Journal of Shellfish Research, Vol. 30, No. 3, 829–844, 2011.

PHYSIOLOGICAL, PATHOLOGICAL, AND DEFENSE ALTERATIONS IN MANILA CLAMS (SHORT-NECK CLAMS), *RUDITAPES PHILIPPINARUM*, INDUCED BY *HETEROCAPSA CIRCULARISQUAMA*

LEILA BASTI,[1]* MAKOTO ENDO[2] AND SUSUMU SEGAWA[1]

[1]*Department of Ocean Sciences, Tokyo University of Marine Science and Technology, Shinagawa, Tokyo 108-8477, Japan;* [2]*Laboratory of Fish Health Management, Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Shinagawa, Tokyo 108-8477, Japan*

ABSTRACT In a laboratory study, we investigated the clearance rates (CRs), respiration rates (RRs), total hemocyte count (THC), pathological alterations, and mucocyte densities in the gills of the short-neck clam *Ruditapes philippinarum* when exposed to the toxic dinoflagellate *Heterocapsa circularisquama*. Within 2 h of exposure to *H. circularisquama* at a concentration of 5–10^3 cells/mL, both the CRs and the RRs were significantly decreased by 43–52% compared with the control CRs and 43–93% compared with the control RRs, respectively (ANOVA, Newman-Keuls, $P < 0.05$). Clams exposed to *H. circularisquama* at 10^3 cells/mL for 96 h showed a set of defensive and degenerative pathologies that were absent in control clams. Within 3 h, the gills exhibited cilia matting, followed within 24 h by heavy hemocytic infiltration in the connective tissue of plicae, distortion of filaments, hyperplasia, and fusion of adjacent filaments with matted cilia. Within 48 h, the gill plicae became contracted and showed multifocal epithelial hyperplasia, increased filament fusion, exfoliation of the frontal cilia, epithelial desquamation, and abnormal epithelial masses. After 72 h, the gills presented extensive necrosis of epithelial and connective tissues, atrophy and fusion of the filaments, large masses of gill debris and mucus, and extensive exfoliation and loss of the frontal, laterofrontal, and lateral cilia. After 96 h, the gills of moribund clams exhibited more advanced stages of necrosis and degeneration. Quantitative analysis of the pathological alterations showed that both the prevalence and intensity of the defensive pathologies increased significantly, reaching their maximal values after 24 h of exposure, then decreasing. Meanwhile, the prevalence and intensity of the degenerative pathologies continued to increase throughout the experiments (ANOVA, Newman-Keuls/Fisher's LSD, $P < 0.05$). In addition, significant decreases in the THC (*t*-test, $P < 0.01$) and the total, acid, and mixed mucocyte densities in the gills (ANOVA, Fisher's LSD, $P < 0.05$) were observed after 48 h of exposure. The current study clearly showed physiological, pathological, and defense alterations induced by *H. circularisquama* in clams, highlighting the occurrence of cytotoxicity and tissue repair failure. Inhibition of feeding and respiration as well as extensive necrosis in the gills, coupled with depression of defense mechanisms resulting from depletion of hemocytes and mucocytes, ultimately resulted in the death of the clams.

KEY WORDS: *Heterocapsa circularisquama*, *Ruditapes philippinarum*, physiology, pathology, hemocytes, mucocytes, cytotoxicity, necrosis

INTRODUCTION

The massive monospecific outgrowths of certain microalgae, commonly referred to as harmful algal blooms (HABs), are ancient and natural phenomena induced by a set of favorable environmental factors (Anderson et al. 1993, Fogg 2002, Landsberg 2002). In addition to harmful impacts in recreational areas where they form, HABs can cause anoxic conditions resulting in mass mortalities among marine organisms, resulting from respiratory failure, hemorrhaging, bacterial infection, starvation resulting from nutritional and size mismatches, and predation (Gainey & Shumway 1991, Rensel 1993, Wikfors & Smolowitz 1993, Wikfors & Smolowitz 1995, Smayda 1997).

A more serious condition of these HABs occurs when the outgrowing species is capable of synthesizing toxic substances that lead to severe damage among marine fauna, including fish, shellfish, birds, marine mammals, and other animals depending on the food web (Geraci et al. 1989, Landsberg & Steidinger 1998, Scholin et al. 2000, Flewelling et al. 2005). In addition, certain HAB species produce potent toxins that can be bioamplified throughout the food web to impact human health via the consumption of contaminated shellfish and fish or through water and aerosol exposure (Shilo 1967, Hansen et al. 2001, Kirkpatrick et al. 2004, Wang 2008). A relatively small number of microalgae, including approximately 80 species of dinoflagellates, diatoms, and cyanobacteria, are capable of causing toxic blooms; the majority of these species are dinoflagellates (Baden et al. 1995, Fleming & Baden 1998). There has been a marked increase in the incidence of HABs worldwide (Matsuyama & Shumway 2009), affecting many industries, notably shellfishery- and fishery-related businesses, and resulting in important economic losses associated with individual outbreaks (Shumway 1990, Anderson et al. 2000).

Among relatively recently reported HAB species, the dinoflagellate *Heterocapsa circularisquama* has been forming recurrent toxic blooms associated with mass mortality of marine bivalves (Matsuyama et al. 1996) in embayments of western and central Japan (Matsuyama 2003a), leading to serious hardship to shellfish industries (Matsuyama 1999). Recently, *H. circularisquama* was identified in water samples from Hong Kong Bay, China (Iwataki et al. 2002), and Bizerte Lac, Tunisia (Turki 2004). In 2004, Spatharis et al. confirmed the presence of *H. circularisquama* in southern Greece at high blooming densities associated with bivalve kills in 2004. The authors found that *H. circularisquama* is an established species in southern Greece, thereby raising concerns about its potential geographical spreading and blooms in the future.

The effects of *H. circularisquama* on several marine organisms were tested under laboratory conditions. This dinoflagellate was

*Corresponding author. E-mail: bastileila@gmail.com
DOI: 10.2983/035.030.0324

found to induce unusual locomotion and death in gastropods, to inhibit feeding and cause death in bivalves, to inhibit feeding in some jellyfish and protozoa, and to cause death among protozoa and dinoflagellates. However, crustaceans, finfish, copepods, and diatoms were not affected (Matsuyama 2003b).

Several works have focused on extracting, purifying, and identifying the toxins associated with *H. circularisquama,* and determining their activity. Initially, unstable toxic substances located on the surface of *H. circularisquama* cells were proposed to be the toxins responsible for the mortality of bivalves (Matsuyama et al. 1997). Subsequently, cell-free ethanol extracts from *H. circularisquama* were found to cause cytotoxicity in HeLa cells and the rotifer *Brachionus plicatilis,* and to exhibit hemolytic toxicity toward rabbit erythrocytes (Oda et al. 2001, Sato et al. 2002). The toxic activity of *H. circularisquama* extracts is photosensitizing, and its potency varies among strains (Kim et al. 2002). The dinoflagellate *H. circularisquama* is suspected to produce as many as 3 toxins, one of which, purified and labeled H2-a, has shown both potent photosensitizing cytotoxic activity against HeLa cells and hemolytic activity in rabbit erythrocytes (Miyazaki et al. 2005). In addition, a new digalactosyl diacylglycerol extracted and characterized from *H. circularisquama* exhibits cytotoxic activity toward oyster heart cell suspensions (Hiraga et al. 2002).

Despite previous efforts, studies of the effects of *H. circularisquama* on bivalves are relatively limited, and the underlying toxicity mechanism is still poorly understood. Available reports have addressed the effects of *H. circularisquama* on the mortality of pearl oysters, *Pinctada fucata;* Pacific oysters, *Crassostrea gigas;* blue mussels, *Mytilus galloprovincialis;* Asian mussels, *Musculista senhousia;* and the short-neck clam, *Ruditapes philippinarum* (Nagai et al. 1996, Matsuyama 2003b, Yamatogi et al. 2005, Nagai 2009, Basti & Segawa 2010); the clearance rates of *C. gigas* and *M. galloprovincialis* (Nagai et al. 1996, Matsuyama et al. 1999); the D-larvae, trochophores, eggs, and sperm of *C. gigas* and *M. galloprovincialis* (Matsuyama 2003b, Matsuyama et al. 2001); the trochophores and D-larvae of *R. philippinarum* (Basti et al. 2011); and valve movement behavior in *P. fucata* (Nagai et al. 2006) and *R. philippinarum* (Basti et al. 2009).

The original intent of this study was to gain a better understanding of the toxicity of *H. circularisquama* in marine bivalves by examining the physiological, pathological, and defense responses of a representative bivalve species, the short-neck clam, *R. philippinarum*, when exposed to this toxic alga under laboratory conditions. The clearance rate, respiration rate, nature, prevalence, and intensity of pathologies observed in the gills as well as the total circulating hemocytes and densities of mucocytes in clam gills were assessed.

MATERIALS AND METHODS

Clam Sampling and Acclimation

Clams of the species *R. philippinarum* were collected from Hakkeijima, southwest Tokyo Bay, Japan (35°20'13″ N, 139°38'04″ E). They were acclimated to the experimental conditions for 2 wk in 70-L tanks filled with filtered and continuously aerated seawater. Clams were fed daily on a single ration of the nontoxic microalga *Isochrysis galbana* (5×10^4 cells/mL). Prior to experiments, healthy clams showing regular filtration and responding to physical stimuli of their mantle and siphons were allowed to clear their gut contents for 24–48 h in static 20-L tanks filled with filtered (GF/C Whatman® pore size, 1.2 μm, Maidstone, UK) and continuously aerated seawater.

Algal Culture

Stock cultures of *I. galbana* and *H. circularisquama* (strain 92HC) were cultured in autoclaved modified SWM-3 medium (Chen et al. 1969, Itoh & Imai 1987), at 25°C, 33‰, and a pH of 7.8–8 under a 12-h light/12-h dark cycle. Illumination was provided by white fluorescent lamps at a light intensity of 120 mol photon/m^2/sec. The algae used for the experiments were collected during the late exponential to early stationary phase and added at desired concentrations to the experimental seawater.

Measurements of Physiological Rates

Clearance Rate

Adult clams ($n = 5$) were placed in preaerated transparent glass vessels containing 800 mL filtered seawater (Millipore Membrane filters, pore size 0.45 μm, Munster, Ireland) at 20°C, 33‰, and a pH of 7.8–8. Five concentrations of *H. circularisquama* were used in the experiments (5, 50, 250, 500, and 10^3 cells/mL). Cultures of *I. galbana* were added to the seawater to a total concentration of 5×10^4 cells/mL. The clearance rate (CR) of clams exposed to *I. galbana* at 5×10^4 cells/mL was defined as the control. This concentration was less than the threshold value for the biodeposition of organic materials as pseudofeces (Foster-Smith 1975, Widdows et al. 1979, Savina & Pouvreau 2004), and was tested *a priori*. Controls without animals regulated at the same alga concentration were used to correct for any spontaneous changes in algal densities resulting from algal settlement or reproduction. All experiments were performed in triplicate. Animals were allowed to resume filtration, and measurements were initiated when all clams opened their shells or extended their siphons.

CR was measured using the method of Coughlan (1969):

$$CR = \ln(F_0/F_t)V/t$$

where CR is the clearance rate (per hour per individual), V is the total volume of ambient water (in liters), F_0 and F_t are the fluorescence values between two sampling times; and t is the time increment (in hours).

Microalgal cell densities were determined based on measurement of chlorophyll fluorescence. Using a syringe, 5-mL samples were collected in triplicate from the center of each vessel. Samples were collected every 30 min for 2 h, and chlorophyll *a* was extracted immediately with 6 mL DMF (N,N-dimethyl-formamide, Tokyo, Japan) and stored at –5°C in complete darkness (Suzuki & Ishimaru 1990) until analysis with a fluorometer (10-AU-005, Turner Designs Inc., Sunnyvale, CA). Any small changes in the fluorescence value of the control were subtracted from the experimental rates.

