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Haemolytic activity and reactive oxygen species production of four harmful algal bloom species

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ABSTRACT

Based on haemolytic activity and reactive oxygen species (ROS) production of *Chattonella marina, Chattonella antiqua, Heterocapsa circularisquama, Alexandrium tamiyavanichii* and *Karenia mikimotoi*, the species were categorized into four types. (1) *H. circularisquama*: haemolytic activity was detected in both cell suspension and cell-free culture supernatant, but with greater activity in cell suspension than in the supernatant suggesting the presence of both cell surface and secreted haemolytic agents. (2) *A. tamiyavanichii*: equal haemolytic activities were detected in both the cell suspension and cell-free culture supernatant suggesting the presence of only secreted haemolytic agents. (3) *K. mikimotoi*: haemolytic activity was detected only in the cell suspension, indicating haemolytic agents occur only on the cell surface. (4) *C. marina* and *C. antiqua*: no significant haemolytic activity was detected in either cell suspension or cell-free culture supernatant, but high ROS were detected in the cell suspensions. *Heterocapsa circularisquama* and *K. mikimotoi* showed lethal effects on rotifers (*Brachionus plicatilis*), whereas *A. tamiyavanichii, C. marina* and *C. antiqua* had no effect. Our results suggest that *H. circularisquama, K. mikimotoi* and *A. tamiyavanichii* produce haemolytic agents with distinct characteristics, whereas *C. marina* and *C. antiqua* have an extremely potent ability to produce ROS.

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KEYWORDS Dinoflagellate; haemolytic agent; harmful algal blooms; raphidophyte; reactive oxygen species; red tide

Introduction

The frequency, magnitude and duration of harmful algal blooms (HABs) are increasing as a result of global warming (Paerl & Huisman, 2008). HABs often cause mass mortality of wild and farmed fish and shellfish, resulting in serious economic losses and pollution of coastal marine habitats. In addition to such direct effects on marine organisms, some HAB-forming species produce toxic substances that can accumulate in shellfish and other marine organisms, which if consumed are a potential threat to human health. The mechanisms and impacts of toxicity tend to vary substantially among species. More than 100 species of toxic marine microalgae have been identified but the mechanisms causing toxicity in these HAB species are mostly still unclear. The major groups of organisms that form HABs are dinoflagellates (*Heterocapsa circularisquama, Karenia mikimotoi* and *Alexandrium* spp.) and raphidophytes (*Chattonella* spp.).

Chattonella spp. are highly toxic to fish, particularly yellowtail (*Seriola quinqueradiata*; Okaichi, 1989). Previous studies have shown that *Chattonella* spp. produce extremely high concentrations of reactive oxygen species (ROS) under normal growth conditions (Shimada *et al.*, 1991; Oda *et al.*, 1997; Kim *et al.*, 2007) and several lines of evidence implicate these in fish mortality (Ishimatsu *et al.*, 1996; Kim *et al.*, 1999; Marshall *et al.*, 2003, 2005; Hiroishi *et al.*, 2005). *Chattonella* spp. are also known to produce several other toxic substances, such as neurotoxins, haemolytic agents and haemagglutinating agents (Ahmed *et al.*, 1995; Khan *et al.*, 1996; Kuroda *et al.*, 2005). However, it has been reported that dead cells of *C. antiqua* and filtrate of the culture medium containing the algae were not toxic to red sea bream (Matsusato & Kobayashi, 1974). Ishimatsu *et al.* (1996) also reported that ruptured *C. marina* cells showed no toxic effects on yellowtail, and there was a clear correlation between the cells' ability to produce O_2^- and fish-killing activity (Ishimatsu *et al.*, 1996). These findings suggest that live cells are essential for the toxicity of *Chattonella* spp., and any toxic factors may be quite unstable. *Heterocapsa*

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circularisquama can be lethal to shellfish, particularly bivalves such as pearl oysters, but is not known to be toxic to fish (Matsuyama, 2012). In a previous study, we showed that *H. circularisquama* has a haemolytic toxin that may be a cause of shellfish mortality (Oda *et al.*, 2001). The bloom-forming *K. mikimotoi* is known to be toxic to both fish and shellfish (Honjo, 1994). Previous studies have demonstrated that *K. mikimotoi* produces various toxic agents, including haemolytic toxins and ROS (Jenkinson & Arzul, 2001; Yamasaki *et al.*, 2004; Satake *et al.*, 2005; Gentien *et al.*, 2007). Some *Alexandrium* spp. are known to produce toxins that cause paralytic shellfish poisoning (PSP) in humans. In addition, some *Alexandrium* spp. are reported to have direct detrimental effects on several marine organisms (Sephton *et al.*, 2007; Galimany *et al.*, 2008; Teegarden *et al.*, 2008). Previous work suggests that *A. tamiyavanichii, A. tamarense* and *A. fraterculus* produce cytotoxic agents (Katsuo *et al.*, 2007; Yamasaki *et al.*, 2008), whereas *A. taylori* secretes a proteinaceous haemolytic toxin (Emura *et al.*, 2004).

