Journal of Shellfish Research, Vol. 30, No. 1, 177–186, 2011.

EFFECTS OF THE TOXIC DINOFLAGELLATE *HETEROCAPSA CIRCULARISQUAMA* ON LARVAE OF THE PEARL OYSTER *PINCTADA FUCATA MARTENSII* (DUNKER, 1873)

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ABSTRACT The effects of the toxic dinoflagellate *Heterocapsa circularisquama* on the activity rate, development rate, prevalence of damage, and survival rate of trochophore and D-shaped larvae of the pearl oyster *Pinctada fucata martensii* were studied in relation to *H. circularisquama* cell densities and exposure duration. In addition, larvae were regularly processed via scanning electron microscopy to investigate morphological damage. The activity rate of both larval stages was significantly decreased after 3–6 h of exposure to *H. circularisquama* at densities ranging from 100 to 2×10^4 cells/mL. The prevalence of damage was significantly high after 3–6 h of exposure to *H. circularisquama* at densities of 100 to 2×10^4 cells/mL and 5×10^3 to 2×10^4 cells/mL for trochophores and D-shaped larvae, respectively. Cytoplasmic discharge, mass mucus production, irregular shape, delayed or inhibited mineralization of the shell, mantle protrusion, the appearance of abnormal masses in the velum, and the exfoliation of the larvae cilia coupled with epithelial desquamation were frequently observed. The activity rate of D-larvae transformed from trochophores exposed to *H. circularisquama* for 12–48 h at densities ranging from 10 to 2×10^4 cells/mL was significantly reduced. The survival of D-shaped larvae plummeted to less than 0.013 for densities $\geq 5 \times 10^3$ cells/mL. The results indicate that *H. circularisquama* blooms have detrimental impacts on bivalves at early life stages. Blooms of *H. circularisquama* occurring during the spawning periods will influence the natural recruitment in *P. fucata martensii* and will have profound impacts on its population biology. Therefore, shellfish farms should not be built in coastal areas where *H. circularisquama* occurs, or genitors should be relocated during potential blooming periods.

KEY WORDS: *Heterocapsa circularisquama*, *Pinctada fucata martensii*, larvae, activity, damage, survivorship

INTRODUCTION

Harmful algal blooms (HABs) often cause serious economic loss worldwide as a result of the contamination and closure of bivalve harvests (Shumway 1990). Among HABs, approximately 58 species of dinoflagellates are known to induce toxic red tides associated with bivalve mortality (Burkholder 1998). Several studies considering the relationship between toxic dinoflagellates and the shellfish industry are available and deal mainly with toxin uptake, anatomic distribution, and depuration (Bricelj & Shumway 1998), but they also have examined the direct effects of the toxic algae on bivalve species. HABs producing dinoflagellates have been shown to induce several detrimental effects in bivalves, interfering with feeding activities (Cucci et al. 1985, Shumway & Cucci 1987, Lesser & Shumway 1993), shell valve behavior (Gainey & Shumway 1988), burrowing abilities (Bricelj et al. 2000, MacQuarrie & Bricelj 2008), and byssus production (Shumway et al. 1987), and compromising growth and survival (Widdows et al. 1979, Nielsen & Strömgren 1991, Luckenbach et al. 1993).

A relatively new toxic dinoflagellate species, *Heterocapsa circularisquama*, forms recurrent toxic efflorescences in Japan and has been associated with mass mortality of natural and cultured bivalves (Matsuyama et al. 1996), leading to serious hardship for shellfish fisheries and aquaculture industries (Matsuyama 1999). The dinoflagellate *H. circularisquama* was shown to induce several deleterious effects in juvenile and adult marine bivalves, ranging from behavioral alteration (Nagai et al. 2006, Basti et al. 2009) and impairments of the basic physiological functions of feeding and respiration (Matsuyama et al. 1997, Basti & Segawa unpublished results) to death (Matsuyama et al. 1992, Nagai et al. 1996, Yamatogi et al. 2004, Basti & Segawa 2010).

Because *H. circularisquama* appears to have established itself as a permanent resident of the Japanese central and western coastal areas, where it is forming recurrent, extensive toxic blooms (Matsuyama 2003a, 2003b), it is plausible that native shellfish species, including the commercially important pearl oyster *Pinctada fucata*, could be exposed at any stage of their life cycle. Red tides of *H. circularisquama* occur more frequently during the summer and autumn seasons (Matsuyama 2003a) and fall along the Japanese coasts at the time when the majority of bivalve species spawn. The timing, density, and geographical extent of *H. circularisquama* could play a role in the recruitment success of bivalves if there are detrimental effects on any particular life history stage.

The current study was designed to establish the causal relationship between *H. circularisquama* densities and persistency in the water, and potential toxic effects for *Pinctada fucata martensii*

TABLE 1.

Morphometric measures for *Pinctada fucata martensii* genitors.

Sex	*n*	Shell Length (mm)	Shell Height (mm)	Body Wet Weight (g)
Male	4	78.42 ± 1.89	29.3 ± 2.76	63.4 ± 7.74
Female	4	79.45 ± 3.05	29.32 ± 2.34	65.72 ± 5.99

Data expressed as mean ± SD.

