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Intracellular haemolytic agents of *Heterocapsa circularisquama* exhibit toxic effects on *H. circularisquama* cells themselves and suppress both cell-mediated haemolytic activity and toxicity to rotifers (*Brachionus plicatilis*)

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ABSTRACT

A harmful dinoflagellate, *Heterocapsa circularisquama*, is highly toxic to shellfish and the zooplankton rotifer *Brachionus plicatilis*. A previous study found that *H. circularisquama* has both light-dependent and -independent haemolytic agents, which might be responsible for its toxicity. Detailed analysis of the haemolytic activity of *H. circularisquama* suggested that light-independent haemolytic activity was mediated mainly through intact cells, whereas light-dependent haemolytic activity was mediated by intracellular agents which can be discharged from ruptured cells. Because *H. circularisquama* showed similar toxicity to rotifers regardless of the light conditions, and because ultrasonic ruptured *H. circularisquama* cells showed no significant toxicity to rotifers, it was suggested that live cell-mediated light-independent haemolytic activity is a major factor responsible for the observed toxicity to rotifers. Interestingly, the ultrasonic-ruptured cells of *H. circularisquama* suppressed their own lethal effect on the rotifers. Analysis of samples of the cell contents (supernatant) and cell fragments (precipitate) prepared from the ruptured *H. circularisquama* cells indicated that the cell contents contain inhibitors for the light-independent cell-mediated haemolytic activity, toxins affecting *H. circularisquama* cells themselves, as well as light-dependent haemolytic agents. Ethanol extract prepared from *H. circularisquama*, which is supposed to contain a porphyrin derivative that displays photosensitising haemolytic activity, showed potent toxicity to *Chattonella marina*, *Chattonella antiqua*, and *Karenia mikimotoi*, as well as to *H. circularisquama* at the concentration range at which no significant toxicity to rotifers was observed. Analysis on a column of Sephadex LH-20 revealed that light-dependent haemolytic activity and inhibitory activity on cell-mediated light-independent haemolytic activity existed in two separate fractions (f-2 and f-3), suggesting that both activities might be derived from common compounds. Our results suggest that the photosensitising haemolytic toxin discharged from ruptured *H. circularisquama* cells has a relatively broad spectrum of phytoplankton toxicity, and that physical collapse of *H. circularisquama* cells can lead not only to the disappearance of its own toxicity, but also to mitigation of the effects of other HABs.

1. Introduction

Heterocapsa circularisquama is a toxic dinoflagellate that has been causing mass mortality of bivalves in the coastal areas of western Japan since 1988 (Horiguchi, 1995; Matsuyama et al.,

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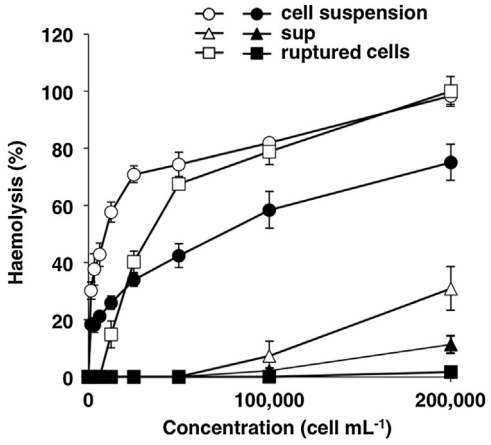

Fig. 1. Haemolytic activities of live cell suspension (○,●), cell-free cultured supernatant (△,▲), and ultrasonically ruptured cells (□,■) of *Heterocapsa circularisquama* on rabbit erythrocytes in the light (○,△,□) and in the dark (●, ▲, ■). Each sample and all erythrocytes were incubated in 96 well-plates in a SWM-3 medium at 26 °C for 5 h, and then the haemolysis was measured as described in the text. Each point represents the average of triplicate measurements. Each bar represents standard deviation.

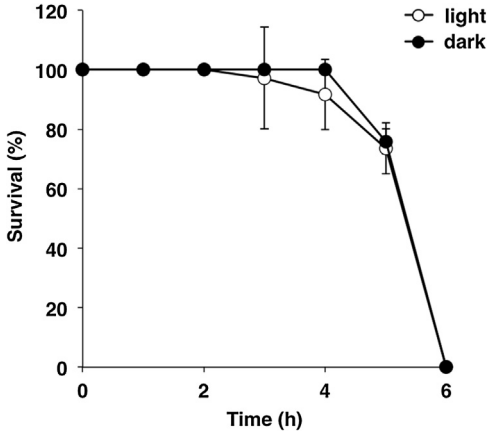

Fig. 2. Toxicity of *Heterocapsa circularisquama* on rotifers in the light (○) and in the dark (●). Rotifers in 48-well plates (10 rotifers well^{-1}) were exposed to *H. circularisquama* (final 1×10^4 cells mL^{-1}) suspended in SWM-3 medium at 26 °C for the indicated periods of time, and then the number of viable rotifers remaining were counted as described in the text. Each point represents the mean of triplicate measurements. Each bar represents standard deviation.