Respiration Rate

Respiration rates (RRs, measured in milligrams of oxygen per hour) were measured for adult clams ($n = 3$) in sealed glass vessels containing 900 mL filtered (0.45-μm Millipore filter) seawater, and the experiments were run in triplicate. The water was preaerated, and the oxygen concentration was initially set

to 6 mg O_2/L. Controls without animals were performed to correct for any change in the oxygen concentration resulting from microrespiration.

The total concentration of food particles was set to 5×10^4 cells/mL, and the *H. circularisquama* concentrations considered as well as water temperature, pH, and salinity were the same as for the CR experiments. The decrease in the concentration of oxygen was measured every 30 min for 2 h with calibrated oxygen microelectrodes inserted in the sealed vessels and connected to oxygen meters (DKK, TOA Corp., Saitama, Japan). RR was then calculated using the following equation:

$$RR = [C_{t_0} - C_{t_i}] \times V \times 60/(t_i - t_0)$$

where RR is the rate of oxygen uptake (measured in milligrams of oxygen per hour), C_t is the concentration of oxygen in the water (measured in milligrams of oxygen per liter) at time t, V is the total volume of water in the sealed chamber (measured in liters), and t_0 and t_i are the initial and end times (measured in hours) for the measurement period.

Standardization of Physiological Rates

To relate the CR and RR to the dry tissue weight, the soft parts of the clams were removed from the shell and dried for 48 h at 80°C to a constant dry weight (Sobral & Widdows 1997, Pouvreau et al. 1999). CR was then expressed in terms of the weight-specific clearance rate (CRws, measure per hour per gram), and RR was corrected for the dry weight of the clams and expressed as the weight-specific rate (RRws, measured in milligrams of oxygen per hour per gram). A total of 90 clams were used to determine CRws, and 54 clams were used for RRws measurements (Table 1).

CRws and RRws were converted to a standard animal of 1 g tissue dry weight using the following equation (Bayne & Newell 1983):

$$Ys = (Ws/We)^b Y$$

where Ys is the physiological rate for an animal of standard weight, Ws is the standard weight (1 g), We is the observed weight of the animal (measured in grams), Y is the measured physiological rate, and b is the weight exponent for the physiological rate function. The average-weight exponents (b) for CRs and RRs calculated for several bivalves showed that they are generally approximately 2/3 and 3/4 for filtration and respiration, respectively (Savina & Pouvreau 2004). Thus, we used these values for CR and RR.

TABLE 1.

Morphometric measures for *Ruditapes philippinarum* clams used in the experiments.

Experimental Sets	*n*	Shell Length (mm)	Body Wet Weight (g)	Body Dry Weight (g)
Clearance rate	90	32.35 ± 1.17	—	0.26 ± 0.10
Respiration rate	54	31.41 ± 1.36	—	0.21 ± 0.07
Histology				
Light microscopy	68	30.69 ± 3.52	6.18 ± 2.55	—
Scanning electron microscopy	35	31.60 ± 4.43	6.34 ± 1.94	—
Mucocyte density	24	32.28 ± 3.27	7.68 ± 3.13	—
Total hemocyte count	20	29.61 ± 2.28	5.60 ± 1.50	—

Data expressed in mean ± SD.
—, not applicable.

Histopathology of Clam Gills

Exposure and Sample Processing

In another set of experiments, clams were exposed to *H. circularisquama* at a concentration of 10^3 cells/mL at 20°C, 33‰, and a pH of 7.8–8, and sampled every 3, 24, 48, 72, and 96 h. The gills were excised and immediately preserved for 2–4 days in either 10% buffered formalin solution or Bouin's solution for light and scanning electron microscopy, respectively. A total of 68 clams and 35 clams were processed for light and scanning electron microscopy, respectively (Table 1).

After dehydration in an ascendant ethanol solution series, bivalve gills were embedded in paraffin, and 5-µm gill sections were stained with hematoxylin–eosin. Slides were observed under a light microscope (Eclipse E600, NIKON, Kanagawa, Japan) to detect histopathological alterations.

For scanning electron microscopy, dehydrated gills were subjected to freeze drying (JFD–300, JEOL, Tokyo, Japan) and ion sputtering (E–1030, HITACHI, Tokyo, Japan), and then mounted on aluminum stabs for observation with an electron microscope (S–4000, HITACHI, Tokyo, Japan).

Quantitative Assessment of the Prevalence and Intensity of Pathologies

The total prevalence of pathologies, total intensity of pathologies, prevalence of each pathology, and intensity of each pathology in clam gills were assessed for each sampling time based on a semiquantitative approach, as follows:

$$\text{Total prevalence of pathology} = \sum (\text{Pathology}_{\text{Indiv.1}}, \ldots, \text{Pathology}_{\text{Indiv.N}})/\text{N}$$

$$\text{Total intensity of pathology} = \sum (\text{Intensity}_{\text{Indiv.1}}, \ldots, \text{Intensity}_{\text{Indiv.N}})/\text{N}$$

$$\text{Prevalence of pathology}_i = \text{Mean}\ (\text{Prevalence}_{\text{Pathologyi, Indiv.1}}, \ldots, \text{Pathology}_{\text{Pathologyi, Indiv.N}})$$

$$\text{Intensity of pathology}_i = \text{Mean}\ (\text{Intensity}_{\text{Pathologyi, Indiv.1}}, \ldots, \text{Intensity}_{\text{Pathologyi, Iindiv.N}})$$

where N is the number of clams processed for each sampling time.

Pathology was either absent (0) or present (1). The intensity of pathology was scored as mild (1), moderate (2) or heavy (3).

For the interpretation of the resultant averaged data, the prevalence was considered based on the following criteria: low, $0 \leq$ prevalence < 0.4; intermediate, $0.4 \leq$ prevalence < 0.8; and high, $0.8 \leq$ prevalence ≤ 1.0. Intensity was considered as follows: mild, $0 \leq$ intensity ≤ 1.00; moderate, $1.00 <$ intensity ≤ 1.90; heavy, $1.90 <$ intensity ≤ 3.00.

After 96 h, few clams (<10%) survived exposure to *H. circularisquama*, and therefore quantitative analysis of the prevalence and intensity of pathology could not be performed.

Defense Responses

Mucocyte Staining and Counting

Clam gills processed for light microscopy were used to determine the effects of *H. circularisquama* on mucocyte density (Table 1). After deparaffination and rehydration, the 5-µm sections were stained using the Alcian Blue–Periodic Acid Schiff

(AB–PAS) protocol described by Benninger et al. (1993), with slight modifications. The AB–PAS technique stains mucocytes in a range of colors from blue to pink, depending on the proportion of acid (AB–positive) and neutral (PAS–positive) mucopolysaccharides (MPS) they contain. The mucocytes were classified into 3 groups: acid (AMPS), which stain blue; mixed (MMPS), which stain with various shades of purple; or neutral (NMPS), which stain pink-reddish (Benninger & St-Jean 1997).

Stained gill sections were used to perform mucocyte counts. For each specimen, 3 gill plicae were chosen at random, and 10 ordinary filaments were chosen at random from each plica. For each filament, 5 random counting zones were considered. The mucocytes from each zone were counted, and the mean densities per zone were calculated for each secretion type and for all mucocytes combined.

Total Hemocyte Count

Clams were exposed to *H. circularisquama* at a concentration of 10^3 cells/mL. The total hemocyte count (THC) was determined for control and exposed clams (Table 1) after 24 h and 48 h of exposure. Hemolymph, typically 200 µL, was withdrawn from the anterior adductor muscle of each clam with a sterile 1-mL plastic syringe fitted with a 25-gauge needle. The syringe contained 200 µL 10 mM L-cysteine (Sigma) in autoclaved seawater, pH 7.5, maintained on ice to avoid hemocyte agglutination. The hemolymph collected from each clam was stored separately and never pooled. THC was determined for each individual using a hemocytometer and direct counting under a light microscope.

Statistical Analyses

Normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene's median test) were checked *a priori*. When the parametric assumptions were met, analysis of variance was performed on the data. Otherwise, data transformations (angular or logarithmic) were considered. To test the effects of *H. circularisquama* densities on the determined physiological rates, 1-way ANOVAs were performed, followed by the Newman-Keuls post hoc test. In addition, factorial ANOVAs were performed, followed by the Newman-Keuls or Fisher's LSD post hoc tests to test the effects of *H. circularisquama* density and exposure time on the prevalence and intensity of the pathologies present. To test the effects on mucocyte density, factorial ANOVAs were performed, followed by Fisher's LSD post hoc test. Student's *t*-test was used to compare THCs between 2 groups.

RESULTS

Effects on Clearance Rate and Respiration Rate

Both CRs and RRs of *R. philippinarum* were significantly affected after 2 h of exposure to *H. circularisquama* (ANOVA, $P < 0.01$). A significant decrease of CRs was observed at all considered concentrations by approximately 43–52% compared with the control. Similarly, RRs decreased significantly for exposure to all *H. circularisquama* cell densities by 43–93% of the control value (Fig. 1).

Exposure to the toxic alga induced a brief, incomplete shell closure and triggered valve adductions and the production of pseudofeces, which were rejected through the exhalant siphon. The pseudofeces were dense, viscous, and contained *H. circularisquama* cystlike cells.

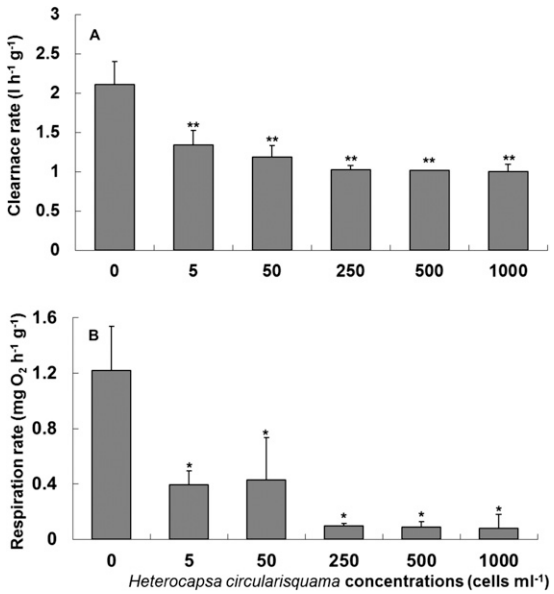

Figure 1. (A, B) Mean (±SE) standardized clearance rate (A) and respiration rate (B) for *Ruditapes philippinarum* fed *Heterocapsa circularisquama* at different cell densities in a mixture with *Isochrysis galbana* (total cell density, 5×10^4 cells/mL). *Significant difference from control (ANOVA, Newman-Keuls, $P < 0.05$). **Significant difference from control (ANOVA, Newman-Keuls, $P < 0.01$).

Histopathology of Clam Gills

Identification of Pathologies

Light and scanning electron micrographs of control and exposed clam gills are shown in Figure 2 and Figure 3, respectively. Gills of *R. philippinarum* exhibit an organization typical of heterorhabdic eulamellibranchiate bivalves. The gill plicae are formed by a variable number of ordinary filaments and principal filaments in the depressions between adjacent plicae (Figs. 2A, 3A). A monolayer of epithelial cells laying on a basal lamina is supported by connective tissue (Fig. 2B). Several blood sinuses provide a constant supply of hemocytes in to and out of the gills. Both ordinary and principal filaments support 3 types of cilia: short frontal and mid-long lateral cilia, and long laterofrontal cilia with microvilli (Figs. 2B, 3B). Adjacent gill lamellae are held together by interlamellar junctions rich in muscle fibers (Fig. 2A).

Exposure of clams to *H. circularisquama* induced several gill anomalies that were not observed in the gills of control animals, which occurred at different exposure durations (Figs. 2C–I, 3C–K). The pathologies identified in clam gills could be grouped into two major categories: defensive pathologies, consisting of hemocytic infiltration in connective and epithelial tissues, hyperplasia, and hypertrophy; and degenerative pathologies, consisting of filament fusion, cilia matting and/or exfoliation, epithelial desquamation, necroses of connective and epithelial tissues, and atrophy.