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DOI: 10.2983/035.030.0125

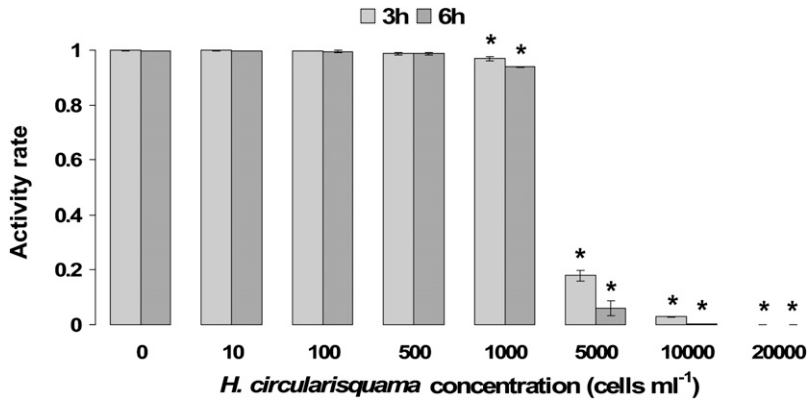

Figure 1. Activity rate of trochophore larvae of *P. fucata martensii* exposed to *H. circularisquama*. An asterisk indicates a significant difference from the relative control (ANOVA, Turkey-HSD, $P < 0.01$).

trochophores and D-shaped larvae. The activity rate, development rate, nature and prevalence of damage, and survival rate of larvae were examined.

MATERIALS AND METHODS

Larval Rearing

Sexually mature adult pearl oysters, *P. fucata* (Table 1), were reared at the K. Mikimoto & Co. Ltd farm, Ago Bay, Mie Prefecture, Japan. Oyster shells were opened with a shell opener and several incisions were made to the gonads. Gametes were obtained by stripping the oysters and filtering the gonad spills through gauze. Eggs and sperm were placed into 2-L and 1-L beakers, respectively, containing 0.75 mM ammonia–seawater solution for activation. The egg density in the suspension was determined by taking 3 samples under agitation. The density was then adjusted to 10^4 eggs/L. Only eggs showing a regular, round shape were used. The sperm quality was checked under a microscope and only those spermatozoa showing high swimming activity were used for fertilization. Eggs were activated for 45 min, and spermatozoa for 10 min. The eggs were then fertilized with spermatozoa for 10 min, washed with filtered (1.0-μm pore size) and UV-treated seawater, and transferred to 30-L tanks maintained at 25°C. Trochophores (12 h postfertilization) and D-shaped larvae (24 h postfertilization) were washed and used for the exposure experiments.

Alga Culture

The toxic *H. circularisquama* (strain HC92) was isolated from Ago Bay, Mie Prefecture, Japan, and cultured at 25°C in F/2 medium, under a 12-h light/dark photoperiod. After counting, the alga cells were added to the experimental seawater at the desired densities.

In Vivo *Exposure of Larvae*

Both trochophores and D-shaped larvae were exposed to *H. circularisquama* in triplicate at 0, 10, 100, 500, 10^3, 5×10^3, 10^4, and 2×10^4 cells/mL in 6- or 12-well plates, depending on the analysis to be performed. For each concentration of resuspended cell medium, 2 or 10 mL was transferred to each well chamber, and the experiments were performed under a 12-h light/dark photoperiod at 25°C for 72 h. The original volume of *H. circularisquama* culture medium was diluted with seawater several times before use. We assume that the alga culture medium has no effects on the pearl oyster larvae (Nagai et al. 1996). The trochophore and D-shaped larvae densities were set to 50 individuals/mL, and food was not provided.

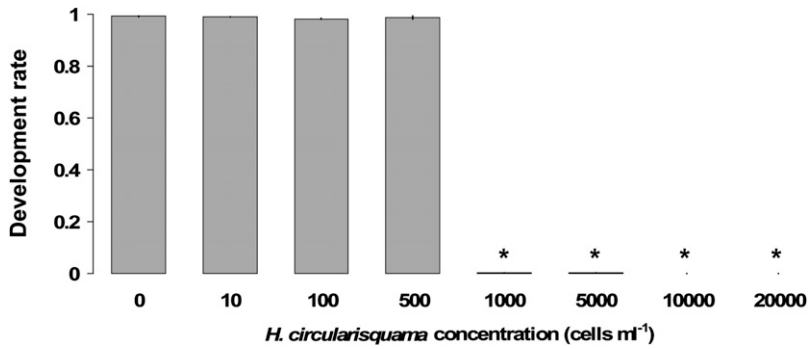

Figure 2. Development rate of trochophore larvae of *P. fucata martensii* exposed to *H. circularisquama* for 24 h. An asterisk indicates a significant difference from the relative control (ANOVA, Turkey-HSD, $P < 0.01$).

