the same conditions as a control. The clearance rate due to clam filtration activity was measured by an indirect method, which was based on determining the time-course decrease in chlorophyll levels in the intact cell suspension and disrupted cell suspension, as a common indicator reflecting the amount of each sample. A quantitative analysis of the concentration of chlorophyll in each sample was carried out as previously described. A histological analysis of tissues of the clams exposed to a flagellate was conducted on a cross-section of gill tissue prepared and stained with Harris’ hematoxylin and eosin as previously described. The stained sections were then examined under an optical microscope. A neutral red (NR) lysosomal retention assay of the hemocytes was conducted by following the method previously described. In brief, hemocytes were withdrawn from a clam in physiological saline. The cell suspension was mixed with a neutral red solution and then incubated in the dark at room temperature for 1 h. The stained cells were examined under an optical microscope to evaluate the NR

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**Fig. 1.** Effects of Live Cell Suspensions (A and C) and Disrupted Cell Suspensions (B and D) of H. circularisquama, H. triqueta, and C. marina on the Viability (A and B) and the Clearance Activity (C and D) of Short-Necked Clams.

A and B. Toxic effects on short-necked clams. A. After exposure to a live cell suspension (3.0–4.0 × 10⁴ cells/mL) of H. circularisquama, H. triqueta (△), C. marina (□), or the ESM medium alone (○), the viability of the clams in each experimental group was examined after the indicated periods of time described in the text. B. After exposure to disrupted cell suspensions prepared from the same cultures of flagellates, the viability of the clams was similarly examined. C and D. Clearance activity of short-necked clams towards live cell suspensions (C) and disrupted cell suspensions (D) of H. circularisquama, H. triqueta, and C. marina. The clams were exposed to a live or disrupted cell suspension of H. circularisquama (○), H. triqueta (△), or C. marina (□), and then the level of each sample in the extra medium was estimated from the chlorophyll content after the indicated periods of time.
Cross-sections are shown of gill tissues prepared from clams exposed to the ESM medium alone (A), or to the H. triquetra (B), C. marina (C), or H. circularisquama (D) cell suspension. Cross-sections of the gill tissue of the clams in each group were prepared after 24 h of exposure and then microscopically examined. An arrow indicates the presence of a mucus-like substance, and the bar represents 20 μm.

Fig. 2. Cross-Section of the Gill Tissue of Short-Necked Clams after Exposure to a Live Cell Suspension of H. circularisquama, H. triquetra, or C. marina.

Cross-sections are shown of gill tissues prepared from clams exposed to the ESM medium alone (A), or to the H. triquetra (B), C. marina (C), or H. circularisquama (D) cell suspension. Cross-sections of the gill tissue of the clams in each group were prepared after 24 h of exposure and then microscopically examined. An arrow indicates the presence of a mucus-like substance, and the bar represents 20 μm.

Retention. At least 50 cells were scored as intact (NR retention by the lysosomes) or destabilized (NR leaking into the cytoplasm with lysosomal abnormality), the data being expressed as the percentage of cells with destabilized lysosomes per clump.

Figure 1A shows the mortality of short-necked clams after their exposure to various flagellate cells. H. circularisquama had a potent lethal effect on the clams, all the clams eventually dying within 60 h of exposure. Although C. marina had a slight toxic effect on the clams, it was evident that C. marina was less toxic than H. circularisquama. The group exposed to H. triquetra even showed a slightly higher survival rate than the control group exposed to the medium alone. This may have been due to a feeding effect of H. triquetra. The ultrasonically disrupted cell suspension of each of these flagellates had no significant toxic effect on the clams (Fig. 1B). The culture supernatant of each flagellate also had no toxic effect on the clams (data not shown). These results suggest that the live cell condition of the flagellates was essential, particularly for the potent clam-killing activity of H. circularisquama. The toxic effect of the live H. circularisquama cell suspension on the clams was also reflected by the significantly reduced clearance rate towards the flagellate cells (Fig. 1C). Consistent with the results of the mortality tests, the clearance rate of the clams for H. circularisquama cells was profoundly delayed, suggesting that H. circularisquama could inhibit the filtering activity of the clams. A slight decrease in the clearance rate for C. marina was observed during the early period of exposure, the delay being evident when compared with that of H. triquetra. The filtration process of a bivalve is generally influenced by such physical features of the target particles as size, density and electric charge, and by the entire morphol-