Exposure to *H. circularisquama* for 3 h triggered hemocytic infiltration in epithelial tissue and connective tissue (Fig. 2C). The cilia became disorganized, lost flexibility, and became matted in a layer of mucus (Fig. 3C, D). After 24 h of exposure, the clam gills continued to exhibit hemocytic infiltration in connective and epithelial tissues, with hypertrophy and necrosis being observed (Fig. 2D). The filaments became loose and distorted, and were

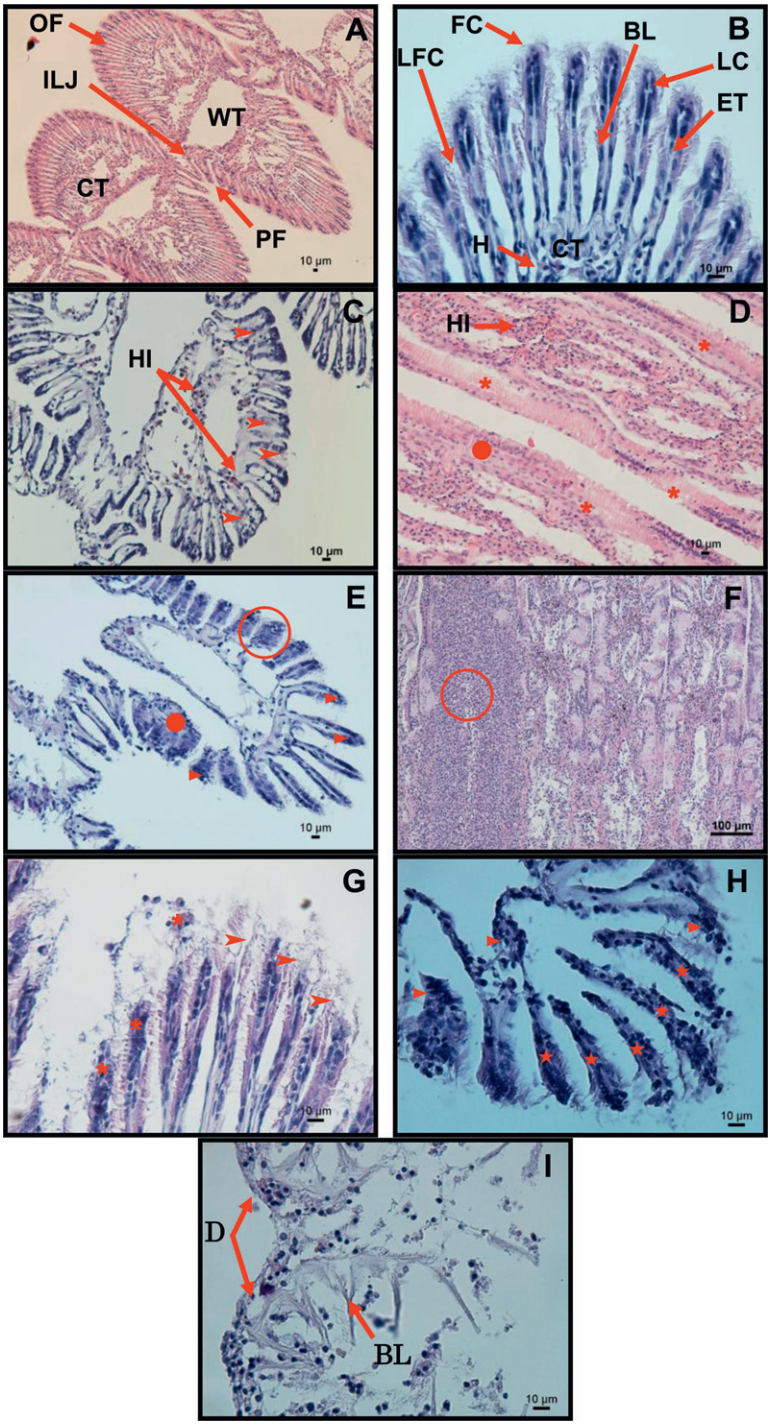

Figure 2. Light micrographs of *Ruditapes philippinarum* gills. (A, B) Control. (C-I) Exposed to *Heterocapsa circularisquama* at 10^3 cells/mL for 3 h (C), for 24 h (D), for 48 h (E, F), for 72 h (G, H), and for 96 h (I). BL, basal lamina; CT, connective tissue; D, degeneration; ET, epithelial tissue; FC, frontal cilia; H, hemocyte; HI, hemocytic infiltration; ILJ, interlamellar junction; LC, lateral cilia; LFC, laterofrontal cilia; OF, ordinary filaments; PF, principal filaments; WT, water tube; ➤, cilia matting; *, epithelial desquamation; ●, hypertrophy; ○ hyperplasia; ►, necrosis; ★, atrophy.

associated with large mucus balls and gill debris (Fig. 3E, F). After 48 h of exposure, the gills became contracted and exhibited multifocal epithelial hyperplasia and filament fusion, detachment of filament cilia with their respective epithelial cells, and additional necrotic nuclei in the epithelium (Fig. 2E, F). Scanning electron micrographs showed large areas of gill filaments devoid of frontal cilia, along with fusion and disorganization of gill filaments. Abnormal epithelial masses were also frequently observed, together with epithelial desquamation (Fig. 3G, H). After 72 h of exposure, the necrotic appearance of the epithelium and connective tissues, and the atrophy of the filaments extended throughout the gills (Fig. 2G, H). The gills were completely

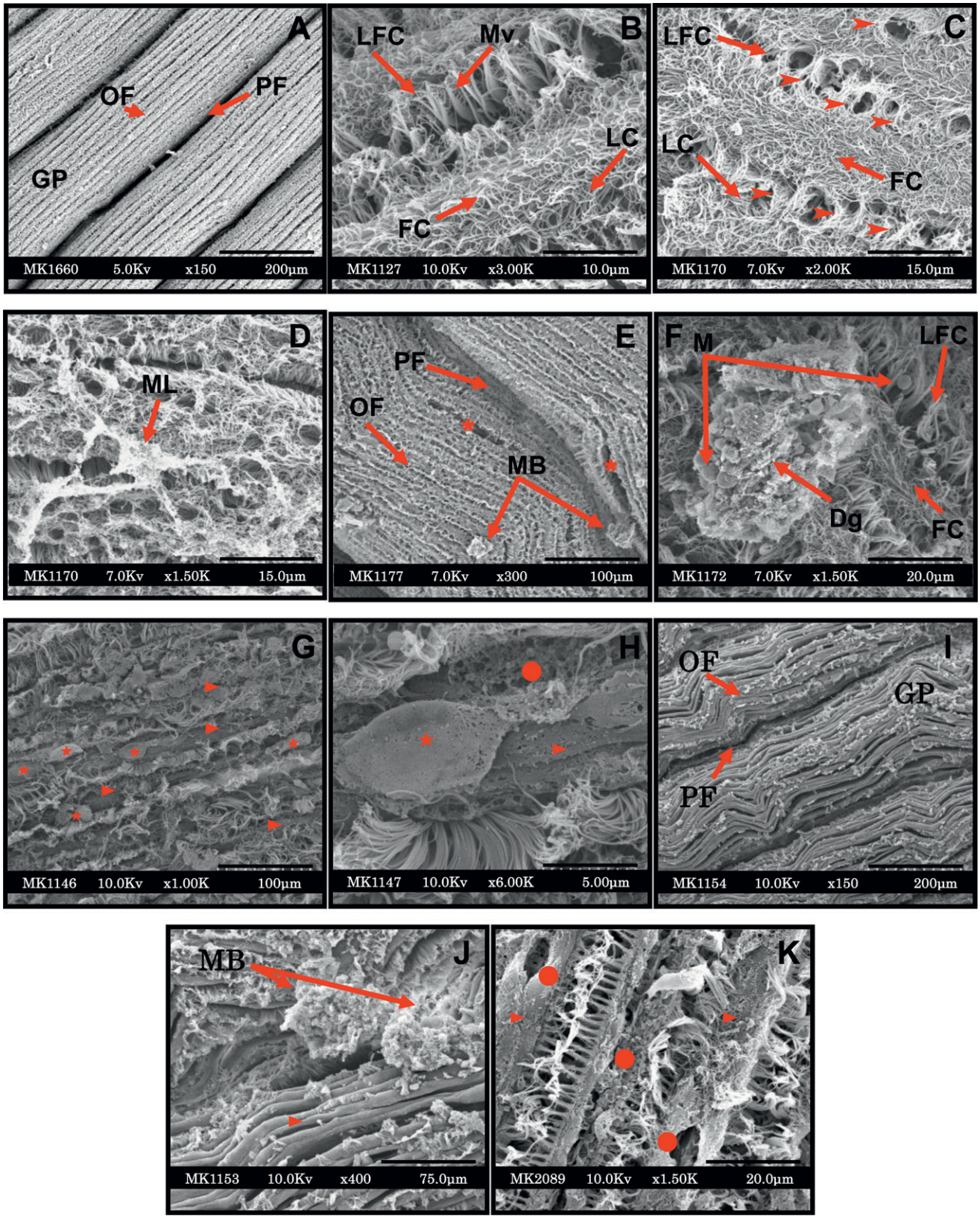

Figure 3. Scanning micrographs of *Ruditapes philippinarum* gills. (A, B) Control. (C–K) Exposed to *Heterocapsa circularisquama* at 10^3 cells/mL for 3 h (C, D), for 24 h (E, F), for 48 h (G, H), for 72 h (I, J), and for 96 h (K). FC, frontal cilia; GP, gill plica; LC, lateral cilia; LFC. laterofrontal cilia; MB, mucus ball; ML, mucus layer; Mv, microvilli; OF, ordinary filaments; PF, principal filaments;. ➤, cilia matting; *, filament distortion; ►, cilia exfoliation; ●, epithelial desquamation; ★ abnormal mass.

denuded from all ciliary structures, including not only the frontal but also the laterofrontal and lateral cilia. Extensive thinning and fusion of gill filaments and lamellae were observed along with extremely large masses of both mucus and gill debris formed by cilia and epithelium debris (Fig. 3I, J). After 96 h, the gills of moribund clams showed an advanced stage of necrosis and degeneration of the epithelial and connective tissues, and an advanced stage of epithelial and connective desquamation (Figs. 2I, 3K).

Quantitative Analysis of Pathologies

The effects of *H. circularisquama* exposure and its duration on the total prevalence and total intensity of pathologies, and the prevalence and intensity of each pathology are presented in Table 2. The total prevalence and total intensity of pathologies were both significantly affected by *H. circularisquama* exposure, the exposure duration, and their interaction. In addition, the prevalence and intensity of all degenerative pathologies were significantly affected by *H. circularisquama* exposure, the exposure duration, and their interaction. The intensity of the defensive pathologies was also affected by *H. circularisquama* exposure, the exposure duration, and their interaction, but their prevalence was not affected by the exposure duration. The prevalence and intensity of hypertrophy were affected only by *H. circularisquama* exposure. The prevalence of hemocytic infiltration in epithelial tissue was affected by *H. circularisquama* exposure, the exposure duration, and their interaction; however, the intensity of this parameter was affected only by *H. circularisquama* exposure. In contrast, the intensity of hemocytic infiltration in connective tissue was affected by *H. circularisquama* exposure, the exposure duration, and their interaction, whereas the prevalence of this parameter was not affected throughout the course of the experiments.

The time-course changes in the prevalence and intensity of pathologies are given in Table 3 and Table 4, respectively. The total prevalence and total intensity of pathologies increased significantly over 24 h to 72 h of exposure compared with the respective controls, with the highest prevalence being observed after 48 h of exposure, whereas the highest intensity was detected after 72 h of exposure. Overall, the pathologies occurred with a low prevalence and low intensity after 3 h of exposure, with an intermediate prevalence and moderate intensity after 24 h of exposure, with a high prevalence and moderate intensity after 48 h of exposure, and with a high prevalence and heavy intensity after 72 h of exposure.