These findings suggest that HAB toxicity results from a wide variety of toxic agents, which vary among species. Haemolytic agents and ROS seem to be important toxic factors. However, the characteristics and biochemical features of the haemolytic toxins and the importance of ROS as a toxic factor remain poorly understood. Furthermore, there is no comparative study on haemolytic and ROS-producing activities among different HAB species so far, so it is not clear whether or not the activity detected in a certain species is significant compared with other species. To gain insight into this point, comparative study on the various HAB species under the same experimental conditions is necessary, which may provide useful information for understanding the major toxic factors of HAB species. In this study, we measured and compared the haemolytic activity and ROS production of *C. marina, C. antiqua, K. mikimotoi, A. tamiyavanichii* and *H. circularisquama* to gain insights into the potentially toxic agents produced by these HAB species.

Materials and methods

Materials

Superoxide dismutase (Cu/Zn-SOD, 3800 U mg^{-1} of protein, from bovine erythrocytes), horseradish peroxidase (100 U mg^{-1} of protein), catalase (5000–15 000 U mg^{-1} of protein), and L-012 were purchased from Wako Pure Chemical Industries, Ltd, Osaka, Japan. *P*-hydroxyphenylacetic acid (PHPA) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). Other chemicals were of the highest grade commercially available.

Cultivation

HAB species were obtained from a variety of sources. *Heterocapsa circularisquama* Horiguchi and *Alexandrium tamiyavanichii* Balech were isolated from Fukuyama Bay and Hiroshima Bay, Japan, respectively. *Chattonella marina* (Subrahmanyan) Hara & Chihara and *C. antiqua* (Hada) Ono were isolated from Kagoshima Bay and Shimabara Bay, Japan, respectively. These *Chattonella* species were genetically closely related (Demura *et al.*, 2009), but they were morphologically distinguishable and hence, we considered them as different species. *Karenia mikimotoi* (Miyake & Kominami *ex* Oda) Gert Hansen & Ø.Moestrup was isolated from Hiroshima Bay, Japan. Cultures of these species were maintained at 26°C in 100 ml flasks containing 60 ml of modified seawater medium (SWM-3) at a salinity of 25 psu (Yamasaki *et al.*, 2007). The modified SWM-3 containing a Tris-HCl buffer system was autoclaved for 15 min at 121°C before use. The cultures were kept under a 12:12-h photoperiod regime using a cool-white fluorescent lamp (200 ± 5 µmol photons m^{-2} s^{-1}). Cell numbers in the cultures were counted using a haemocytometer (Erma Inc., Tokyo, Japan). The rotifer *Brachionus plicatilis* Müller was provided by Dr A. Hagiwara (Faculty of Fisheries, Nagasaki University, Japan) and was cultured with *Nannochloropsis oculata* (Droop) D.J.Hibberd. All samples were cultured using sterilized instruments. Each culture was used throughout the experiments at late exponential growth phase unless otherwise specified.

Measurement of haemolytic activity

For each HAB species, a cell-free culture supernatant was obtained from 1 ml of cell suspension of each species in the late exponential growth phase by centrifugation at 5000 × *g* for 5 min at 4°C. Triplicate 50 µl aliquots of serial two-fold dilutions (using modified SWM-3) of intact cell suspension and cell-free culture supernatant were then added to round-bottom 96-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA). Haemolytic activity was measured using rabbit erythrocytes isolated from rabbit blood (Nippon Bio-Test Laboratories, Tokyo, Japan). Erythrocytes were washed three times with phosphate-buffered saline (PBS) and adjusted to a final concentration of 4% (v/v) in modified SWM-3. To each well containing test samples, 50 µl of the 4% (v/v) erythrocyte suspension was added. The well plates were gently shaken. After incubation for 5 h at 26°C under illumination from a fluorescent lamp (200

μmol photons m^{-2} s^{-1}), the plates were centrifuged at 900 × *g* for 5 min. Aliquots (70 μl) of supernatant were withdrawn from the wells and transferred to flat-bottom 96-well plates (Becton-Dickinson). Released haemoglobin was determined by measuring absorbance at 560 nm using a microplate reader (Multiskan GO, Fisher Scientific Inc., Massachusetts, USA). Negative controls (zero haemolysis) and positive controls (100% haemolysis) were prepared using erythrocytes suspended in modified SWM-3 alone and in modified SWM-3 containing 1% Triton X-100 (v/v), respectively.