Fig. 3. Lysosomal Destabilization Rates of Hemocytes Prepared from Clams Exposed to the Live Cell Suspension (A), Disrupted Cell Suspension (B), and Cell-Free Culture Supernatant (C) of H. circularisquama, H. triquetra, or C. marina.

After exposure to the live cell suspension (A) (3.0–4.0 x 10^5 cells/mL) of H. circularisquama (A), H. triquetra (B), C. marina (C), or to the ESM medium alone (D), hemocytes were prepared from the clams in each experimental group, and a lysosomal destabilization assay was conducted as described in the text. After exposure to the disrupted cell suspension (B) or cell-free culture supernatant (C) prepared from the same cultures of flagellates just described, a lysosomal destabilization assay on the hemocytes was conducted in a similar manner.

ogy. Since H. circularisquama and H. triquetra are morphologically quite similar, the different clearance rates by the clams for these flagellates was obviously due to their different toxic potential towards the clams. The gills and such related tissues as ctenidia and labial palps of bivalves are also known to be involved in sorting the captured particles. There is thus the possibility that inhaled harmful flagellate cells might damage gill tissue which may lead to eventual death. In fact, as shown in Fig. 2, abnormally swollen gill tissue with the presence of a mucus-like substance was found in the clams exposed to H. circularisquama cells, while the gills of the clams exposed to the other flagellate cells was histologically almost indiscernible from the control gills. To our knowledge, this is the first evidence to show that histological change in the gill tissue of the clam was induced by H. circularisquama.

Lysosomes, which are typically involved in cellular defense, tissue repair and nutrition, can become destabilized through exposure to a variety of stressors. When lysosomal membranes are destabilized, the contents leak into the cytoplasm of the cells which ultimately causes
cell death.\textsuperscript{28,29} Lysosomal destabilization is therefore considered to be a well-established and sensitive biomarker of cellular stress in shellfish,\textsuperscript{30} for example, an elevated lysosomal destabilization rate has been demonstrated in oysters exposed to the raphidophycean flagellate, \textit{Heterosigma akashiwo}.\textsuperscript{22}

Figure 3 shows a gradual increase in the lysosomal destabilization rate observed in the clams exposed to the live cell suspension of \textit{H. circularisquama}, together with the exposure time, whereas \textit{C. marina} and \textit{H. triqueta} caused no significant increase in lysosomal destabilization. The disrupted cell suspension and culture supernatant prepared from each of these flagellates had no significant effect. These results suggest that only the live cell suspension of \textit{H. circularisquama} could induce significant stress in the clams among the samples tested.

It seems likely that the underlying toxic mechanisms of \textit{H. circularisquama} and \textit{C. marina} causing harmful effects on surrounding organisms are quite different. \textit{H. circularisquama} probably induced its lethal effect on the clams through certain bivalve-specific toxins located on the cell surface, while \textit{C. marina} might have caused gill tissue damage via reactive oxygen species or still unknown factors; fish, especially the yellowtail, may be highly susceptible to such gill tissue damage. Further studies are required to clarify these propositions.

In conclusion, our results suggest that \textit{H. circularisquama} caused gill tissue damage that may have led to general physiological stresses and eventual death. The living cell condition of the flagellate was essential to manifest the potent lethality towards clams among the clams than \textit{H. circularisquama} under the conditions used. Since no significant toxic effect of the disrupted flagellate cells was apparent, our results also suggest that physically disrupting the flagellate cells may lead to mitigating the harmful effects of red tide, at least against shellfish that can filter the ruptured cell debris. A combination of mechanically disrupting red tide flagellate cells and introducting appropriate shellfish cultures into a red tide field may be a promising mitigation strategy.

References