Both the prevalence and intensity of the defensive pathologies increased significantly, exhibiting maximal values after 24 h of exposure and then decreasing. Meanwhile, the prevalence and intensity of the degenerative pathologies continued to increase throughout the experiment. After 48 h of exposure, all the examined clams were characterized by the onset of degenerative pathologies (Table 3). The intensity of the degenerative pathologies was maximal after 72 h of exposure (Table 4).

Exposure to *H. circularisquama* for 3 h only induced a significant increase in the prevalence of light cilia matting (Tables 3 and 4). Exposure to *H. circularisquama* for 24 h induced a significant increase in the prevalence of all degenerative pathologies and defensive pathologies, except for hemocytic infiltration in connective tissue (Table 3). The intensity of all defensive pathologies and degenerative pathologies, except for epithelial desquamation, increased significantly compared with both the respective controls and the 3-h exposure group. A high prevalence was observed for moderate hemocytic infiltration in epithelial tissue, heavy hyperplasia, moderate filament fusion, light cilia matting and exfoliation, and moderate necrosis of epithelial tissue. In this group, light hypertrophy, epithelial desquamation,

TABLE 2.

Factorial ANOVA results for the effects of *Heterocapsa circularisquama* density (10^3 cells/mL) and exposure duration on the prevalence and intensity of pathology in gills of *Ruditapes philippinarum*.

Pathology	Prevalence of Pathology			Intensity of Pathology		
	H. circularisquama	Time	*H. circularisquama* × Time	*H. circularisquama*	Time	*H. circularisquama* × Time
Total	***	***	***	***	***	***
Defensive	***	NS	*	***	***	***
Hemocytic infiltration in connective tissue	NS	NS	NS	***	*	**
Hemocytic infiltration in epithelial tissue	***	***	***	***	NS	NS
Hyperplasia	***	**	***	***	**	**
Hypertrophy	**	NS	NS	*	NS	NS
Degenerative	***	***	***	***	***	***
Filament fusion	***	***	***	***	***	***
Cilia matting, exfoliation	***	***	***	***	***	***
Epithelial desquamation	***	***	***	***	***	***
Necrosis of connective tissue	***	***	***	***	***	***
Necrosis of epithelial tissue	***	***	***	***	***	***
Atrophy	***	***	***	***	***	***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
NS, nonsignificant effects.

TABLE 3.

Time-course changes in the prevalence of pathology in the gills of *Ruditapes philippinarum* exposed to *Heterocapsa circularisquama* at 10^3 cells/mL.

Pathology	Exposure Duration (h)			
	3	24	48	72
Total	0.20 ± 0.00^{a}	$0.76 \pm 0.00^{b,**}$	$0.93 \pm 0.02^{c,**}$	$0.85 \pm 0.04^{b,c,**}$
Defensive	0.45 ± 0.27^{a}	$0.85 \pm 0.12^{b,**}$	$0.81 \pm 0.15^{b,c,**}$	$0.62 \pm 0.14^{a,d}$
Hemocytic infiltration in connective tissue	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.87 ± 0.13
Hemocytic infiltration in epithelial tissue	0.80 ± 0.20^{a}	$1.00 \pm 0.00^{b,**}$	$1.00 \pm 0.00^{b,**}$	$0.75 \pm 0.16^{c,**}$
Hyperplasia	0.00 ± 0.00^{a}	$0.90 \pm 0.10^{b,**}$	$0.91 \pm 0.09^{c,**}$	$0.75 \pm 0.16^{a,c}$
Hypertrophy	0.00 ± 0.00^{a}	$0.50 \pm 0.16^{b,**}$	$0.36 \pm 0.15^{c,b,*}$	$0.13 \pm 0.12^{a,c}$
Degenerative	0.03 ± 0.03^{a}	$0.70 \pm 0.09^{b,**}$	$1.00 \pm 0.00^{c,**}$	$1.00 \pm 0.00^{c,**}$
Filament fusion	0.00 ± 0.00^{a}	$0.90 \pm 0.10^{b,**}$	$1.00 \pm 0.00^{b,**}$	$1.00 \pm 0.00^{b,**}$
Cilia matting, exfoliation	$0.20 \pm 0.20^{a,**}$	$0.90 \pm 0.10^{b,**}$	$1.00 \pm 0.00^{b,**}$	$1.00 \pm 0.00^{b,**}$
Epithelial desquamation	0.00 ± 0.00^{a}	$0.40 \pm 0.16^{b,**}$	$1.00 \pm 0.00^{c,**}$	$1.00 \pm 0.00^{c,**}$
Necrosis of connective tissue	0.00 ± 0.00^{a}	$0.50 \pm 0.16^{b,**}$	$1.00 \pm 0.00^{c,**}$	$1.00 \pm 0.00^{c,**}$
Necrosis of epithelial tissue	0.00 ± 0.00^{a}	$0.90 \pm 0.10^{b,**}$	$1.00 \pm 0.00^{b,**}$	$1.00 \pm 0.00^{b,**}$
Atrophy	0.00 ± 0.00^{a}	$0.60 \pm 0.16^{b,**}$	$1.00 \pm 0.00^{c,**}$	$1.00 \pm 0.00^{c,**}$

* Significant difference from the respective control (ANOVA, $P < 0.05$).
** Significant difference from the respective control (ANOVA, $P < 0.01$).
Data expressed in mean ± SE.
Data marked with the same letter indicate no significant difference between the exposed groups for each pathology (Newman-Keuls or Fisher's LSD, $P < 0.05$).

necrosis of connective tissue, and atrophy all occurred at an intermediate prevalence. Although the prevalence of hemocytic infiltration of connective tissue was maximal and was not significantly different from the respective control, its intensity became significantly greater.

Exposure to *H. circularisquama* for 48 h induced a significant increase in the prevalence of all degenerative and defensive pathologies compared with the respective controls, except for hemocytic infiltration in connective tissue. Compared with the 24-h exposure group, the prevalence of epithelial desquamation, necrosis of connective tissue, and atrophy increased significantly, whereas the prevalence of hypertrophy decreased significantly. The intensity of all pathologies in this group was significantly higher than the respective controls. In addition, the intensity of all degenerative pathologies increased significantly to high intensities, whereas the intensity of both hemocytic infiltration of the epithelial tissue and hypertrophy decreased, compared with the 24-h exposure group.

TABLE 4.

Time-course changes in the intensity of pathology in the gills of *Ruditapes philippinarum* exposed to *Heterocapsa circularisquama* at 10^3 cells/mL.

Pathology	Exposure Duration (h)			
	3	24	48	72
Total	0.22 ± 0.02^{a}	$1.19 \pm 0.17^{b,**}$	$1.76 \pm 0.12^{c,**}$	$1.93 \pm 0.11^{c,**}$
Defensive	0.50 ± 0.30^{a}	$1.65 \pm 0.39^{b,**}$	$1.36 \pm 0.30^{b,**}$	$0.90 \pm 0.28^{a,**}$
Hemocytic infiltration in connective tissue	1.20 ± 0.20^{a}	$2.40 \pm 0.27^{b,**}$	$2.00 \pm 0.24^{b,**}$	$1.00 \pm 0.19^{c,**}$
Hemocytic infiltration in epithelial tissue	0.80 ± 0.20^{a}	$1.50 \pm 0.17^{b,**}$	$1.36 \pm 0.16^{a,**}$	1.00 ± 0.27^{a}
Hyperplasia	0.00 ± 0.00	$2.10 \pm 0.38^{b,**}$	$1.54 \pm 0.29^{b,**}$	$1.50 \pm 0.38^{b,**}$
Hypertrophy	0.00 ± 0.00^{a}	$0.60 \pm 0.22^{b,*}$	$0.54 \pm 0.26^{b,*}$	0.13 ± 0.12^{b}
Degenerative	0.03 ± 0.03^{a}	$0.88 \pm 0.15^{b,**}$	$2.03 \pm 0.08^{c,**}$	$2.60 \pm 0.10^{d,**}$
Filament fusion	0.00 ± 0.00^{a}	$1.20 \pm 0.20^{b,**}$	$2.36 \pm 0.21^{c,**}$	$3.00 \pm 0.00^{d,**}$
Cilia matting, exfoliation	0.20 ± 0.20^{a}	$0.90 \pm 0.10^{b,**}$	$2.18 \pm 0.19^{c,**}$	$2.50 \pm 0.19^{d,**}$
Epithelial desquamation	0.00 ± 0.00^{a}	0.40 ± 0.16^{a}	$1.91 \pm 0.17^{b,**}$	$2.38 \pm 0.18^{c,**}$
Necrosis of connective tissue	0.00 ± 0.00^{a}	$0.80 \pm 0.29^{b,**}$	$2.00 \pm 0.24^{c,**}$	$2.62 \pm 0.18^{d,**}$
Necrosis of epithelial tissue	0.00 ± 0.00^{a}	$1.40 \pm 0.22^{b,**}$	$1.82 \pm 0.24^{c,**}$	$2.75 \pm 0.16^{d,**}$
Atrophy	0.00 ± 0.00^{a}	$0.60 \pm 0.16^{b,*}$	$1.90 \pm 0.22^{c,**}$	$2.37 \pm 0.18^{d,**}$

* Significant difference from the respective control (ANOVA, $P < 0.05$).
** Significant difference from the respective control (ANOVA, $P < 0.01$).
Data expressed in mean ± SE.
Data marked with different letters indicate significant difference between the exposed groups for each pathology (Newman-Keuls or Fisher's LSD, $P < 0.05$).

With the exception of hemocytic infiltration of epithelial tissue, the prevalence of all defensive pathologies in the gills of clams exposed to *H. circularisquama* for 72 h was not significantly different from the respective controls or the 48-h exposure group. However, the prevalence of all degenerative pathologies remained significantly higher than the respective controls and was significantly different from the 48-h exposure group (Table 4).

Among the defensive pathologies, the intensity of hemocytic infiltration in connective tissue and hyperplasia were significantly different from the respective controls. The intensity of all degenerative pathologies was significantly higher than the respective controls and the 48-h exposure group.

Defense Responses

Effects on Gill Mucocyte Density

The average total mucocyte density in control clam gills was 3.27 ± 0.09. Three types of mucocytes were identified in clam gills: blue-stained acid mucocytes (AMPS), which accounted for 60.78 ± 1.43% of the total mucocytes; purple-stained mixed mucocytes (MMPS), accounting for 35.29 ± 1.40% of the total mucocytes; and pink-stained neutral mucocytes (NMPS), which were rarely observed and accounted for only 3.88 ± 0.80% of the total mucocytes.

ANOVA showed that the total and acid mucocyte densities were significantly affected by *H. circularisquama* exposure, the exposure duration, and their interaction. The density of *H. circularisquama* and the exposure duration had significant effects on the mixed mucocyte density, although their interaction did not. The neutral mucocyte density was not affected throughout the experiments (Table 5). Exposure to *H. circularisquama* for 48 h induced a significant decrease in the density of total, acid, and mixed mucocytes by approximately 47%, 51%, and 39%, respectively. Exposure to *H. circularisquama* for 72 h also induced a significant decrease in the density of total, acid, and mixed mucocytes by approximately 48%, 48%, and 52%, respectively (Fig. 4).

Effects on Total Hemocyte Counts

THC increased significantly after 24 h of exposure to *H. circularisquama* at a concentration of 10^3 cells/mL to more than double the THC of control clams (Table 6). It then decreased significantly after 48 h of exposure to levels comparable with the control THC.

TABLE 5.

Factorial ANOVA results for the effects of *Heterocapsa circularisquama* concentration (10^3 cells/mL) and the exposure duration on mucocyte density in the gills of *Ruditapes philippinarum*.

Factor	Total	Acid	Mixed	Neutral
H. circularisquama density	*	*	*	NS
Exposure duration	*	*	*	NS
H. circularisquama density × exposure duration	*	*	NS	NS

*$P < 0.01$.
NS, nonsignificant effect.