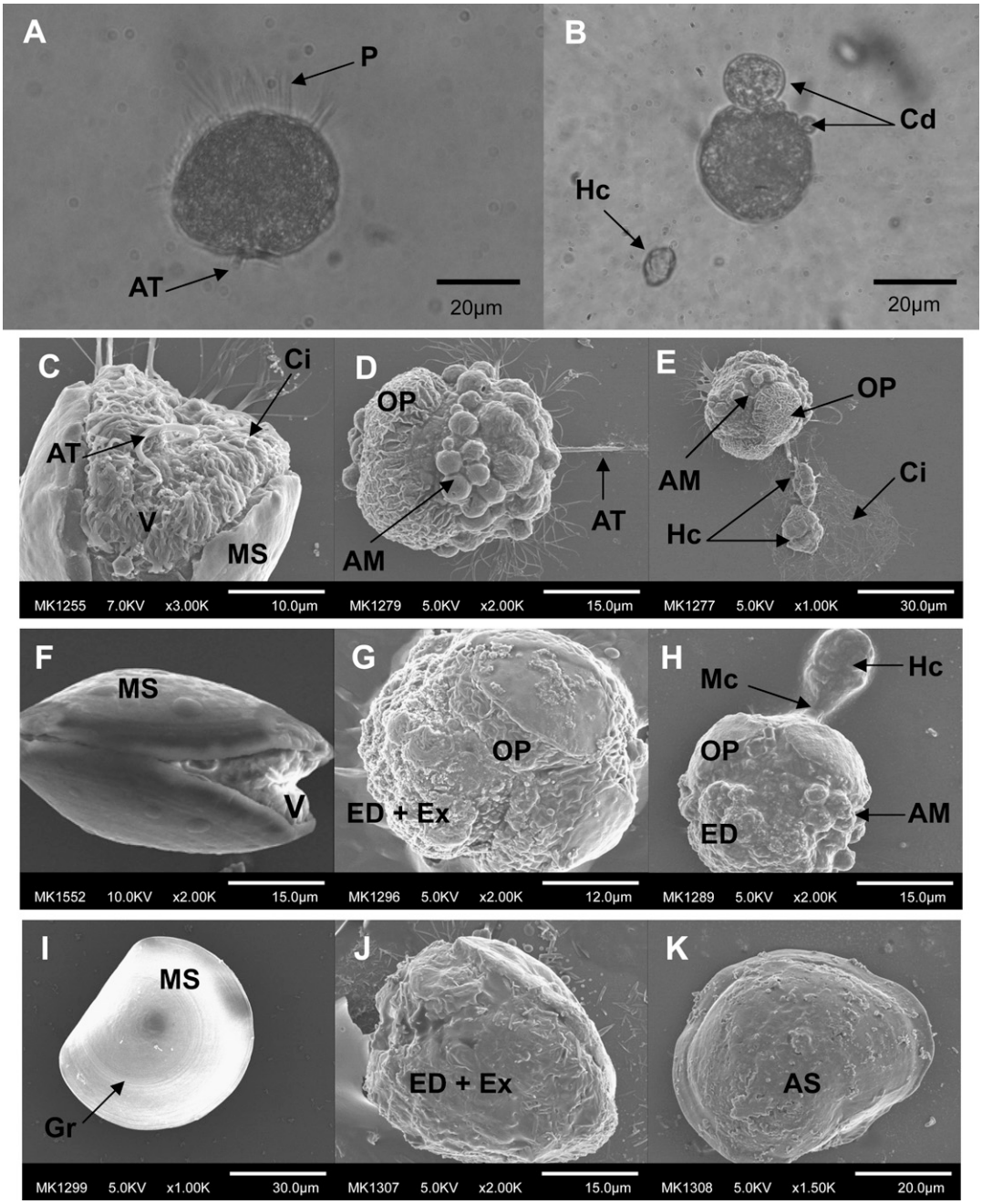

Figure 3. Light (LM) and scanning electron micrographs (SEM) of control and exposed *P. fucata martensii* trochophore larvae to *H. circularisquama* at 10^3 cells/mL. (A) Control trochophore 2 h postexposure (LM) showing normal body shape with the prototroch and apical tuft. (B) Trochophore exposed for 2 h (LM) showing cytoplasmic discharges. (C) Control trochophore (SEM) 3 h postexposure showing the animal pole developing into a sail of velum with the apical tuft in the center, and mineralization of the shell. (D, E) Trochophores (SEM) 3 h postexposure showing incomplete mineralization of the organic pellicle, abnormal masses in the velum, and *H. circularisquama* cells trapped in the detached cilia (F) Control trochophore (SEM) 6 h postexposure showing a fully developed velum and a further mineralized shell. (G, H) Trochophores (SEM) 6 h postexposure showing exfoliation of the velum cilia, epithelial desquamation, and hypersecretion of mucus entrapping *H. circularisquama* cell. (I) Control trochophore (SEM) 24 h postexposure fully developed into a perfectly shaped D-larva with a straight hinge and concentric growth rings. (J, K). Trochophores (SEM) 24 h postexposure showing heavier exfoliation of the velum cilia and epithelial desquamation, and abnormally shaped D-larva with incomplete mineralization of the shell. AM, abnormal mass; AS, abnormal shell; AT, apical tuft; Cd, cytoplasmic discharge; Ci, cilia; ED, epithelial desquamation; Ex, exfoliation; Gr, growth rings; Hc, *H. circularisquama* cell; Mc, mucus; MS, mineralized shell; OP, organic pellicle; P, prototroch; V, velum.

Effects on Trochophores

Trochophores exposed to *H. circularisquama* were regularly observed or sampled and fixed in a 5% formalin solution to determine the following:

Trochophore activity rate = number of actively swimming trochophores/total number of trochophores

Prevalence of abnormalities = number of abnormal trochophores/total number of trochophores

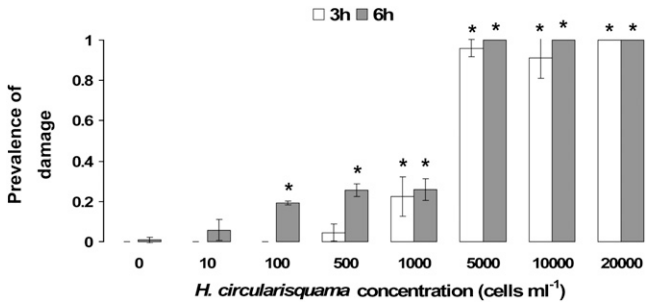

Figure 4. Prevalence of damage among trochophore larvae of *P. fucata martensii* exposed to *H. circularisquama*. An asterisk indicates a significant difference from the relative control (ANOVA, Turkey-HSD, $P < 0.01$).