To examine the effect of heat on haemolytic activity, cell-free supernatant prepared from a *H. circularisquama* culture (1×10^5 cells ml^{-1}) and an *A. tamiyavanichii* culture (2×10^3 cells ml^{-1}) was subjected to a range of temperatures (0, 27, 37 and 60° C) for 1, 2, 3 and 18 h. Following this, haemolytic activity was measured following the procedure described above. To estimate the molecular size of the haemolytic agents in the supernatants from *H. circularisquama* and *A. tamiyavanichii*, supernatants were passed through ultrafiltration units (3000~100,000 cut filters, Merck KGaA, Darmstadt, Germany). The haemolytic activity of each filtrate was measured as described above.

To gain insight into which molecules the haemolytic agents in the supernatants of *H. circularisquama* and *A. tamiyavanichii* recognized, haemolytic activity was examined in the presence of various carbohydrates (final 10 mM).

Rotifer exposure experiment

Rotifer exposure tests were conducted in 48-well plates (Becton-Dickinson). One hundred μl of modified SWM-3 containing 10 rotifers was added to each well in a 48-well plate which contained 900 μl of cell suspension from each HAB species. The final concentration of cells was adjusted to 1×10^4 cells ml^{-1} for *C. marina, C. antiqua* and *K. mikimotoi*, 2×10^3 cells ml^{-1} for *A. tamiyavanichii* and 1×10^5 cells ml^{-1} for *H. circularisquama*. As a negative control, 10 individual rotifers were cultured in 1 ml of the modified SWM-3 alone. The number of dead rotifers was counted every hour over the course of a 7-h incubation using a stereomicroscope.

Measurement of O_2^-

Production of superoxide anions (O_2^-) by each HAB species was measured at 26°C using the chemiluminescence method, with L-012 as a superoxide-specific chemiluminescent probe as described by Kadomura *et al.* (2006). The probe was dissolved in distilled water and stored at −80°C until use. After the addition of L-012 to each flagellate cell suspension in the presence or absence of SOD, the chemiluminescence response was recorded immediately with a Bio-Orbit Luminometer (1254-001, Bio-Orbit Oy, Turku, Finland). The reaction mixtures typically consisted of 145 μl of each flagellate cell suspension, 50 μl of L-012 (final 10 μM) and 5 μl of SOD solution (final 100 U ml^{-1}) or 5 μl of modified SWM-3. As a control, chemiluminescence response in modified SWM-3 containing each probe was measured in the absence of flagellate cells.

Measurement of H_2O_2

The PHPA assay method at 26°C was used to detect H_2O_2 in the flagellate cell suspensions (Hyslop & Sklar, 1984). After the addition of PHPA (final 1 mM) and horseradish peroxidase (final 100 μg ml^{-1}) to the flagellate cell suspension in the presence or absence of catalase (final 100 U ml^{-1}), any increase in fluorescence intensity during the first min of incubation was measured with a fluorescent spectrophotometer (Model 650-60, Hitachi High-Tech Science Co., Tokyo, Japan) at an excitation wavelength of 317 nm and an emission wavelength of 400 nm. Each measurement was repeated three times. H_2O_2 concentration was estimated using a standard curve of H_2O_2 in cell-free modified SWM-3 medium. The standard solution of H_2O_2 in modified SWM-3 was prepared using reagent H_2O_2. Under these conditions the increase in fluorescence intensity was proportional to H_2O_2 concentration.

Statistical analysis

All experiments were carried out in triplicate. Data were expressed as mean ± standard deviation (SD), and paired Student's *t*-tests were used to test for significant differences, with $p = 0.05$ considered to be the threshold for statistical significance.

Results

Haemolytic activity

Haemolytic activity in *H. circularisquama* cell suspension was positively related to cell concentration, approaching its maximum at 5×10^4 cells ml^{-1} (Fig. 1A). The cell-free culture supernatant prepared from *H. circularisquama* culture showed reduced haemolytic activity in comparison. The cell concentration of *A. tamiyavanichii* at late exponential growth phase was around 2×10^3 cells ml^{-1}, much lower than that of *H. circularisquama*. Despite this, significant cell-concentration dependent haemolytic activity was observed (Fig. 1B). Haemolytic activity in *A. tamiyavanichii* cell-free culture supernatant differed little from that in the cell suspension. *Karenia mikimotoi*