DISCUSSION

Studies on interactions between the toxic dinoflagellate *H. circularisquama* and bivalves have focused primarily on mortality, CRs, and valve movement responses for use in early biological warning systems (Nagai et al. 1996, Matsuyama et al. 1997, Matsuyama et al. 1998, Matsuyama 1999, Matsuyama et al. 1999, Matsuyama 2003a, Matsuyama 2003b, Nagai et al. 2006, Basti et al. 2009, Basti & Segawa 2010). Although this toxic alga has been known to cause mass mortalities of different bivalve species for more than 3 decades, the process by which *H. circularisquama* affects the survival of marine bivalves remains unclear.

In the current work, we showed that *H. circularisquama* causes a set of physiological, pathological, and defense alterations in the short-neck clam *R. philippinarum* (summarized in Table 7, along with alterations reported in the literature for some other toxic dinoflagellates). This is the first study to describe the pathological and defense alterations caused by *H. circularisquama* in bivalves in detail and to quantify them in a time-dependant manner.

A short-term, 2-h exposure to *H. circularisquama* at 5–10^3 cells/mL resulted in a decrease in both CR and RR of *R. philippinarum* associated with a brief shell valve closure, increased mucus secretion, production of pseudofeces containing *H. circularisquama* cystlike cells, and cilia matting. Previous studies have shown that CRs of the blue mussel *M. galloprovincialis* and the Pacific oyster *C. gigas* were significantly decreased after 10–30 min of exposure to *H. circularisquama* at cell densities ranging from 50–10^3 cells/mL (Matsuyama et al. 1997, Matsuyama et al. 1999). These decreases were associated with mantle retraction, violent valve clapping, shell valve closure, and inhibition of byssus production (Matsuyama et al. 1997). The effects of *H. circularisquama* on the RRs of marine bivalves have not been investigated previously. Reduction or inhibition of CRs of several bivalve species in the presence of toxic dinoflagellates is a well-known deleterious effect attributable to avoidance behaviors (i.e., valve closure, burrowing) that effectively isolate shellfish from the external environment (Ray & Aldrich 1967, Shumway & Cucci 1987, Bricelj et al. 1990, Shumway et al. 1990, Bricelj et al. 1991, Nagai et al. 2006, Estrada et al. 2007a, Hégaret et al. 2007, Tran et al. 2010). The effects of HABs on the oxygen consumption of bivalves vary with the bivalve species and HAB species. For instance, Shumway et al. (1985) found that *Alexandrium tamarense* induces a decrease in the oxygen consumption of *P. magellanicus* and *Spisula solidissima*, an increase in the oxygen consumption of *M. arenaria*, and an increase or no change for *M. edulis*. Basti et al. (2009) reported that *R. philippinarum* initiated a short but incomplete closure reaction when *H. circularisquama* was added to seawater. Although the total duration of valve opening estimated for 24 h of exposure was not affected by the presence of the toxic alga, it is possible that the short closure reaction observed in this study, which occurred within less than 2 h of exposure, contributed to the decreased CRs and RRs.

Increased mucus secretion in the gills, as observed in the clams in this study, has also been reported for other bivalves

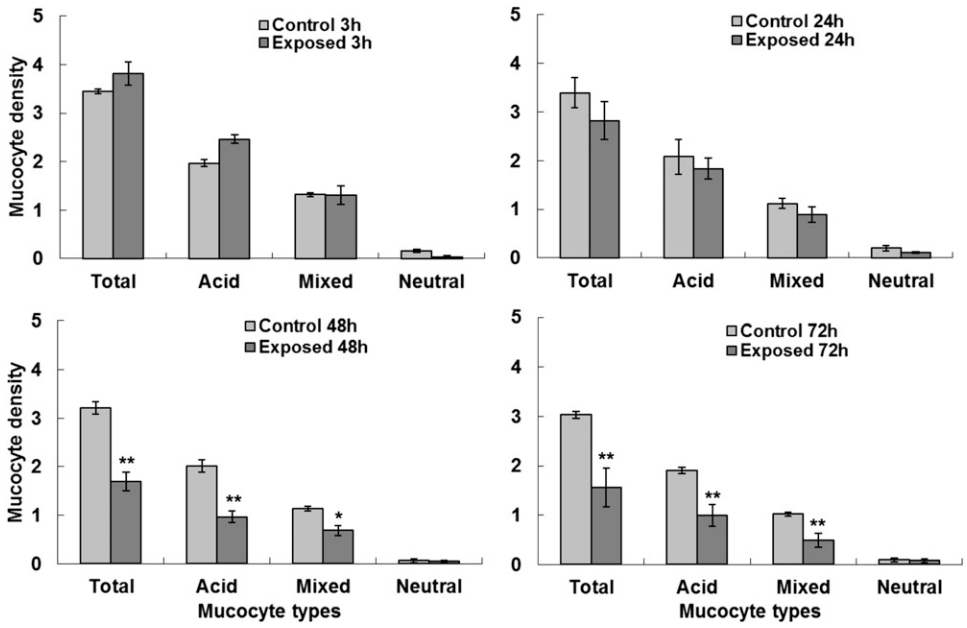

Figure 4. Time-course changes in total, acid, mixed, and neutral mucocyte densities in *Ruditapes philippinarum* gills exposed to *Heterocapsa circularisquama*. Data expressed as the mean ± SE. *Significant difference from respective control (ANOVA, Fisher's LSD, $P < 0.05$). **Significant difference from respective control (ANOVA, Fisher's LSD, $P < 0.01$).

exposed to *Alexandrium minutum* (Haberkorn et al. 2010), *A. tamarense* (Shumway & Cucci 1987), *Alexandrium fundyense* (Galimany et al. 2008a), *Cochlodinium polykrikoides* (Gobler et al. 2008), *Karenia mikimotoi* (Smolowitz & Shumway 1997), and *Prorocentrum minimum* (Hégaret et al. 2010). Mucus associated with gills may serve as a barrier to cell contact with toxic alga. In addition, it contains several lytic enzymes that play an important role in defense mechanisms against various stressors (Fisher 1992, Brun et al. 2000). The production of pseudofeces by exposed clams coupled with the presence of *H. circularisquama* cystlike cells in the pseudofeces, as observed in this study and reported in a previous work (Basti & Segawa 2010), suggest that clams reject *H. circularisquama* cells preferentially to avoid the ingestion of toxic food particles (Ward et al. 1997).

Moreover, exposure of clams to *H. circularisquama* at a concentration of 10^3 cells/mL for 3 h induced light cilia matting, which could also explain the alterations observed in CRs and RRs. Indeed, gill cilia are known to play a major role in feeding and particle processing. Alterations in gill cilia would be expected to alter particle transport, which is ensured by the frontal cilia; water pumping, which is ensured by the lateral cilia; and particle retention and filtration, which are functions of the laterofrontal cilia (Riisgård 1988, Benninger et al. 1992, Benninger et al. 1993, Benninger et al. 1997). Therefore, the decreased CRs and RRs detected in this study appear to be results of ciliary dysfunction combined with brief shell valve closure, pseudofecal production, and the mucus load.

Exposure to *H. circularisquama* for 24 h triggered a set of degenerative pathologies in the gills of *R. philippinarum* that occurred at an intermediate prevalence with light to moderate intensity. These pathologies consisted of filament fusion, cilia matting and exfoliation, epithelial desquamation, and necrosis of connective and epithelial tissues— all of which underlie cytotoxicity. In addition, the large balls of mucus and gill debris resulting from the detachment of cilia and epithelial tissue constituted a heavy load for the gills and would have further altered the activity of the remaining cilia at this stage. At the same time, a nonspecific inflammatory reaction was observed to reach its highest prevalence and intensity, coupled with an increase in the number of circulating hemocytes. The inflammatory reaction consisted of hemocytic infiltration of connective and epithelial tissues, hyperplasia, and hypertrophy. The toxic dinoflagellates *A. minutum*, *A. fundyense*, *P. minimum*, *K. mikimotoi*, and *C. polykrikoides* were previously reported to cause one or more of the degenerative pathologies described here for *H. circularisquama* in gills of bivalves, but at relatively higher cell densities and over longer exposure durations (Table 7). In bivalves, gill hyperplasia, in which the epidermis thickens by producing extra cells, is a nonspecific initial response to an array of irritants (Smolarz et al. 2006), including toxic dinoflagellates. In fact, gill hyperplasia, which is generally associated with hypertrophy, was previously described in the hard clam *Mercenaria mercenaria* exposed to *P. minimum* (Hégaret et al. 2010), the bay scallops *Argopecten irradians* and Pacific oysters *C. gigas* exposed to *C. polykrikoides*

TABLE 6.

Total hemocyte count (mean ± SE, $\times 10^6$ cells/mL) in *Ruditapes philippinarum* exposed to *Heterocapsa circularisquama* at 10^3 cells/mL.

	Treatments	
Exposure Duration (h)	**Control**	**Exposed**
24	2.19 ± 0.49^{a}	$4.91 \pm 0.32^{*,a}$
48	2.39 ± 0.44^{a}	3.01 ± 0.25^{b}

* Significant difference to the respective control (*t*-test, $P < 0.01$).
Data marked with different letters indicate significant difference between the control groups or the exposed groups (*t*-test, $P < 0.01$).

TABLE 7.

Effects of *Heterocapsa circularisquama* and other toxic dinoflagellates on the gill tissues and hemocytes of marine bivalves.

Causative Agent	Toxin	Density (cells/mL)	Exposure Duration	Physiological Alterations		Histopathological Alterations (gills)	Mucocytes (gills)	Hemocyte Count	References
				CR	RR				
Heterocapsa circularisquama		5–10^3	2–96 h	D	D	Hemocytic infiltration, hyperplasia, hypertrophy, filament fusion, cilia exfoliation, epithelial desquamation, atrophy, necrosis	D (total, acid, mixed)	I + D	Current study
	Cytotoxic, hemolytic	50–10^3	10–30 min	D	NA	NA	Excess mucus	NA	Matsuyama et al. (1997, 1999), Oda et al. (2001), Hiraga et al. (2002), Sato et al. (2002), Miyazaki et al. (2005), Kim et al. (2008)
Alexandrium minutum	PST, cytotoxic, hemolytic*	5×10^3	96 h	D	NA	Hemocytic infiltration, necrosis	Excess mucus	I	Haberkorn et al. (2010)
Alexandrium tamarense		100	6 days	D	I/D/NE	NA	Excess mucus	NA	Shumway and Cucci (1987), Li et al. (2002)
Alexandrium fundyense		50–700	40–60 min	D	NA	Cilia exfoliation, necrosis	Excess mucus (blue-secretory mucocytes)	D/NE	Bricelj et al. (1990), Galimany et al. (2008a), Hégaret et al. (2007)
Alexandrium monilatum		550	2 h	D	NA	NA	NA	NA	May et al. (2010)
Prorocentrum minimum	Enterotoxic	2×10^4	5 days	D	NA	Hemocytic infiltration and aggregates (granulome, encapsulation), hyperplasia, vaccuolation of water tubular epithelial cells, thrombi, neoplasia	NA	NE/I	Wikfors and Smolowitz (1993), Landsberg (1996, 2002), Hégaret and Wikfors (2005a, 2005b) Galimany et al. (2008b), Hégaret et al. (2010).
Karenia brevis (formerly *Gymnodinium breve*)	Brevetoxins	100–10^3	<24 h	D	NA	NA	NA	NA	Poli et al. (1986), Shimizu et al. (1990), Baden et al. (1995), Leverone et al. (2007)
Karenia mikimotoi (formerly *Gyrodinium aureolum*)	Exotoxins, glycolipids, hemolytic, cytotoxins	500–10^3	10–20 min	D	NA	Hemocytic infiltration, inflammation, necrosis	NA	NA	Gentien and Arzul (1990), Arzul et al. (1995), Smolowitz and Shumway (1997), Matsuyama et al. (1998, 1999) Jenkinson and Arzul (2001), Satake et al. (2002, 2005)
Gymnodinium catenatum	PST	450–1,650	2–7 h	D	NA	Hemocytic infiltration	NA	NA	Oshima et al. (1993), Estrada et al. (2007a, 2007b)

continued on next page

TABLE 7.
continued

Causative Agent	Toxin	Density (cells/mL)	Exposure Duration	Physiological Alterations		Histopathological Alterations (gills)	Mucocytes (gills)	Hemocyte Count	References
				CR	RR				
Cochlodinium polykrikoides	Reactive oxygen species, polysaccharides, neurotoxic, hemolytic, hemagglutinative, PST	$100–5 \times 10^4$	1–9 days	NA	NA	Hemocytic infiltration, filament fusion, hyperplasia, epithelial desquamation, apoptosis, hemorrhaging, inflammation, apoptosis	NA	NE	Onoue and Nozawa (1989a, 1989b), Kim et al. (1999, 2000, 2002), Gobler et al. (2008)

CR, clearance rate; D, decrease; I, increase; NA, not available; NE, no effect; PST, paralytic shellfish toxin; RR, respiration rate.
* Matsuyama et al. (2001), Emura et al. (2004), Juhl et al. (2007), Katsuo et al. (2007), Ford et al. (2008), and Hégaret et al. (2008).