Development rate = number of D-shaped larvae/total number of trochophores

Activity rate of transformed trochophores = number of actively swimming D-shaped larvae/total number of transformed D-shaped larvae

Effects on D-Shaped Larvae

D-shaped larvae exposed to *H. circularisquama* were regularly observed or sampled and fixed in a 5% formalin solution to determine the following:

D-shaped larvae activity rate = number of actively swimming D-shaped larvae/total number of D-shaped larvae

Prevalence of abnormalities = number of abnormal D-shaped larvae/total number of D-shaped larvae

Survival rate = number of alive D-shaped larvae/total number of larvae

Scanning Electron Microscopy

Trochophores and D-shaped larvae samples were fixed with 4% glutaraldehyde solution in 0.15 M sodium cacodylate trihydrate buffer, which contained 0.28 M sucrose (pH, 7.2), for 2 h at room temperature (18–20°C). After fixation, samples were dropped on 12-mm-round coverslips (Fisher Scientific, Germany) coated with a 0.01% poly-L-lysine solution (Sigma-Aldrich, St. Louis, MO). After the 30–40 min necessary for larvae to adhere to the coverslips, the samples were dehydrated in the following graded series of ethanol solutions for 30 min each: 25%, 50%, 70%, 85%, 90%, 95%, 100%, and 100%. The samples were then washed for 15 min each in a 50:50 solution of 100% ethanol and hexamethyldisilazane (HMDS; TCI, Japan). Last, 2 washes of 15 min each in 100% HMDS were conducted. Excess HMDS was removed by gentle pipetting, and samples were allowed to air-dry overnight at room temperature (16–18°C) (Dalo et al. 2008). After coating with an ion sputter (E-1030; Hitachi, Japan), samples were observed with a scanning electron microscope (S-4000; Hitachi) for morphological abnormalities.

Statistical Analysis

The normality and homogeneity of variance were tested *a priori* using the Kolmogorov-Smirnov test and Bartlett test, respectively. Data expressed as a rate were transformed by the angular transformation (arcsin$\sqrt{}$percentage) to ensure normality. The effects of *H. circularisquama* concentration and exposure duration were tested using factorial ANOVA. To determine the concentrations causing significant effects, the Newman-Keuls, Turkey-HSD or Bonferroni post hoc tests were used.

RESULTS

Effects on Trochophores

The activity rate of the trochophores significantly decreased after the 3-h exposure to *H. circularisquama* at densities $\geq 10^3$ cells/mL (Fig. 1), and the development rate was inhibited by exposure to the same densities for 24 h (Fig. 2).

The trochophores exhibited several anomalies, including cytoplasmic discharges, mass mucus production, delayed or inhibited mineralization of the shell, irregular shell shape, appearance of abnormal masses along the trochophore body, and exfoliation of the larval cilia with epithelial desquamation (Fig. 3). Cells of *H. circularisquama* were frequently observed attaching to trochophores, shedding their cell walls, and transforming into round, temporary cysts. The algal cells formed a heavy load for the trochophores, thereby altering their swimming behavior.

The prevalence of damage increased significantly after a 3-h and 6-h exposure to *H. circularisquama* densities $\geq 10^3$ cells/mL and ≥ 100 cells/mL, respectively (Fig. 4).

The activity rate of the D-shaped larvae that were transformed from the trochophores exposed to *H. circularisquama* decreased significantly after 12 h of exposure to densities $\geq 10^3$ cells/mL, 24 h of exposure to densities ≥ 100 cells/mL, and 48 h of exposure to densities ≥ 10 cells/mL (Fig. 5).

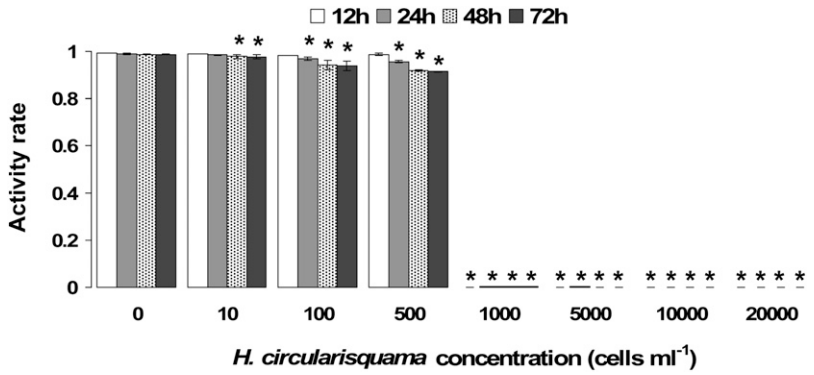

Figure 5. Activity rate of *P. fucata martensii* D-shaped larvae transformed from trochophore larvae exposed to *H. circularisquama*. An asterisk indicates a significant difference from the relative control (ANOVA, Turkey-HSD, $P < 0.01$).

TABLE 2.

Factorial ANOVA results for the effects of *H. circularisquama* densities (*H*) and exposure duration (*E*) on the activity rate, the prevalence of damage, the development rate, and the activity rate of transformed trochophores for *P. fucata martensii* trochophores.

	SS	df	MS	F	*P*
Activity rate					
H	13.552	7	3.358	1.936	0.000*
E	0.025	1	0.043	0.025	0.000*
$H \times E$	0.032	7	0.004	0.004	0.000*
Prevalence of damage					
H	18.834	7	2.690	274.44	0.000*
E	0.425	1	0.425	43.39	0.000*
$H \times E$	0.264	7	0.037	3.85	0.004*
Development rate					
H	7.791	7	1.113	10,013	0.000*
Activity rate of transformed trochophores					
H	30.799	7	4.399	21,128.8	0.000*
E	0.0.24	3	0.008	38.2	0.000*
$H \times E$	0.062	21	0.003	14.2	0.000*

* $P < 0.01$. SS, sum or squares; MS, mean of squares.

A factorial ANOVA and a 1-way ANOVA showed that both *H. circularisquama* concentration, exposure duration, and their interaction had significant effects on the activity rate, development rate, prevalence of damage, and activity rate of transformed trochophores (Table 2). The experiment duration had no effects on the control trochophores for any parameter of assessment.

Effects on D-Shaped Larvae

The activity rate of D-shaped larvae decreased significantly after 3 h of exposure to *H. circularisquama* at densities $\geq 10^3$ cells/mL. After 48 h and 72 h of exposure, the activity rate decreased for densities ≥500 cells/mL and ≥100 cells/mL, respectively (Fig. 6).