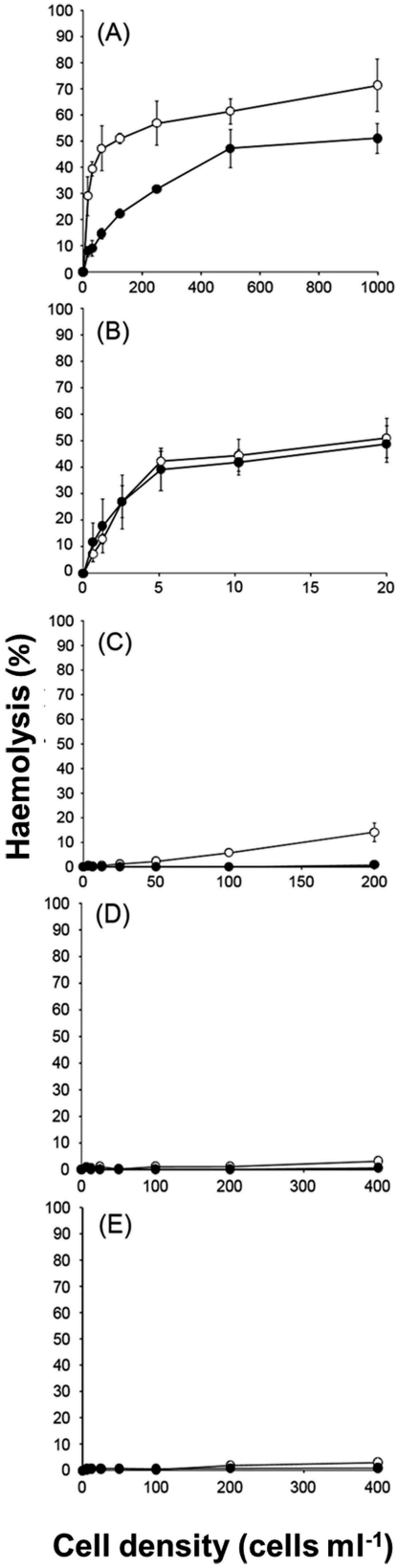

Fig. 1. Haemolytic activities of cell suspension (○) and cell-free culture supernatant (●) of *Heterocapsa circularisquama* (A), *Alexandrium tamiyavanichii* (B), *Karenia mikimotoi* (C), *Chattonella marina* (D) and *C. antiqua* (E). Cell suspensions or supernatants equivalent to the indicated cell concentration of each species were mixed with rabbit erythrocytes. After 5 h incubation at 26°C, the extents of haemolysis were measured. Each point represents the mean of triplicate measurements, ± SD (%).

showed a positive relationship between cell concentration and haemolytic activity, although even at 2×10^4 cells ml^{-1} activity was less than that of *H. circularisquama* at 3×10^2 cells ml^{-1} or *A. tamiyavanichii* at 1.25×10^2 cells ml^{-1} (Fig. 1C). No significant haemolytic activity was observed in either cell suspensions or cell-free supernatants of *C. marina* and *C. antiqua* at concentrations of up to 4×10^4 cells ml^{-1} (Figs 1D, E).

Biochemical characterization of H. circularisquama *and* A. tamiyavanichii *haemolytic agents*

The haemolytic constituents of cell-free supernatants from *H. circularisquama* (1×10^5 cells ml^{-1}) and *A. tamiyavanichii* (2×10^3 cells ml^{-1}) were heat labile; haemolysis was reduced by 70% after 1 h of incubation at 60°C (Figs 2A, B). The haemolytic properties of *H. circularisquama* were slightly more heat-stable than those of *A. tamiyavanichii* supernatant; *H. circularisquama* haemolysis showed almost no reduction after 18 h of incubation at 0°C, whereas that of *A. tamiyavanichii* decreased by nearly 50%. In both *H. circularisquama* and *A. tamiyavanichii*, haemolytic activities of the supernatants declined by 90% after being passed through the 100 000 cut-filter, suggesting that the haemolytic agents may have molecular size greater than 100 000. There was no haemolytic activity detected in the filtrates obtained from other filters.

Effects of various saccharides on the haemolytic activities of the cell-free supernatants of H. circularisquama *and* A. tamiyavanichii

The inhibition profiles obtained in the presence of saccharides differed between *H. circularisquama* and *A. tamiyavanichii* (Fig. 3). Haemolysis of supernatant of *A. tamiyavanichii* was most inhibited by mannitol, whereas that of *H. circularisquama* was most inhibited by fructose, although this effect was comparatively small.

Superoxide (O_2^-) and hydrogen peroxide (H_2O_2)

The strongest chemiluminescence response was observed in *C. antiqua* (1×10^4 cells ml^{-1}), and a weaker but significant response was observed in *C. marina* (1×10^4 cells ml^{-1}) (Fig. 4A). In the presence of SOD (100 U ml^{-1}), these responses decreased to the background level observed in the medium alone. A weak response, only slightly above the background level, was observed in *K. mikimotoi* (Fig. 4B). In *H. circularisquama* and *A. tamiyavanichii*, the responses were almost background level (Figs 4A, B).