1996). Harmful algal blooms (HABs) of *H. circularisquama* have been rapidly increasing since early 1990. A characteristic feature of this dinoflagellate is that it is known to be highly toxic to bivalves such as the pearl oyster (*Pinctada fucata*), short-necked clam (*Ruditapes philippinarum*), and oyster (*Crassostrea gigas*). Harmful effects on wild and cultured finfish, other marine vertebrates, and public health have not been reported so far (Matsuyama et al., 1992; Yamamoto and Tanaka, 1990). Pearl oysters exposed to >10^6 *H. circularisquama* cells L^{-1} in laboratory exposures immediately contracted their mantles and closed their valves, became paralysed, and then eventually died (Nagai et al., 1996). These symptoms closely resembled those of previous field observations (Matsuyama et al., 1996). In addition, paralytic shellfish poisoning (PSP) and diarrheic shellfish poisoning (DSP) toxins in the *H. circularisquama* cells have not been detected by direct HPLC analysis yet (Matsuyama et al., 1997).

In addition to their effect on bivalves, it has been reported that *H. circularisquama* exhibits lethal effects on a microzooplankton tintinnid ciliate *Favella taraikaensis* in a cell density-dependent manner (Kamiyama, 1997; Kamiyama and Arima, 1997). Frequent

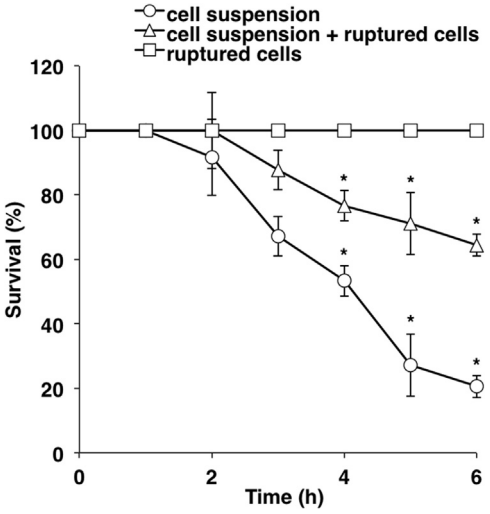

Fig. 3. Effects of ultrasonically ruptured cells of *Heterocapsa circularisquama* on the toxicity of *H. circularisquama* against rotifers in the light. Rotifers in 48-well plates (10 rotifers well^{-1}) were exposed to *H. circularisquama* (final 1×10^4 cells mL^{-1}) suspended in SWM-3 medium in the presence (△) or absence (○) of ruptured cells of *H. circularisquama* (1×10^5 cells mL^{-1}) at 26 °C for the indicated periods of time, and then the numbers of viable rotifers were counted as described in the text. Toxicity of the ruptured cells of *H. circularisquama* (1×10^5 cells mL^{-1}) on rotifers (□) was examined under the same conditions. Asterisks denote significant differences between the absence and the presence of ruptured cells. ($p < 0.05$).

contact of *H. circularisquama* cells with the cytoplasm around the oral plug of *F. taraikaensis* and subsequent morphological changes of *F. taraikaensis* were observed at high flagellate cell concentrations (Kamiyama and Arima, 1997). We have also found that a microzooplankton rotifer (*Brachionus plicatilis*) is similarly susceptible to *H. circularisquama* (Kim et al., 2000).

It has been speculated that unstable toxic substances located on the cell surface of *H. circularisquama* may be responsible for its toxicity to bivalves (Matsuyama et al., 1997). Although no such toxic substances have been successfully isolated and identified from *H. circularisquama* yet, it has been observed that an influx of Ca^{2+} was induced in the trochophore larvae of short-necked clams (*Ruditapes philippinarum*) after exposure to *H. circularisquama* (Matsuyama 1999). Based on these findings, a schematic toxic mechanism of *H. circularisquama* against bivalve molluscs has been proposed (Matsuyama, 2012).