D-shaped larvae exposed to *H. circularisquama* showed several anomalies, including abnormal protrusion of the velum and mantle with *H. circularisquama* cells attaching to the velum, an irregular shell shape and hinge, abnormal masses in the velum, and exfoliation of the velum cilia with epithelial desquamation (Fig. 7). D-shaped larvae typically closed their shells when encountering *H. circularisquama* cells. However, the cells of *H. circularisquama* were frequently observed attaching to the velum or entrapped inside the shell, thereby altering the swimming behavior of the D-shaped larvae.

The prevalence of damage among D-shaped larvae increased significantly after 3, 12, and 24 h of exposure to *H. circularisquama* at densities $\geq 5 \times 10^3$ cells/mL, $\geq 10^3$ cells/mL, and ≥500 cells/mL, respectively (Fig. 8).

The survival rate of D-shaped larvae after 72 h of exposure was significantly lower than the control value for the *H. circularisquama* density of 10^3 cells/mL, but remained higher than 0.8. For densities $\geq 5 \times 10^3$, the survival rate plummeted to less than 0.013 (Fig. 9).

A factorial ANOVA and a 1-way ANOVA showed that both *H. circularisquama* concentration, exposure duration, and their interaction had significant effects on the activity rate, prevalence of damage, and survival rate of D-shaped larvae (Table 3). The duration of the experiment had no effects on the activity rate or the prevalence of damage in the control D-shaped larvae.

DISCUSSION

Susceptibility to H. circularisquama *Densities and Exposure Duration*

In the current experiments, *H. circularisquama* induced deleterious effects on the trochophore and D-shaped larva stages of the pearl oyster *P. fucata martensii*. The effects ranged from a reduced activity rate, to extensive damage, reduced or inhibited development, and a reduced survival rate. These effects were directly related to the cell densities of *H. circularisquama* and the exposure duration, which had synergistic effects.

Our results showed that an *H. circularisquama* density of 10^3 cells/mL was critical for trochophore larvae, inducing decreased activity after only 3 h and increasing damage, leading to the inhibition of development. The lower densities of 100–500 cells/mL had no effects on the activity and development, but induced some damage within 6 h and altered the activity rate of the transformed trochophores after 24 h. The lowest density of 10 cells/mL appeared safe for the trochophores, but the activity rate was altered for the transformed trochophores after 72 h.

For D-shaped larvae, an *H. circularisquama* density of 10^3 cells/mL was also critical and induced a decreased activity rate (3 h), increased damage (12 h), and decreased survival rate (72 h). The lower density of 500 cells/mL had no effect on the

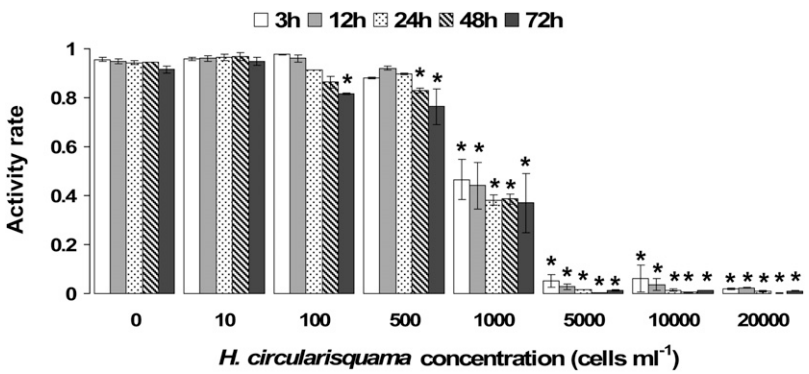

Figure 6. Activity rate of *P. fucata martensii* D-shaped larvae exposed to *H. circularisquama*. An asterisk indicates a significant difference from the relative control (ANOVA, Newman-Keuls, $P < 0.01$).