The highest H_2O_2 concentration was observed in *C. marina* cell suspension, and lower but significant

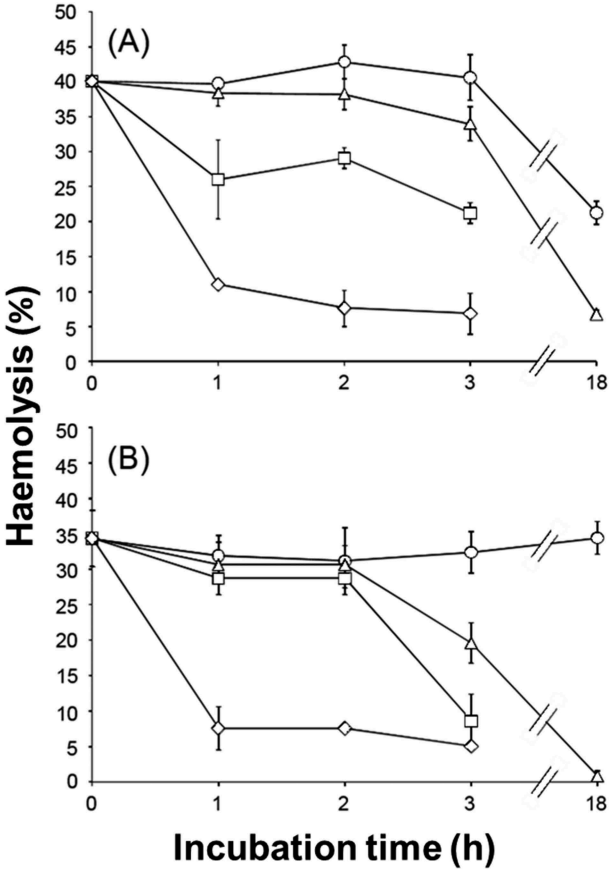

Fig. 2. Heat-stability of the haemolytic activities of cell-free culture supernatant of *A. tamiyavanichii* (A) and *H. circularisquama* (B). The cell-free supernatant of each species was treated at 0 (○), 27 (△), 37 (□) and 60°C (◇) for the indicated periods of time, and then the haemolytic activities were measured. Each point represents the mean of triplicate measurements, ± SD (%).

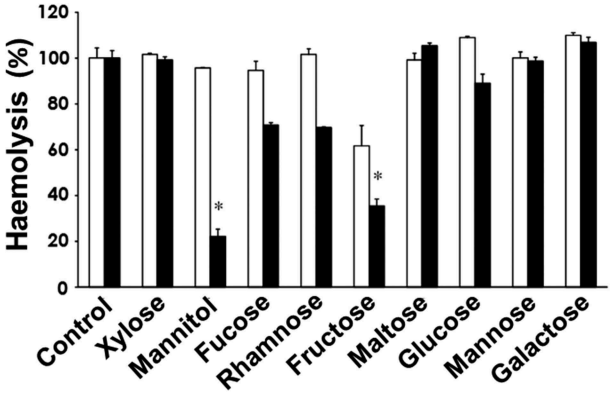

Fig. 3. Effects of various saccharides on the haemolytic activities of the cell-free culture supernatants of *H. circularisquama* (□) and *A. tamiyavanichii* (■). In the absence (control) or presence of the indicated saccharides at 10 mM, the haemolytic activities of the cell-free culture supernatants of each species were measured. The activity was expressed as % of control (100%) without the saccharides. Data are means ± SD (%) of triplicate measurements. Asterisks indicate significant differences between the absence (control) and presence of saccharides ($^{*}p < 0.05$).

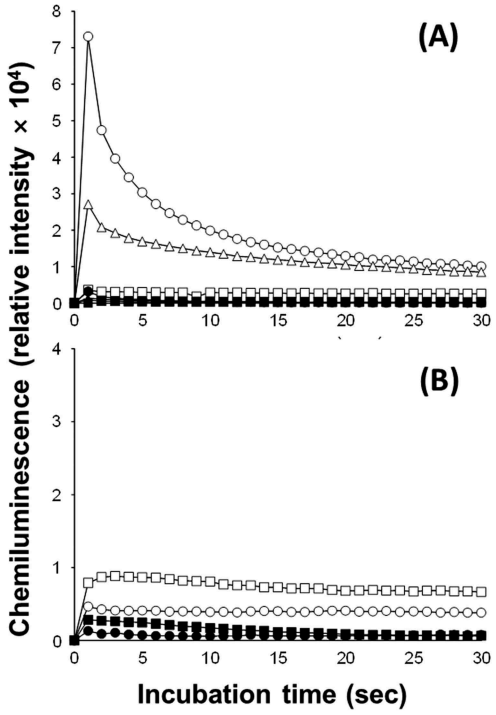

Fig. 4. L-012-mediated chemiluminescence responses of *C. marina* (△, ▲), *C. antiqua* (○, ●) and *H. circularisquama* (□, ■) (A), *K. mikimotoi* (□, ■) and *A. tamiyavanichii* (○, ●) (B). Cell suspensions of *C. marina* (1×10^4 cells ml^{-1}), *C. antiqua* (1×10^4 cells ml^{-1}), *H. circularisquama* (10^5 cells ml^{-1}), *A. tamiyavanichii* (2×10^3 cells ml^{-1}), and *K. mikimotoi* (1×10^4 cells ml^{-1}) were subjected to L-012-mediated chemiluminescence analysis in the presence (●,▲,■) or absence (○,△,□) of SOD (100 U ml^{-1}).