(Gobler et al. 2008), and *C. virginica* exposed to *K. mikimotoi* (Smolowitz & Shumway 1997). Hemocytic infiltration of gill tissues induced by *H. circularisquama* has also been described for other marine bivalves exposed to *A. minutum* (Haberkorn et al. 2010), *P. minimum* (Hégaret et al. 2010), *Prorocentrum rhathymum* (Pearce et al. 2005), *Gymnodinium sanguinea* (Bricelj et al. 1992), *Gymnodinium catenatum* (Estrada et al. 2007b), *K. mikimotoi* (Smolowitz & Shumway 1997), and *C. polykrikoides* (Gobler et al. 2008). The hemocytic infiltration of clam gills indicates that hemocytes reacted to the presence of the harmful alga or to its toxins or metabolites to protect tissues from contact with the toxic alga or to accomplish tissue repair (Hégaret et al. 2010). An increased number of circulating hemocytes is generally considered to be an immune response to pathogens or parasites (Ford et al. 1993, Anderson et al. 1995, Cáceres-Martínez et al. 2000, Villalba et al. 2004). It has also been reported in bivalves exposed to *A. minutum* and *P. minimum* (Table 7). Regardless of whether the toxic alga in perceived as an invader, the observed activation of immune responses in clams investigated in this study strongly suggests that they reacted to the physiological impairment induced by the algal toxins in their gills, notably through necrosis.

Both the numbers of circulating hemocytes and the inflammatory reaction decreased after 48 h of exposure. At the same time, the density of total, acid, and mixed mucocytes also decreased, whereas the degenerative pathologies continued to increase in both prevalence and intensity, with severe ciliary exfoliation, epithelial desquamation, necrosis, and atrophy extending throughout the gills being observed. Necrosis impairs particle collection and respiratory functions, which leads to further reductions of food intake and respiration, and contributes to the general physiological weakening of animals. Epithelial desquamation would also leave the clams vulnerable to secondary infections caused by opportunistic pathogens. In addition, it explains the reduction of mucocyte density through the loss of epithelial masses and, thus, mucocyte cells that are embedded in the epithelium. Mucus secreted by mucocytes is known to play an important role in bivalve feeding physiology as well as defense mechanisms. Decreased mucocyte density induces a decrease in mucus production and renders the gills more susceptible to *H. circularisquama*.

The necrotic activity of *H. circularisquama* was suspected, but not demonstrated, previously. In 2002, a new digalactosyl diacylglycerol and two known monogalactosyl diacylglycerols were extracted from *H. circularisquama* and shown to induce cytotoxicity in heart cells of oysters (Hiraga et al. 2002). In addition, H2-a was recently shown to induce cytotoxicity against HeLa cells with a high potency (Kim et al. 2008). H2-a tended to accumulate in the plasma membrane of HeLa cells, and necrosis was proposed as the most plausible mechanism leading to cell death (Kim et al. 2008). Our work confirms the necrotic activity of suspended *H. circularisquama* cells in bivalve gills and shows that it leads progressively to degeneration of gill tissues and losses of filament structures, causing the rapid death of bivalves in the presence of this toxic alga.

The decreases in the number of circulating hemocytes and in both the prevalence and intensity of hemocytic infiltrations of gill tissues indicate that the immune system of the clams became overwhelmed by the potent cytotoxic and, possibly, hemolytic toxins of *H. circularisquama*. Indeed, *H. circularisquama* has been shown to induce hemolysis in mammalian erythrocytes,

and this hemolytic activity was found to be light dependant (Oda et al. 2001, Sato et al. 2002). One hemolytic agent, H2-a, was extracted and purified, and chemical and structural analysis revealed that it is a porphyrin derivative with a similar structure to pyropheophorbide *a* methyl ester, a well-known photosensitizing hemolytic compound (Oda et al. 2001, Sato et al. 2002, Miyazaki et al. 2005). Thus, the sudden suppression of the immune response in the gills of clams might be related to hemolytic activity of *H. circularisquama*, although further hematoimmunological experiments are required to confirm these findings.

CONCLUSION

This is the first study to demonstrate clearly the cytotoxicity of *H. circularisquama* in *R. philippinarum* clams and in bivalves in general. Under the experimental conditions used in this work, short-term exposure of 2–3 h to the harmful alga caused a significant decrease in CRs and RRs of clams as a result of cilia matting, brief valve closure, and mucus and pseudofecal production. Within 24 h of exposure, the toxic alga triggered an inflammatory reaction consisting of hemocytic infiltration, hyperplasia, and hypertrophy of gill tissues, coupled with an increase in the number of circulating hemocytes. At the same time, a significant cytotoxic effect on the gill tissues consisting of filament fusion, cilia exfoliation, epithelial desquamation, necrosis, and atrophy was observed and continued to increase in both prevalence and intensity throughout the experiments. The observed inflammation, number of circulating hemocytes, and gill mucocyte densities decreased significantly after 48 h of exposure. Ultimately, the clams died as a result of necrosis and failure of their defensive mechanisms to overcome the extensive cytotoxicity caused by *H. circularisquama*.

ACKNOWLEDGMENTS

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

LITERATURE CITED

Anderson, R. S., E. M. Burreson & K. T. Paynter. 1995. Defense responses of hemocytes withdrawn from *Crassostrea virginica* infected with *Perkinsus marinus*. *J. Invertebr. Pathol.* 66:82–89.

Anderson, D. M., S. B. Galloway & J. D. Joseph. 1993. Marine biotoxins and harmful algae: a national plan. Woods Hole Oceanographic Institution technical report WHOI-93-02. Woods Hole, MA: Woods Hole Oceanographic Institution. 44 pp.

Anderson, D. M., P. Hoagland, Y. Kaoru & A. W. White. 2000. Estimated annual economic impacts from harmful algal blooms (HABs) in the United States. Woods Hole Oceanographic Institution technical report, WHOI-2000-11. Woods Hole, MA: Woods Hole Oceanographic Institution. 99 pp.

Arzul, G., S. Turki, A. Hamza, P. Daniel & M. Mercaron. 1995. Fish kills induced by phycotoxins. *Toxicon* 33:1119.

Baden, D. G., L. A. Flemming & J. A. Bean. 1995. Marine toxins. In: F. A. DeWolf, editor. Handbook of clinical neurology, vol. 21. New York: Elsevier. pp. 1–34.

Basti, L. & S. Segawa. 2010. Mortalities of the short-neck clam *Ruditapes philippinarum* induced by the toxic dinoflagellate *Heterocapsa circularisquama*. *Fish. Sci.* 76:625–631.

Basti, L., K. Nagai, Y. Shimasaki, Y. Oshima, T. Honjo & S. Segawa. 2009. Effects of the toxic dinoflagellate *Heterocapsa circularisquama* on the valve movement behavior of the Manila clam *Ruditapes philippinarum*. *Aquaculture* 291:41–47.

Basti, L., G. Jiyoji, K. Higuchi, K. Nagai & S. Segawa. 2011. Effects of the toxic dinoflagellate *Heterocapsa circularisquama* on larvae of the pearl oyster *Pinctada fucata martensii* (Dunker, 1873). *J. Shellfish Res.* 30:177–186.

Bayne, B. L. & R. C. Newell. 1983. Physiological energetics of marine molluscs. In: K. M. Wilbur & A. S. M. Salenddin, editors. The Mollusca, vol. 4: physiology, part I. London: Academic Press. pp. 406–515.

Benninger, P. G., S. C. Dufour & J. Bourque. 1997. Particle processing mechanisms of the eulamellibranch bivalves *Spisula solidissima* and *Mya arenaria*. *Mar. Ecol. Prog. Ser.* 150:157–169.

Benninger, P. G. & S. D. St-Jean. 1997. Particle processing on the labial palps of *Mytilus edulis* and *Placopecten magellanicus* (Mollusca: Bivalvia). *Mar. Ecol. Prog. Ser.* 147:117–127.

Benninger, P. G., S. D. St-Jean, Y. Poussart & J. E. Ward. 1993. Gill function and mucocyte distribution in *Placopecten magellanicus* and *Mytilus edulis* (Mollusca: Bivalvia): the role of mucus in particle transport. *Mar. Ecol. Prog. Ser.* 98:275–282.

Benninger, P. G., J. E. Ward, B. A. MacDonald & R. J. Thompson. 1992. Gill function and particle transport in *Placopecten magellanicus* (Mollusca: Bivalvia) as revealed using video endoscopy. *Mar. Biol.* 114:281–288.

Bricelj, V. M., J. H. Lee & A. D. Cembella. 1991. Influence of dinoflagellate cell toxicity on uptake and loss of paralytic shellfish toxins in the northern quahog *Mercenaria mercenaria*. *Mar. Ecol. Prog. Ser.* 74:33–46.

Bricelj, V. N., J. H. Lee, A. D. Cembella & D. M. Anderson. 1990. Uptake of *Alexandrium fundyense* by *Mytilus edulis* and *Mercenaria mercenaria* under controlled conditions. In: E. Granéli, B. Sundstorm, L. Edler & D. M. Anderson, editors. Toxic marine phytoplankton. New York: Academic Press. pp. 41–51.

Bricelj, V. M., S. E. Ford, F. J. Borrero, F. O. Perkins, G. Rivara, R. E. Hillman, R. A. Elston & J. Chang. 1992. Unexplained mortalities of hatchery-reared juvenile oysters, *Crassostrea virginica* (Gmelin). *J. Shellfish Res.* 11:331–347.

Brun, N. T., N. W. Ross & A. D. Boghen. 2000. Changes in electrophoretic profiles of gill mucus proteases of the Eastern oyster *Crassostrea virginica* in response to infection by the turbellarian *Urastoma cyprine*. *J. Invertebr. Pathol.* 75:163–170.

Cáceres-Martínez, J., R. Vásquez-Yeomans, Y. G. Rentería, S. Curiel-Ramírez, J. A. Valdez & G. Rivas. 2000. The marine mites *Hyadesia* sp. and *Copidognathus* sp. associated with the mussel *Mytilus galloprovincialis*. *J. Invertebr. Pathol.* 76:216–221.

Chen, L. C. M., T. Edelstein & J. MacLachlan. 1969. *Bonnemaisonia hamifera* Hariot in nature and in culture. *J. Phycol.* 5:211–220.

Coughlan, J. 1969. The estimation of filtering rate from the clearance of suspensions. *Mar. Biol.* 2:356–358.

Emura, A., Y. Matsuyama & T. Oda. 2004. Evidence for the production of a novel proteinaceous haemolytic exotoxin by dinoflagellate *Alexandrium taylori*. *Harmful Algae* 5:665–677.