Some phytoplankton species produce multiple toxins, and some of such toxins exhibit haemolytic activity. For instance, palytoxin (Habermann et al., 1989) and maitotoxin (Igarashi et al., 1999) are known to induce a Ca^{2+} influx into mammalian erythrocytes, eventually causing haemolysis. A previous study found that *H. circularisquama* cell suspension causes marked haemolysis in rabbit erythrocytes in a cell density-dependent manner (Oda et al., 2001; Sato et al., 2002). Furthermore, a comparative study of the haemolytic activity of several strains of *H. circularisquama* isolated from different localities in Japan suggests that haemolytic activity and toxicity to shellfish are well-correlated (Kim et al., 2002). Since the haemolytic test is a simple and small-scale semiquantitative assay, it is useful not only for searching for toxic agents of *H. circularisquama* but also for estimating its own potential toxicity.

It has been reported that live *H. circularisquama* cells must come into direct contact with bivalves in order for there to be lethal effects on the bivalves, which indicates the effect may be the result of certain toxins located on the cell surface (Matsuyama, 2012). Based on the findings, it seems likely that the haemolytic substance on the cell surface of *H. circularisquama* is a toxin responsible for the shellfish-killing mechanism. As mentioned above, *H. circularisquama* shows a lethal effect on a rotifer (*Brachionus plicatilis*) in

a cell density-dependent manner (Kim et al., 2000), and our previous study suggested that haemolytic activity was involved in the toxicity to rotifers (Kim et al., 2000).

Regarding the compound responsible for the haemolytic activity of *H. circularisquama*, it was found that an ethanol extract prepared from the flagellate cells showed haemolytic activity, and that this activity was light-dependent (Oda et al., 2001; Sato et al., 2002). Purification and characterisation studies suggested that one such photosensitising haemolytic agent (named H2-a) has structural similarity to pyropheophorbide a methyl ester (PME), a well-known photosensitising haemolytic agent (Miyazaki et al., 2005). Comparative studies on the cytotoxicity of H2-a and PME to HeLa cells suggested that H2-a induces necrotic cell death, whereas PME triggers apoptosis (Kim et al., 2008). Although the exact reason for this difference in types of cell death induced is still unclear, it is speculated that the relatively high affinity of H2-a to the plasma membrane might result in quick membrane damage, leading to the collapse of targeted cells without induction of apoptotic intracellular signal transduction (Kim et al., 2008). The biological significance of the presence of a photosensitising haemolytic agent like H2-a in *H. circularisquama* for the organism itself besides a possible toxic effect on surrounding organisms of other species remains to be studied. Hence, in this study, a detailed analysis on the effects of ultrasonic-ruptured *H. circularisquama* cells on the cells themselves was conducted through a combination of haemolytic assays and rotifer-exposure experiments. The results obtained suggest the presence of intracellular agents in *H. circularisquama* cells which can not only be toxic to *H. circularisquama* cells themselves, but can also suppress live cell-mediated haemolytic activity and toxicity to rotifers. Possible impacts of ruptured *H. circularisquama* cells on HAB species, including *H. circularisquama*, are discussed.

2. Materials and methods

2.1. Plankton and rotifer cultures

Heterocapsa circularisquama was isolated from Ago Bay, Japan, and was kindly provided by Dr. Y. Matsuyama (Seikai National Fisheries Institute, Japan). *Chattonella marina* and *Chattonella antiqua* were isolated from Kagoshima Bay and Shimabara Bay, Japan, respectively. *Karenia mikimotoi* was isolated from Hiroshima Bay, Japan. These species were maintained at 26 °C in 100 mL flasks containing 60 mL of a modified seawater medium (SWM-3) at a salinity of 25 (Yamasaki et al., 2007) in a 12:12 h photoperiod which was maintained by using a cool-white fluorescent lamp (200 ± 5 μmol m^{-2} s^{-1}). The modified SWM-3 contained a Tris-HCl buffer system and was autoclaved for 15 min at 121 °C before use. The cell numbers of the cultures were counted microscopically using a haemocytometer (Erma Inc., Tokyo, Japan). Each flagellate culture used throughout the experiment was in its late exponential growth phase unless otherwise specified. The rotifer *Brachionys plicatilis* was kindly provided by Dr. A. Hagiwara (Faculty of Fisheries, Nagasaki University, Japan) and was cultured with *Nannochloropsis oculata* using the same method as described previously (Zou et al., 2010). All the cultures were prepared using sterilised instruments. A cell-free culture supernatant of *H. circularisquama* was prepared from a cell suspension in its late exponential growth phase ($4–5 \times 10^5$ cells mL^{-1}) through centrifugation at $5000 \times g$ for 10 min at 4 °C. The ruptured cell suspension was prepared by performing an ultrasonic treatment on the cell suspension ($4–5 \times 10^5$ cells mL^{-1}) in an ultrasonic apparatus for 1–3 min at 20 °C. Microscopic observation confirmed that all cells were ruptured by the treatment. Cell contents (supernatant) and cell fragments (precipitate) were prepared from the ruptured *H. circularisquama* cells through centrifugation at $15{,}000 \times g$ for 10 min at 4 °C. The cell-