concentration was detected in that of *C. antiqua* (Fig. 5). H_2O_2 concentrations in cell suspensions of *H. circularisquama* and *K. mikimotoi* were low compared with those of *Chattonella* spp. H_2O_2 was undetectable in *A. tamiyavanichii* cell suspension (Fig. 5).

Effects on rotifers

As shown in Fig. 6, *H. circularisquama* had a potent lethal effect on rotifers; the number of live rotifers gradually decreased with incubation time until all rotifers were dead after 6 h. *Karenia mikimotoi* also showed toxic effects on rotifers. In contrast, *C. marina, C. antiqua* and *A. tamiyavanichii* showed no significant toxic effects on rotifers.

Discussion

Several haemolytic compounds have been isolated from phytoplankton, most of which are fatty acids, such as polyunsaturated fatty acids (PUFAs) (Landsberg, 2002). Examples include digalactosyl monoacylglycerol and octadecapentaenoic acid, which were isolated from *K. mikimotoi* and shown

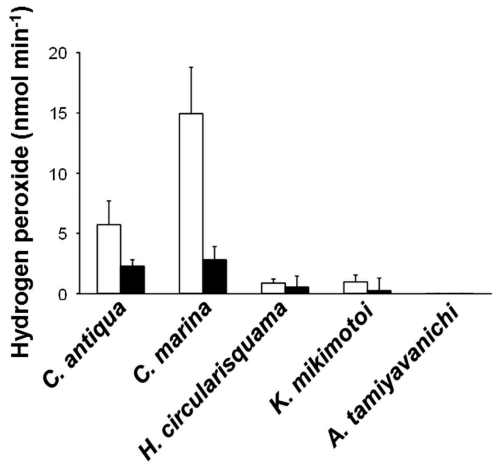

Fig. 5. Hydrogen peroxide (H_2O_2) levels in the cell suspensions of *C. marina* (1×10^4 cells ml^{-1}), *C. antiqua* (1×10^4 cells ml^{-1}), *H. circularisquama* (1×10^5 cells ml^{-1}), *K. mikimotoi* (1×10^4 cells ml^{-1}) and *A. tamiyavanichii* (2×10^3 cells ml^{-1}). Cell suspension of each species was subjected to PHPA-H_2O_2 detection assay in the absence (□) or presence (■) of catalase (100 U ml^{-1}). Each column represents the mean of triplicate measurements ± SD.

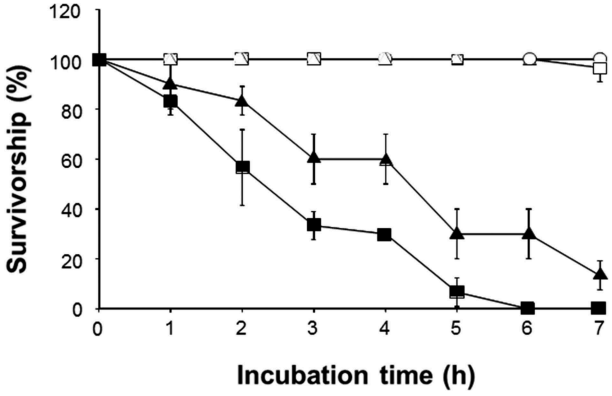

Fig. 6. Effects of *C. marina, C. antiqua, H. circularisquama, K. mikimotoi* and *A. tamiyavanichii* on the rotifer *Brachionus plicatilis*. After exposure to cell suspension of *C. marina* (1×10^4 cells ml^{-1}) (○), *C. antiqua* (1×10^4 cells ml^{-1}) (△), *H. circularisquama* (1×10^5 cells ml^{-1}) (■), *A. tamiyavanichii* (2×10^3 cells ml^{-1}) (□) or *K. mikimotoi* (1×10^4 cells ml^{-1}) (▲), the number of viable rotifers was counted at the indicated times. Each point represents the mean of triplicate measurements ± SD.