Estrada, N. A., N. Lagos, C. García, A. N. Maeda-Martínez & F. Ascencio. 2007a. Effects of the toxic dinoflagellate *Gymnodinium catenatum* on uptake and fate of paralytic shellfish poisons in the Pacific giant lion-paw scallop *Nodipecten subnodosus*. *Mar. Biol.* 151: 1205–1214.

Estrada, N., M. J. Romeao, A. Campa-Córdova, A. Luna & F. Ascencio. 2007b. Effects of the toxic dinoflagellate, *Gymnodinium catenatum* on hydrolytic and antioxidant enzymes, in tissues of the giant lions-paw scallop *Nodipecten subnodosus*. *Comp. Biochem. Physiol. C* 146: 502–510.

Fisher, W. S. 1992. Occurrence of agglutinins in the pallial cavity mucus of oysters. *J. Exp. Mar. Biol. Ecol.* 162:1–13.

Fleming, L. E. & D. G. Baden. 1998. Neurotoxic shellfish poisoning: public health and human health effects. White Paper for the Proceedings of the Texas Conference on Neurotoxic Shellfish Poisoning, Proceedings of the Texas NSP Conference, Corpus Christi, Texas. pp. 27–34.

Flewelling, L. J., J. P. Naar, J. P. Abbott, D. G. Baden, N. B. Barros, G. D. Bossart, M. Y. Bottein, D. G. Hammond, E. M. Haubold, C. A. Heil, M. S. Henry, H. M. Jacocks, T. A. Leighfield, R. H. Pierce, T. D. Pitchford, S. A. Rommel, P.S. Scott, K. A. Steidinger, E.W. Truby, F. M. VanDolah & J. H. Landsberg. 2005. Brevetoxicosis: red tides and marine mammal mortalities. *Nature* 435:755–756.

Fogg, G. E. 2002. Harmful algae: a perspective. *Harmful Algae* 1: 1–4.

Ford, S. E., V. M. Bricelj, C. Lambert & C. Paillard. 2008. Deleterious effects of a non PST bioactive compound from *Alexandrium tamarense* on bivalve hemocytes. *Mar. Biol.* 154:241–253.

Ford, S. E., S. A. Kanaley & D. T. J. Littlewood. 1993. Cellular responses of oysters infected with *Haplosporidium nelson:* changes in circulating and tissue-infiltrating hemocytes. *J. Invertebr. Pathol.* 61: 49–57.

Foster-Smith, R. L. 1975. The role of mucus in the mechanisms of feeding in three filter-feeding bivalves. *Proc. Malacol. Soc. Lond.* 41:571–588.

Gainey, L. F. J. & S. E. Shumway. 1991. The physiological effects of *Aureococcus anophagefferens* (''brown tide'') on the lateral cilia of bivalve molluscs. *Biol. Bull.* 181:298–306.

Galimany, E., I. Sulina, H. Hégaret, M. Ramón & G. H. Wikfors. 2008a. Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: histology, immune responses, and recovery. *Harmful Algae* 7:702–711.

Galimany, E., I. Sulina, H. Hégaret, M. Ramón & G. H. Wikfors. 2008b. Pathology and immune responses of the blue mussel (*Mytilus edulis* L.) after an exposure to the harmful dinoflagellate *Prorocentrum minimum*. *Harmful Algae* 7:630–638.

Gentien, P. & G. Arzul. 1990. Exotoxins production by *Gyrodinium* cf. *aureolum* (Dinophycea). *J. Exp. Mar. Biol. Assoc. UK* 70:571–581.

Geraci, J. R., D. M. Anderson, R. J. Timperi, D. J. St Aubin, G. A. Early, J. H. Prescott & C. A. Mayo. 1989. Humpback whales (*Megaoetera novaeangliae*) fatally poisoned by dinoflagellate toxin. *Can. J. Fish. Aquat. Sci.* 46:1895–1898.

Gobler, C. J., D. L. Berry, O. R. Anderson, A. Burson, F. Koch, B. S. Rodgers, L. K. Moore, J. A. Goleski, B. Allam, P. Browser, Y. Tang & R. Nuzzi. 2008. Characterization, dynamics, and ecological impacts of harmful *Cochlodinium polykrikroides* blooms on eastern Long Island, NY, USA. *Harmful Algae* 7:293–307.

Haberkorn, H., C. Lambert, N. Le Goïc, M. Guéguen, J. Moal, E. Palacios, P. Lassus & P. Soudant. 2010. Effects of *Alexandrium minutum* exposure upon physiological and hematological variables of diploid and triploid oysters, *Crassostrea gigas*. *Aquat. Toxicol.* 97:96–108.

Hansen, G., J. Truquet, J. P. Quod, L. Ten-Hage, C. Lugomela, M. Keywalyanga, M. Hurbungs, P. Wawiye, B. Ogongo, S. Tunje & H. Rakotoarinjanahary. 2001. Potentially harmful microalgae of the western Indian ocean. IOC manuals and guides 41. Paris: UNESCO. 105 pp.

Hégaret, H. & G. H. Wikfors. 2005a. Effects of natural and field-simulated blooms of the dinoflagellate *Prorocentrum minimum* upon hemocytes of Eastern oysters, *Crassostrea virginica*, from two different populations. *Harmful Algae* 4:201–209.

Hégaret, H. & G. H. Wikfors. 2005b. Time-dependant changes in hemocytes of Eastern oysters, *Crassostrea virginica*, and northern bay scallops, *Argopecten irradians irradians*, exposed to a culture strain of *Prorocentrum minimum*. *Harmful Algae* 4:187–199.

Hégaret, H., S. E. Shumway, G. H. Wikfors, S. Pate & J. M. Burkholder. 2008. Potential transport of harmful algae through relocation of bivalve mollusks. *Mar. Ecol. Prog. Ser.* 361:169–181.

Hégaret, H., R. M. Smolowitz, I. Sunila, S. E. Shumway, J. Alix, M. Dixon & G. H. Wikfors. 2010. Combined effects of a parasite, QPX, and the harmful-alga, *Prorocentrum minimum* on the northern quahogs, *Mercenaria mercenaria*. *Mar. Environ. Res.* 69:337–344.

Hégaret, H., G. H. Wikfors & S. E. Shumway. 2007. Diverse feeding responses of five species of bivalve mollusc when exposed to three species of harmful algae. *J. Shellfish Res.* 26:549–559.

Hiraga, Y., K. Kaku, D. Omoda, K. Sugihara, H. Hosoya & M. Hino. 2002. A new digalactosyl diacylglycerol from a cultured marine dinoflagellate *Heterocapsa circularisquama*. *J. Nat. Prod.* 65:1494–1496.

Itoh, K. & I. Imai. 1987. Rafido-So. A guide for studies of red tide organisms. Shuwa, Tokyo: Japan Fisheries Resource Conservation Association. pp. 122–130.

Iwataki, M., M. WahWong & Y. Fukuyo. 2002. New record of *Heterocapsa circularisquama* (Dinophyceae) from Hong Kong. *Fish. Sci.* 68:1161–1163.

Jenkinson, I. & G. Arzul. 2001. Mitigation by cysteine compounds of rheotoxicity, cytotoxicity and fish mortality caused by the dinoflagellates, *Gymnodinium mikimotoi* and *G.* cf. *maguelonnense*. In: G. M. Hallegraeff, S. I. Blackburn, C. J. Bolch & R. J. Lewis, editors. Harmful algal blooms. Paris: UNESCO. pp. 461–464.

Juhl, A. R., C. A. Martins & D. M. Anderson. 2007. Toxicity of *Alexandrium lusitanicus* to gastropod larvae is not caused by paralytic-shellfish-poisoning toxins. *Harmful Algae* 7:567–573.

Katsuo, D., D. Kim, K. Yamaguchi, Y. Matsuyama & T. Oda. 2007. A new and simple method for the detection of cytotoxic substances produced by harmful red tide phytoplankton. *Harmful Algae* 6:790–798.

Kim, C. S., S. G. Lee & G. H. Kim. 2000. Biochemical responses of fish exposed to a harmful dinoflagellate *Cochlodinium polykrikoides*. *J. Exp. Mar. Biol. Ecol.* 254:131–141.

Kim, C. S., S. G. Lee, G. H. Kim & J. Jung. 1999. Reactive oxygen species as causative agents in the ichthyotoxicity of the red tide dinoflagellate *Cochlodinium polykrikoides*. *J. Plankton Res.* 21:2105–2115.

Kim, D., Y. Miyazaki, T. Nakashima, T. Iwashi, T. Fujita, K. Yamaguchi, K. S. Choi & T. Oda. 2008. Cytotoxic action mode of a novel porphyrin derivative isolated from harmful red tide dinoflagellate *Heterocapsa circularisquama*. *J. Biochem. Mol. Toxicol.* 22:158–165.

Kim, D., T. Oda, T. Muramatsu, D. Kim, Y. Matsuyama & T. Honjo. 2002. Possible factors responsible for the toxicity of *Cochlodinium polykrikoides*, a red tide phytoplankton. *Comp. Biochem. Physiol. C* 132:415–423.

Kirkpatrick, B., L. E. Fleming, D. Squiccinarini, L. C. Backer, R. Clark, W. Abrajam, J. Bensom, Y. S. Cheng, D. Johnson, R. Pierce, J. Zaias, G. D. Bossart & D. G. Baden. 2004. Literature review of Florida red tide: implications for human health effects. *Harmful Algae* 3:99–115.

Landsberg, J. H. 1996. Neoplasia and biotoxins in bivalves: is there a connection? *J. Shellfish Res.* 15:203–230.

Landsberg, J. H. 2002. The effects of harmful algal blooms on aquatic organisms. *Rev. Fish. Sci.* 10:113–390.

Landsberg, J. H. & K. Steidinger. 1998. A historical review of red tide events caused by *Gymnodinium breve* as related to mass mortalities of the endangered manatee (*Trichechus manatus latirostris*) in Florida, USA. In: B. Reguera, J. Blanco, M. L. Fernandez & T. Wyatt, editors. Harmful microalgae. IOC of UNESCO and Xunta de Galicia, Santiago de Compostela, Spain. pp. 97–100.

Leverone, J. R., S. E. Shumway & N. J. Blake. 2007. Comparative effects of the toxic dinoflagellate *Karenia brevis* on clearance rates in juveniles of four bivalve molluscs from Florida, USA. *Toxicon* 49:634–645.

Li, S. C., W. X. Wang & D. P. H. Hsieh. 2002. Effects of the toxic dinoflagellate *Alexandrium tamarense* on the energy budgets and growth of two marine bivalves. *Mar. Environ. Res.* 53:145–160.

Matsuyama, Y. 1999. Harmful effect of dinoflagellate *Heterocapsa circularisquama* on shellfish aquaculture in Japan. *Jpn. Agric. Res. Q.* 33:283–293.

Matsuyama, Y. 2003a. Physiological and ecological studies on harmful dinoflagellate *Heterocapsa circularisquama:* I. Elucidation of environmental factors underlying the occurrence and development of *H. circularisquama* red tide. *Bull. Fish. Res. Agen.* 7:24–105. (in Japanese, with English abstract).

Matsuyama, Y. 2003b. Physiological and ecological studies on harmful dinoflagellate *Heterocapsa circularisquama:* II. Clarification on toxicity of *H. circularisquama* and its mechanisms causing shellfish kills. *Bull. Fish. Res. Agen.* 9:13–117. (in Japanese, with English abstract).

Matsuyama, Y. & S. E. Shumway. 2009. Impacts of harmful algal blooms on shellfisheries aquaculture. In: G. Allan & G. Burnell, editors. New technologies in aquaculture: Improving production efficiency, quality and environmental management. Cambridge: Woodhead Publishing. pp. 580–609.

Matsuyama, Y., T. Uchida & T. Honjo. 1997. Toxic effects of the dinoflagellate *Heterocapsa circularisquama* on clearance rate of the blue mussel *Mytilus galloprovincialis*. *Mar. Ecol. Prog. Ser.* 146: 73–80.