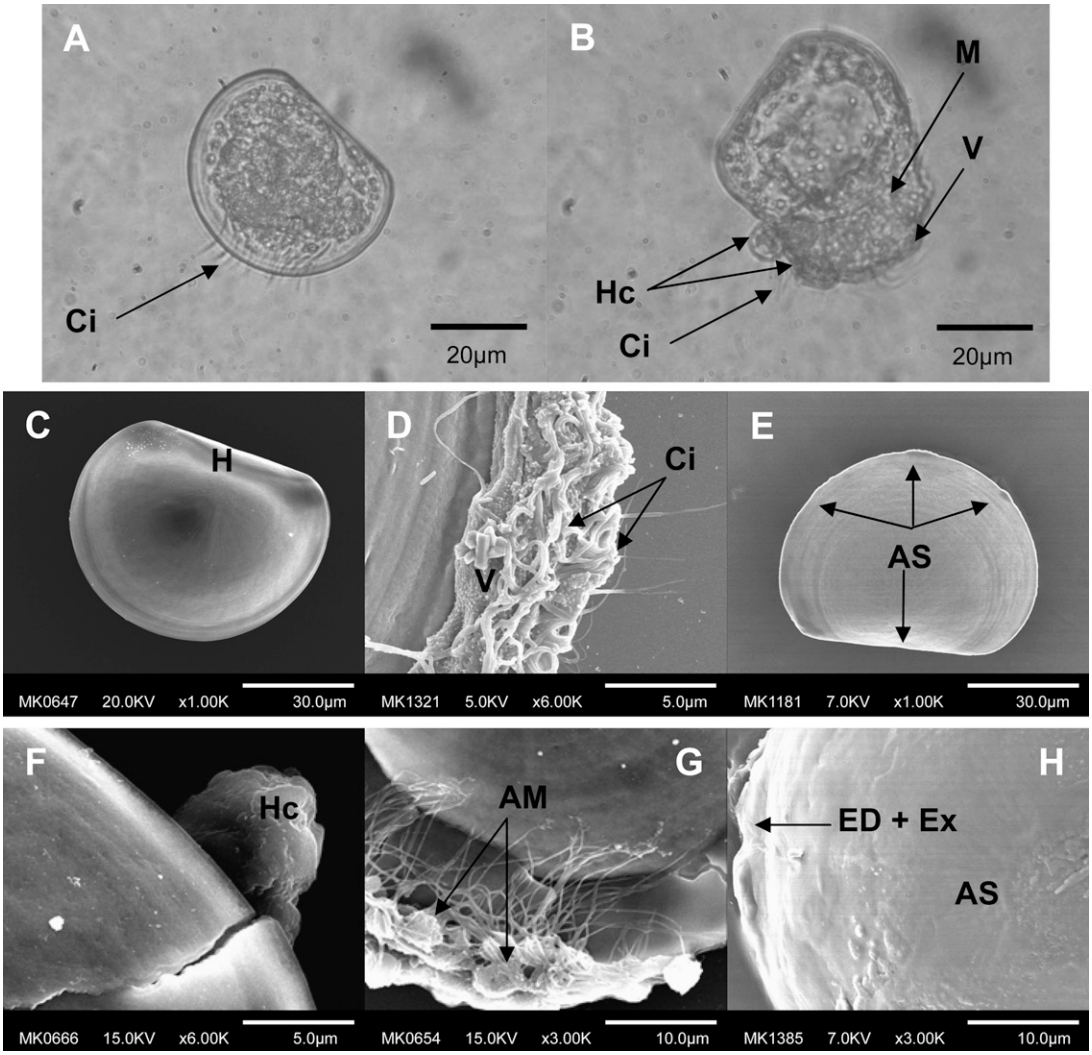

Figure 7. Light (LM) and scanning electron micrographs (SEM) of control and exposed *P. fucata martensii* D-shaped larvae to *H. circularisquama* at 10^3 cells/mL. (A) Control D-shaped larva 6 h postexposure (LM) showing normal D-shaped body with velum cilia slightly extending from the shell. (B) D-shaped larva exposed for 6 h (LM) showing abnormal protrusion of the velum and mantle, with *H. circularisquama* cells attached to the velum. (C, D) Control D-shaped larva (SEM) 24 h postexposure showing perfectly shaped larva with a straight hinge, and normal velum with the outer and inner band of cilia. (E, F) D-shaped larva (SEM) 24 h postexposure showing an abnormal shell, and *H. circularisquama* cells trapped inside the larva. (G, H) D-shaped larva (SEM) 48 h postexposure showing abnormal masses in the velum, and exfoliation of the velum cilia with epithelial desquamation and abnormal mineralization of the shell. AM, abnormal mass; AS, abnormal shell; Ci, cilia; ED, epithelial desquamation; Ex, exfoliation; Hc, *H. circularisquama* cell; M, mantle; V, velum.

survival rate but induced some damage (24 h), thereby altering the activity rate of D-shaped larvae (48 h). Thereafter, *H. circularisquama* densities of 100 cells/mL and 500–10^3 cells/mL were determined to be the critical concentrations for *P. fucata martensii* trochophores and D-shaped larvae, respectively.

Several studies previously established the deleterious effects of some toxic dinoflagellates on marine bivalve larvae. For instance, *Alexandrium tamarense* decreased the survival rate of larvae of the Japanese scallop *Chlamys farreri* after 6 days of exposure to 3×10^3 cells/mL (Yan et al. 2001), and decreased the activity rate of the bay scallop *Argopecten irradians concentricus* D-shaped larvae after 48 h of exposure to 10^4 cells/mL (Yan et al. 2003). *Karenia brevis* decreased the survival rate of larvae of the northern quahog *Mercenaria mercenaria*, the Eastern oyster *Crassostrea virginica*, and *A. irradians*, by exposure to 10^3–5×10^3 cells/mL, and decreased the development rate of *M. mercenaria* and *C. virginica* by exposure to 10^3 cells/mL (Leverone et al. 2006). Matsuyama et al. (2001) reported lethal effects of *A. tamarense*, *Alexandrium taylori*, *Gymnodinium mikimotoi*, and *H. circularisquama* on larvae of the Pacific oyster *Crassostrea gigas* over the cell density range of 100–10^3 cells/mL, which is the same critical range of cell densities for *H. circularisquama* reported in this study. In a previous work, Nagai et al. (1996) showed that densities of 2×10^4 cells/mL and 10^4 cells/mL corresponded to the LD_{50} levels for 2-mo postfertilization juveniles of *P. fucata martensii* exposed to the same strain of *H. circularisquama* used in our experiments. Therefore, the susceptibility of *P. fucata martensii* early life stages to *H. circularisquama* seems to decrease along the developmental process, making the trochophore larvae the most susceptible to *H. circularisquama* blooms.

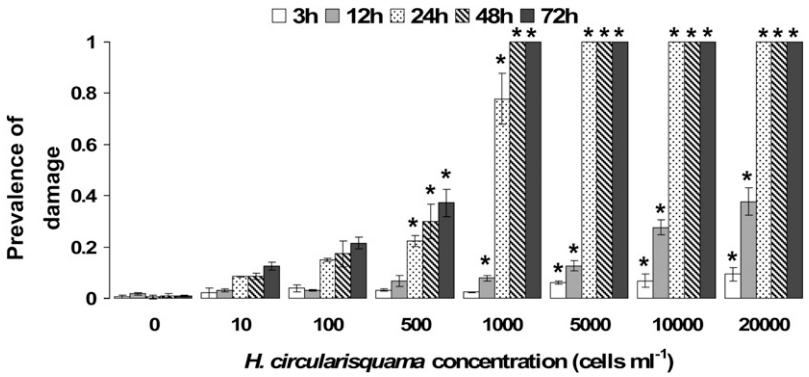

Figure 8. Prevalence of damage among D-shaped larvae of *P. fucata martensii* exposed to *H. circularisquama*. An asterisk indicates a significant difference from the relative control (ANOVA, Bonferroni, $P < 0.01$).