to be haemolytic and ichthyotoxic (Yasumoto *et al.*, 1990). Eicosapentaenoic acid (EPA) is the most potent of PUFAs (Takagi *et al.*, 1984; Jüttner, 2001; Marshall *et al.*, 2003). It has been reported that EPA causes harmful effects on zooplankton (Jüttner, 2001), phytoplankton (Arzul *et al.*, 1998), and fish (Marshall *et al.*, 2003). These findings suggest that haemolytic fatty acids may play important roles in the harmful effects of HABs on aquatic organisms. However, these fatty acid-related haemolytic agents are generally isolated and identified by organic solvent-extraction from the cells. Hence, the mechanism of how hydrophobic water-insoluble fatty acids can release from HAB species and attach to the surrounding organisms in the seawater environment is still unclear. Previous studies on the haemolytic properties of dinoflagellate cell suspensions suggested the presence of water-soluble high molecular weight haemolytic compounds that may be involved in the toxicity (Emura *et al.*, 2004; Yamasaki *et al.*, 2008), although there is only a limited available information on the high molecular weight haemolytic agents. It seems likely that assays that directly detect haemolytic activity in phytoplankton cell suspensions or cell-free supernatants, without the need for extraction or isolation procedures, may provide important tools for finding the toxic factors truly responsible for the harmful effects of phytoplankton.

In this study, we found that the strength of the haemolytic effects of cell suspensions of *H. circularisquama, A. tamiyavanichii* and *K. mikimotoi* on rabbit erythrocytes depended on cell density and varied among species. In contrast, *C. marina* and *C. antiqua* showed no significant haemolytic activity (Fig. 1). Although cell size differs among species, *A. tamiyavanichii* showed significant haemolytic activity even at quite low cell densities (less than 1×10^3 cells ml^{-1}) compared with the other species (around 1×10^4–1×10^5 cells ml^{-1}). Comparison of the haemolytic activity of the cell suspensions and the cell-free supernatants suggests that the haemolytic properties of the HAB species studied can be divided into four categories, defined in terms of location and activity (Fig. 7). There seem to be two types of haemolytic agents, those occurring on the cell surface and those secreted extracellularly. Category 1 contains both secreted and cell surface-associated haemolytic agents and is represented by *H. circularisquama*. Category 2 contains only secreted agents, and is represented by *A. tamiyavanichii*. Category 3 is typified by *K. mikimotoi*, and contains only cell surface-associated haemolytic agents. The 4th category is represented by *C. marina* and *C. antiqua*, which have no haemolytic activity. Although the exact biological significance of these categories is still unclear, *H. circularisquama* and *K. mikimotoi*, which have cell surface-associated haemolytic agents, are known to be toxic to shellfish and zooplankton (Zou *et al.*, 2010; Matsuyama, 2012). Previous studies have suggested that direct contact between *H. circularisquama* cells and the soft tissues of bivalve molluscs (Matsuyama *et al.*, 1997) or microzooplankton (Kamiyama, 1997; Kamiyama & Arima, 1997) is important for a lethal effect to occur. Our previous study also showed that *K. mikimotoi* exerted a lethal effect on zooplankton rotifers through direct contact (Zou *et al.*, 2010). Consistent with these findings, *H. circularisquama* and *K. mikimotoi* had potent lethal effects on rotifers in exposure experiments, whereas *A. tamiyavanichii, C. marina*

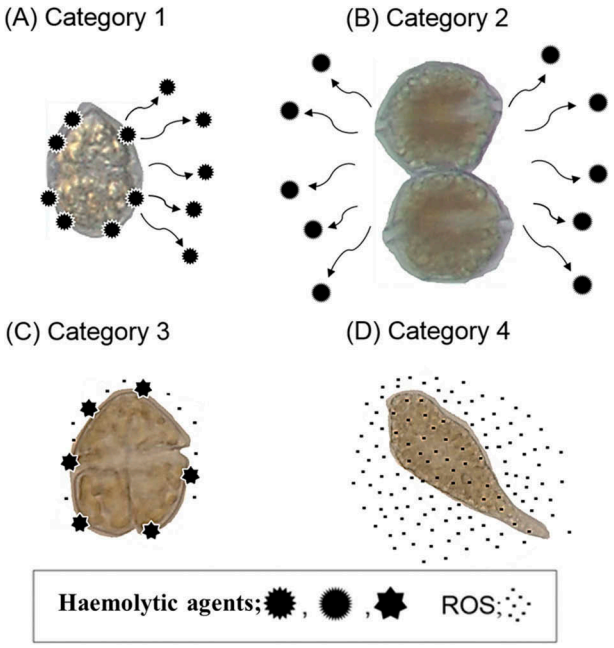

Fig. 7. Schematic illustrations of the presence of the haemolytic agents and ROS production in *H. circularisquama* (A), *A. tamiyavanichii* (B), *K. mikimotoi* (C) and *Chattonella* spp. (D). In *H. circularisquama*, some of the haemolytic agents are secreted extracellularly and some are bound on the cell surface. In *A. tamiyavanichii*, almost all the haemolytic agents are secreted extracellularly. In *K. mikimotoi*, almost all the haemolytic agents are bound on the cell surface, and low levels of ROS are produced. In *Chattonella* spp., there is no haemolytic agent, but high levels of ROS are produced.