Matsuyama, Y., T. Uchida & T. Honjo. 1998. The harmful effect of red tide dinoflagellates, *Heterocapsa circularisquama* and *Gymnodinium mikimotoi* on the clearance rate and survival of the blue mussel, *Mytilus galloprovincialis*. In: B. Reguera, J. Blanco, M. Fernandez & T. Wyatt, editors. Harmful algae. Santiage de Compostela: UNESCO. pp. 422–424.

Matsuyama, Y., T. Uchida & T. Honjo. 1999. Effects of the harmful dinoflagellates, *Gymnodinium mikimotoi* and *Heterocapsa circularisquama*, red-tide on filtering rate of bivalve molluscs. *Fish. Sci.* 65:248–253.

Matsuyama, Y., T. Uchida, K. Nagai, M. Ishimaru, A. Nishimaru, M. Yamaguchi & T. Honjo. T. 1996. Biological and environmental aspects of noxious dinoflagellate red tides by *Heterocapsa circularisquama* in the west Japan. In: T. Yasumoto, Y. Oshima & Y. Fukuyo, editors. Harmful and toxic algal blooms. Paris: UNESCO. pp. 247–250.

Matsuyama, Y., H. Usuki, T. Uchida & Y. Kotani. 2001. Effects of harmful algae on the early planktonic larvae of the oyster, *Crassostrea gigas*. In: G. M. Hallegraeff, S. Blackburn, C. Bolck & R. Lewis, editors. Harmful algal blooms. Paris: UNESCO. pp. 411–414.

May, S. P., J. M. Burkholder, S. E. Shumway, H. Hgaret, G. H. Wikfors & D. Frank. 2010. Effects of the toxic dinoflagellate *Alexandrium monilatum* on survival, grazing and behavioral response of three ecologically important bivalve mollusks. *Harmful Algae* 9:281–293.

Miyazaki, Y., T. Nakashima, T. Iwashita, T. Fujita, K. Yamaguchi & T. Oda. 2005. Purification and characterization of photosensitizing hemolytic toxin from harmful red tide phytoplankton *Heterocapsa circularisquama*. *Aquat. Toxicol.* 73:382–393.

Nagai, K. 2009. Research on means of alleviating damage by *Heterocapsa circularisquama* red tides and reddening adductor disease, causes of the mass mortalities of Japanese pearl oysters (*Pinctada fucata martensii*). PhD diss., Kyushu University. 281 pp. (In Japanese).

Nagai, K., T. Honjo, J. Go, H. Yamashita & S. J. Oh. 2006. Detecting the shellfish killer *Heterocapsa circularisquama* (Dinophyceae) by measuring bivalve valve activity with a Hall element sensor. *Aquaculture* 255:395–401.

Nagai, K., Y. Matsuyama, T. Uchida, M. Yamaguchi, M. Ishimaru, A. Nishimaru, S. Akamatsu & T. Honjo. 1996. Toxicity and LD_{50} levels of the red tide dinoflagellate *Heterocapsa circularisquama* on juvenile pearl oysters. *Aquaculture* 144:149–154.

Oda, T., Y. Sato, D. Kim, T. Muramatsu, Y. Matsuyama & T. Honjo. 2001. Hemolytic activity of *Heterocapsa circularisquama* (Dinophyceae) an its possible involvement in shellfish toxicity. *J. Phycol.* 137:509–516.

Onoue, Y. & K. Nozawa. 1989a. Separation of toxins from harmful red tides occurring along the coast of Kagoshima Prefecture. In: T. Okaichi, D. M. Anderson & T. Nemoto, editors. Red tides: biology, environmental science and toxicology. New York: Elsevier. pp. 371–374.

Onoue, Y. & K. Nozawa. 1989b. Zinc-bound PSP toxins separated from *Cochlodinium* red tide. In: S. Natori, K. Hashimoto & Y. Ueno, editors. Mycotoxins and phycotoxins 88. Amsterdam: Elsevier. pp. 359–366.

Oshima, Y., H. Itakura, K. C. Lee, T. Yasumoto, S. Blackburn & G. M. Hallegraeff. 1993. Toxin production by the dinoflagellate *Gymnodinium catenatum*. In: T. Smayda & Y. Shimizu, editors. Toxic phytoplankton blooms in the sea. New York: Elsevier. pp. 907–912.

Pearce, I., J. Handlinger & G. M. Hallegraeff. 2005. Histopathology in Pacific oyster (*Crassostrea gigas*) spat caused by the dinoflagellate *Prorocentrum rhathymum*. *Harmful Algae* 4:61–74.

Poli, M., T. J. Mende & D. G. Baden. 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels bind to specific sites in rat brain synaptosomes. *Mol. Pharmacol.* 30:129–135.

Pouvreau, S., G. Jonquière & D. Buestel. 1999. Filtration by the pearl oyster, *Pinctada margaritifera*, under conditions of low seston load and small particle size in tropical lagoon. *Aquaculture* 176:295–314.

Ray, S. M. & D. V. Aldrich. 1967. Ecological interactions of toxic dinoflagellates and mollusks in the Gulf of Mexico. In: F. E. Russels & P. R. Sauders, editors. Animal toxins: 1st International symposium on animal toxins. New York: Pergamon Press. pp. 75–83.

Rensel, J. E. 1993. Severe blood hypoxia of Atlantic salmon (*Salmo salar*) exposed to marine diatom *Chaetoceros concavicornis*. In: T. J. Smayda & Y. Shimizu, editors. Toxic phytoplankton blooms in the sea. Amsterdam: Elsevier Science Publishers. pp. 625–630.

Riisgård, H. U. 1988. Particle retention and filtration rate in 6 species of Northeast American bivalves. *Mar. Ecol. Prog. Ser.* 45:217–223.

Satake, M., M. Shoji, Y. Oshima, H. Naoki, T. Fujita & T. Yasumoto. 2002. Gymnocin-A, a cytotoxic polyether from the notorious red tide dinoflagellate, *Gymnodinium mikimotoi*. *Tetrahedron Lett.* 43:5829–5832.

Satake, M., Y. Tanaka, Y. Ishikura, Y. Oshima, N. Naoki & T. Yasumoto. 2005. Gymnocin-B with the largest contiguous polyether rings from the red tide dinoflagellate, *Karenia* (formerly *Gymnodinium*) *mikimotoi*. *Tetrahedron Lett.* 46:3537–3540.

Sato, Y., T. Oda, T. Muramatsu, Y. Matsuyama & T. Honjo. 2002. Photosensitizing hemolytic toxin in *Heterocapsa circularisquama*, a newly identified harmful red tide dinoflagellate. *Aquat. Toxicol.* 56:191–196.

Savina, M. & S. Pouvreau. 2004. A comparative ecophysiological study of two infaunal filter-feeding bivalves: *Paphia rhomboïds* and *Glycymeris glycymeris*. *Aquaculture* 239:289–306.

Scholin, C. A., F. Gulland, G. J. Doucette, S. Benson, M. Busman, F. P. Chavez, J. Cordaro, R. DeLong, A. De Vogelaere, J. Harvey, M. Haulena, K. Lefebvre, T. Lipscomb, S. Loscutoff, L. J. Lowenstine, R. Marin, P. E. Miller, W. A. McLellan, P. D. R. Moeller, C. L. Powell, T. Rowles, P. Silvagni, M. Silver, T. Spraker, V. Trainer & F. M. Van Dolah. 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature* 430:80–84.

Shilo, M. 1967. Formation and mode of action of algal toxins. *Bacteriol. Rev.* 31:180–193.

Shimizu, Y., S. Gupta & C. Hong-Nong. 1990. Biosynthesis of red tide toxins. In: S. Hall & G. Strichart, editors. Marine toxins: origin, structure and molecular pharmacology. Washington, DC: American Chemical Society. pp. 21–28.

Shumway, S. E. 1990. A review of the effects of algal blooms on shellfish and aquaculture. *J. World Aquacult. Soc.* 21:65–104.

Shumway, S. E., J. Barter & S. Sherman-Caswell. 1990. Auditing the impact of toxic algal blooms on oysters. *Environ. Auditor* 2:41–56.

Shumway, S. E. & T. L. Cucci. 1987. The effects of the toxic dinoflagellate *Protogonyaulax tamarensis* on the feeding and behavior of bivalve mollusks. *Aquat. Toxicol.* 10:9–27.

Shumway, S. E., T. L. Cucci, R. C. Newell & C. M. Yenrsch. 1985. Particle selection, ingestion and absorption in filter-feeding bivalves. *J. Exp. Mar. Biol. Ecol.* 91:77–92.

Smayda, T. J. 1997. What is a bloom? A commentary. *Limnol. Oceanogr.* 42:1132–1136.

Smolarz, K., M. Wołowicz & M. Stachnik. 2006. First record of the occurrence of "gill disease" in *Mytilus edulis trossulus* from the Gulf of Gdańsk (Baltic Sea, Poland). *J. Invertebr. Pathol.* 93:207–209.

Smolowitz, R. & S. E. Shumway. 1997. Possible cytotoxic effects of the dinoflagellate, *Gyrodinium aureolum*, on juvenile bivalve mollusks. *Aquacult. Int.* 5:291–300.

Sobral, P. & J. Widdows. 1997. Influence of hypoxia and anoxia on the physiological responses of the clam *Ruditapes decussatus* from southern Portugal. *Mar. Biol.* 127:455–461.

Spatharis, S., N. P. Dolapsakis, A. Economou-Amilli, G. Tsirtsis & D. B. Danielidis. 2009. Dynamics of potentially harmful microalgae in a confined Mediterranean Gulf: assessing the risk of bloom formation. *Harmful Algae* 8:736–743.

Suzuki, R. & T. Ishimaru. 1990. An improved method for the determination of phytoplankton chlorophyll using N,N-dimethylformamide. *J. Oceanogr.* 46:190–194.

Tran, D., H. Haberkorn, P. Soudant, P. Ciret & J. C. Massabuau. 2010. Behavioral responses of *Crassostrea gigas* exposed to the harmful algae *Alexandrium minutum*. *Aquaculture* 298:338–345.

Turki, S. 2004. Suivi des microalgues planktoniques toxiques dans les zones de production, d'élevage des mollusques bivalves et d'exploitation des oursins du Nord de la Tunisie. *Bull. Inst. Natn. Scien. Techn. Mer de Salammbo* 31:83–96. (in French, with English abstract).

Villalba, A., K. S. Reece, M. C. Ordas, S. M. Casas & A. Figueras. 2004. Perkinsosis in molluscs: a review. *Aquat. Living Resour.* 17:411–432.

Wang, D. Z. 2008. Neurotoxins from marine dinoflagellates: a brief review. *Mar. Drugs* 6:349–371.

Ward, J. E., J. S. Levinton, S. E. Shumway & T. Cucci. 1997. Site of particle selection in bivalve molluscs. *Nature* 390:131–132.

Widdows, J., M. N. Moore, D. M. Lowe & P. N. Salkeld. 1979. Some effects of a dinoflagellate bloom (*Gyrodinium aureolum*) on mussel, *Mytilus edulis*. *J. Mar. Biol. Assoc. UK* 5:522–524.

Wikfors, G. H. & R. M. Smolowitz. 1993. Detrimental effects of a *Prorocentrum minimum* isolate upon hard clams and bay scallops in laboratory feeding studies. In: T. J. Smayda & Y. Shimizu, editors. Toxic phytoplankton blooms in the sea. New York: Elsevier Science. pp. 477–452.

Wikfors, G. M. & R. M. Smolowitz. 1995. Do differences in dinoflagellate damage depend upon ingestion? *J. Shellfish Res.* 14:282.

Yamatogi, T., M. Sakaguchi, M. Matsuda, S. Iwanaga, M. Iwataki & K. Matsuoka. 2005. Effect on bivalve molluscs of a harmful dinoflagellate *Heterocapsa circularisquama* isolated from Omura Bay, Japan and its growth characteristics. *Nippon Suisan Gakkaishi* 71:746–754. (in Japanese with English abstract).