The effects observed for both larval stages occurred rapidly compared with the previously mentioned results of other studies dealing with other toxic dinoflagellate species. These observed effects prove that *H. circularisquama* is a highly potent HAB species (Kim et al. 2002). The deleterious effects were observed within a matter of a few hours, depending on the densities. Blooms of *H. circularisquama* in nature reportedly last several days, reaching up to 25×10^5 cells/mL (Matsuyama, 2003a). Consequently, the effects of this toxic alga on the early larva stages of bivalves could be extensive in the wild.

Harming Mechanisms

The toxic *H. circularisquama* may have affected both trochophores and D-shaped larvae by 3 possible processes: internal injuries resulting from consumption of the toxic alga (Wikfors & Smolowitz 1995), contact with toxins excreted in the water (Thain & Watt 1987, Yan et al. 2001), or direct cell-to-cell contact with the alga (Kamiyama & Arima 1997, Matsuyama et al. 1997).

Because trochophore larvae are unable to ingest food particles, only the effects observed for D-shaped larvae could be explained by the consumption of the toxic alga cells. The D-shaped larvae of *Mytilus galloprovincialis* were shown to ingest several toxic dinoflagellate species (*Alexandrium affine*, *Cochlodinium polykrikoides*, *Lingulodinium polyedrum*, *Prorocentrum minimum*, *Prorocentrum micans*, and *Scrippsiella trochoidea*) with mean equivalent spherical diameters of 12–38 μm. However, the feeding began 9–13 days postfertilization (Jeong et al. 2004). Similarly, early D-shaped larvae of the scallop species *A. irradians concentricus* and *C. farreri* were unable to feed on *A. tamarense* cells because of its relatively large size (Yan et al. 2001, Yan et al. 2003), which is similar to the *H. circularisquama* cell size of 20–28 μm (Horiguchi 1995). Consequently, the observed toxic effects on *P. fucata martensii* larvae could not be related to the internal toxicity arising from consumption of the alga, because trochophores do not ingest food, and 1 to 3-day-old D-shaped larvae are unable to graze on food particles as large as *H. circularisquama*.

The effects observed on the larvae could be related to toxic mechanisms through the contact with exotoxins secreted by *H. circularisquama* in the water. However, neither water filtrates of *H. circularisquama* culture nor its suspension of lysed cells induced any detrimental effect on marine bivalves (Matsuyama et al. 1997, Matsuyama 2003b), even though potent cytotoxic effects against mammalian cell lines were detected in the cell-free culture supernatant of *H. circularisquama* (Katsuo et al. 2007). In fact, *H. circularisquama* have 2 flagella and a theca with multiple cellulosic walls overlying the cell membrane (Horiguchi 1995). In this study, the cells of *H. circularisquama* were frequently observed attaching to the body of trochophores or the velum of D-shaped larvae. When the algal cells were swimming in the seawater, the contact between larvae and algal cells occurred. The algal cells then became inactive, liberated their cell wall into the larvae, and transformed into temporary, round cysts. These observations are supported by previous studies that revealed that *H. circularisquama* toxins are located

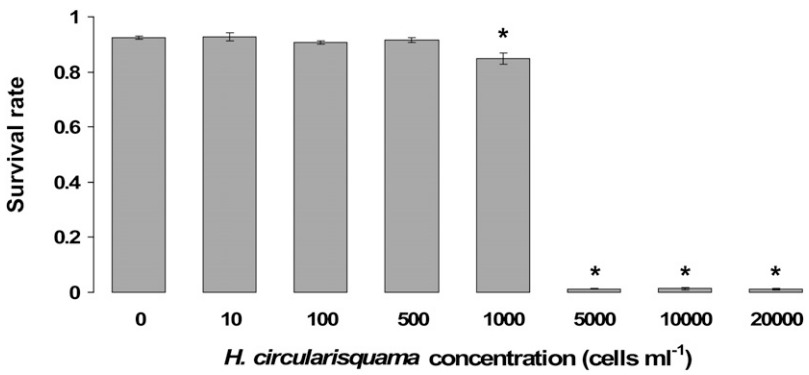

Figure 9. Survival rate of *P. fucata martensii* D-shaped larvae exposed to *H. circularisquama* for 72h. An asterisk indicates a significant difference from the relative control (ANOVA, $P < 0.01$).

TABLE 3.

Factorial ANOVA results for the effects of *H. circularisquama* densities (*H*) and exposure duration (*E*) on the activity rate and prevalence of damage for *P. fucata martensii* D-shaped larvae.

	SS	df	MS	F	P
Activity rate					
H	23.441	7	3.358	1,719.23	0.000**
E	0.171	4	0.043	22.04	0.000**
$H \times E$	0.118	28	0.004	2.16	0.012*
Prevalence of damage					
H	17.130	7	2.447	1,745.07	0.000**
E	14.293	4	3.573	2,548.12	0.000**
$H \times E$	6.651	28	0.237	169.39	0.000**
Survival rate					
H	4.963	7	0.709	2,282.23	0.000**

* $P < 0.05$. * * $P < 0.01$. SS, sum of squares; MS, mean of squares.

on the cell wall and are not excreted into the water, suggesting that the alga induces toxicity by cell contact with bivalve soft tissues (Matsuyama et al. 1997, Kamiyama & Arima 1997). In addition, the results showing that the D-shaped larvae seemed less vulnerable to *H. circularisquama* could be related to the fact that their soft body is protected by the larval shell, thus minimizing the chances of contact with the algal toxins. The early trochophore larvae have no such protective shell, and the ciliary apparatus augments the ratios of surface area to volume, thereby increasing the chances for contact and adherence between the toxic alga and larva. This might explain why the early trochophores are more susceptible to the toxicity of *H. circularisquama*, as suggested previously for the trochophore of *A. irradians* exposed to *Heterosigma akashiwo* (Wang et al. 2006).