and *C. antiqua* had no effect (Fig. 6). Taken together, these results suggest that cell-surface associated haemolytic agents observed in *H. circularisquama* and *K. mikimotoi* may play an important role in their toxicity to bivalves and zooplankton. Furthermore, *A. tamiyavanichii* had no toxic effect on rotifers in spite of having far higher haemolytic activity than *K. mikimotoi*. This suggests that the type of haemolytic agent (i.e. extracellular vs. surface associated) may have a greater influence on toxicity of dinoflagellates than haemolytic potency, at least in the species analysed in this study. In addition, the mechanisms by which bivalves and zooplankton recognize HAB species may be related to cell contact with phytoplankton (Matsuyama *et al.*, 1997; Kim *et al.*, 2000; Zou *et al.*, 2010). Probably, the toxic mechanisms of dinoflagellates are complicated and quite different depending on species, and even on the victimized organisms. Further studies are required to clarify these points.

The inhibitory effects of various saccharides on the haemolytic activity of cell-free supernatants differed between *H. circularisquama* and *A. tamiyavanichii* (Fig. 3), suggesting molecular recognition mechanisms differ between these two HAB species. It seems likely that the haemolytic agents of these species may recognize different saccharide chains in the rabbit erythrocytes. Mannitol had a large inhibitory effect on the haemolytic activity of *A. tamiyavanichii*, but not on that of *H. circularisquama* (Fig. 3). Heat-stability and ultrafiltration analyses indicate that the haemolytic agents secreted from *H. circularisquama* and *A. tamiyavanichii* are heat-labile, high molecular weight compounds (Fig. 2).

In the case of unstable exotoxins like haemolytic agents of these dinoflagellates, it is essential to keep the activity stable for purification and characterization. Further studies are required to identify the molecules in erythrocytes important for recognition in these dinoflagellates as well as the biochemical characterization of the haemolytic agents. In that context, it is advantageous that the activity of the haemolytic agent secreted from *H. circularisquama* was stable at 0°C at least for 18 h. We are now planning to purify the haemolytic agent from *H. circularisquama* by a combination of several chromatographic procedures at 0°C.

Previous studies demonstrated that *Chattonella* spp. produce significant levels of ROS and suggested that these might be responsible for fish mortality, although this is still debated (Ishimatsu *et al.*, 1996). Chemiluminescence and fluorescence analyses confirmed that *C. marina* and *C. antiqua* produce ROS in far greater quantities than the other species tested. Other species of raphidophycean flagellates, such as *Heterosigma akashiwo, Fibrocapsa japonica* and *Olisthodiscus luteus*, are known to produce ROS (Oda *et al.*, 1997). Hence, ROS production may be a common characteristic of raphidophycean flagellates. *Chattonella antiqua* produced more superoxide, but less hydrogen peroxide than *C. marina* (Figs 4, 5). The reason for this is still unclear, but our previous study indicated that the locations and underlying mechanisms of superoxide and hydrogen peroxide generation were different (Kim *et al.*, 2007). Fluorescent microscopic analysis using ROS-specific probes suggested that superoxide is produced on the surface of *Chattonella* cells, while hydrogen peroxide is produced in intracellular compartments (Kim *et al.*, 2007). In addition, it has been reported that *Hymenomonas carterae*, a marine phytoplankter, produces extracellular hydrogen peroxide without involvement of superoxide generation (Palenik *et al.*, 1987). Hence it seems likely that the metabolic or enzymatic systems responsible for hydrogen peroxide and superoxide generation are independent, and they may differ somehow between *C. marina* and *C. antiqua*. Since these raphidophytes showed no toxic effects on rotifers, it is unlikely that ROS are toxic to rotifers.

Recently we have found that *Chattonella marina* and the *Chattonella antiqua* used in this study showed different fish-killing activities and ROS-producing activities, and both activities were well correlated (Cho *et al.*, 2016), suggesting that ROS may be a factor

responsible for the fish-killing activity of *Chattonella* spp.

Our results, together with previous findings, suggest that the cell surface structures of HAB species, which may be deeply involved in the haemolytic activity in some dinoflagellates or superoxide production in raphidophytes, play an important role in the toxicity to surrounding organisms. Based on the results obtained in this study, we consider that detailed analysis of live cell suspension or culture supernatant, especially focusing on cell surface structure or high molecular weight compounds, is necessary to clarify the toxic mechanisms of HAB species.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contributions

K. Cho: wrote the paper. T. Kasaoka and M. Ueno: performed the experiments and analysed the data; revised the final version of the manuscript. L. Basti and Y. Yamasaki: revised the final version of the manuscript. D. Kim and T. Oda: original concept; revised final version of the manuscript.

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