The damage, revealed in our study through scanning electron microscopy, is proof of the cytotoxic effects of *H. circularisquama* in *P. fucata martensii* trochophores and D-shaped larvae. For both larval stages, there was a loss of the larval cilia, abnormal masses growing along the trochophore body or in the velum of the D-shaped larvae, and extensive exfoliation of the cilia with epithelial desquamation leading to death. In 2002, a new digalactosyl diacylglycerol was extracted from *H. circularisquama* and shown to induce cytotoxicity in the heart cells of oysters (Hiraga et al. 2002). In addition, the toxin Ha–2, extracted and purified from *H. circularisquama*, was recently shown to induce cytotoxicity against HeLa cells with high potency (Kim et al. 2008). Ha–2 tended to accumulate in the plasma membrane, and necrosis was proposed as the most plausible mechanism leading to cell death (Kim et al. 2008).

Many algal toxins are known to disrupt cellular ion homeostasis by specifically binding to certain membrane receptors involved in the regulation of cytosolic ions, notably Ca^{2+} (Blumenthal 1995). Saxitoxins, brevetoxin and its derivatives, and ciguatoxin bind to different sites of voltage-dependant sodium channels, resulting in either an inhibition or a persistent activation of the channels, with consequent increase in intracellular calcium (Kao & Walkwe 1982, Gutierrez et al. 1997, Hallegraeff et al. 1998, Dechraoui et al. 1999, Mattei et al. 1999, Van Dolah 2000, LePage et al. 2003). Domoic acid acts as an analog of the neurotransmitter L-glutamate that binds to the glutamate receptor, inducing a persistent activation of the receptor and also resulting in elevation of intracellular Ca^{2+} (Hampson & Manalo 1998, Scholin et al. 2000, Berman et al. 2002). In addition, both maitotoxin and azaspiracid-1 bind to voltage-gated calcium channels, resulting in increased cytosolic Ca^{2+} (Estacion 2000, Román et al. 2002, James et al. 2003, Alfonso et al. 2005, Kakizaki et al. 2006). Yessotoxin binds to voltage-gated calcium/sodium channels, also resulting in increased cytosolic Ca^{2+} (de la Rosa et al. 2001, Perez-Gomez et al. 2006). In addition, Perovic et al. (2000) showed that supernatants of many species of *Alexandrium* induce an increase in intracellular Ca^{2+}.

Matsuyama (2003b) reported an influx of Ca^{2+} in trochophore larvae of the short-neck clam *Ruditapes philippinarum* after exposure to *H. circularisquama*. Ca^{2+} is a critical signaling ion that plays a pivotal role in numerous physiological and biochemical processes of the cell—notably, signal transduction pathways, neurotransmitter release and synaptic plasticity, contraction of all muscle cell types, enzyme regulation, fertilization, shell formation, and death through apoptosis/necrosis, with the latter being associated with elevation of cytosolic Ca^{2+} (McConkey 1998, Berridge et al. 2000, Berridge et al. 2003, Orrenius et al. 2003). The current study showed that shell formation and survival of the pearl oyster larvae are both severely compromised by *H. circularisquama*, which further supports the hypothesis that *H. circularisquama* toxins must affect, either directly or indirectly, cell membrane integrity/permeability, thus interfering with the regulation of intracellular ions—notably, Ca^{2+}. This hypothesis can explain the extensive physiological and pathological alterations observed in the larvae, which ultimately die through apoptosis or necrosis.

Ecological Implications

Recurrent toxic blooms of *H. circularisquama* form along the western and central Japanese coastal area (Matsuyama et al. 1999) mainly during the summer–autumn season (Matsuyama, 2003a). These blooms coincide with the spawning period of almost all bivalves, and the blooms reach high cell densities and last for several days (Matsuyama 2003a, Matsuyama 2003b). The extensive damage observed in this study occurred rapidly and at lower densities than generally reported in the field. Therefore, *H. circularisquama* blooms will potentially affect the population biology of the pearl oyster, and possibly other marine bivalves, along the Japanese coast. The toxic alga was shown to induce the loss of ciliary structure and to cause extensive irreversible cytotoxicity to the larvae, thereby reducing their activity and food intake, and altering their sensory abilities, which ultimately result in starvation, susceptibility to predation, and death (Yan et al. 2003). It has been suggested that several toxic HAB species might produce the toxic substances as a strategy to protect their population from grazing species (Wang et al. 2006), to maintain their population at the ultimate blooming conditions, and to contribute to the decline of shellfish populations (Yan et al. 2001). In any case, *H. circularisquama* could have extensive detrimental effects on the recruitment of *P. fucata martensii* and other bivalves.

CONCLUSION

The toxic dinoflagellate *H. circularisquama* was found to induce adverse effects in the trochophore larvae and D-shaped larvae of *P. fucata martensii* at 100 cells/mL and 500–10^3 cells/mL,

respectively. The impacts ranged from decreased activity, to inhibition of development, increased damage, and decreased survival rate. The damage included exfoliation of larval cilia, epithelial desquamation, abnormal shells, and delayed mineralization of the shell. The harmful mechanism is likely to be direct cell contact.

Blooms of *H. circularisquama* are expected to have extensive toxic effects on pearl oyster larvae and will affect the recruitment of the species along the Japanese coast, causing further hardship to shellfish fisheries and aquaculture industries. Additional studies are required to assess the toxicity mechanisms in larvae at the cellular and molecular levels.

ACKNOWLEDGMENTS

This work was supported by a grant from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Japan (no. 17310027).

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