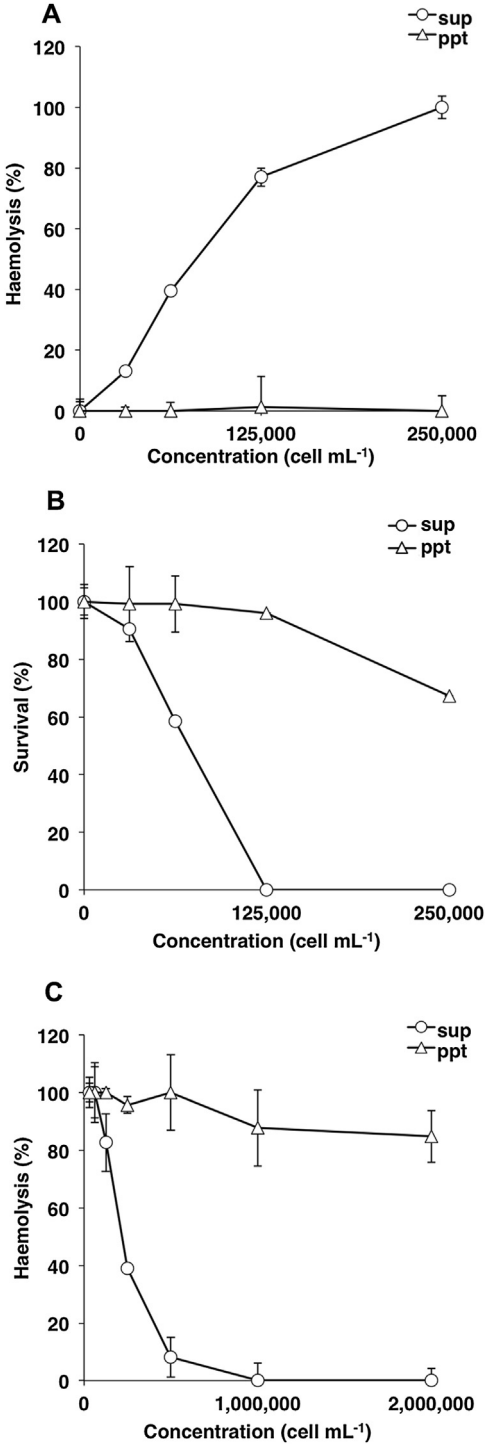

Fig. 4. Bioactivities of supernatant (sup) and precipitate (ppt) fractions prepared from ultrasonically ruptured *Heterocapsa circularisquama*. (A) Haemolytic activities of sup and ppt fractions in the light. Sup (○) or ppt (△) fractions equivalent to indicated cell concentration were mixed with rabbit erythrocytes, and incubated in 96-well plates in SWM-3 medium at 26 °C for 2.5 h in the light, and then the haemolysis was measured as described in the text. Each point represents the mean of triplicate measurements. Each bar represents 1 standard deviation. (B) Toxic effects of sup and ppt fractions on *H. circularisquama* in the light. Sup (○) or ppt (△) fraction equivalent to indicated cell concentration was mixed with *H. circularisquama* (final 1×10^4 cells mL^{-1}), and incubated in 96-well plates in SWM-3 medium at 26 °C for 24 h in the light, and then the numbers of viable *H. circularisquama* cells were counted as described in the text. (C) Effects of sup and ppt fractions on the live *H. circularisquama* cell (final 2×10^5 cells mL^{-1})-mediated haemolytic activities on rabbit erythrocytes in the dark. Sup (○) or ppt (△) fractions equivalent to indicated cell concentration were mixed with both *H. circularisquama* and rabbit erythrocytes, and then incubated in 96-well plates in SWM-3 medium at 26 °C for 5 h in the dark. The haemolysis was then measured as described in the text. Each point represents the mean of triplicate measurements. Each bar represents standard deviation.

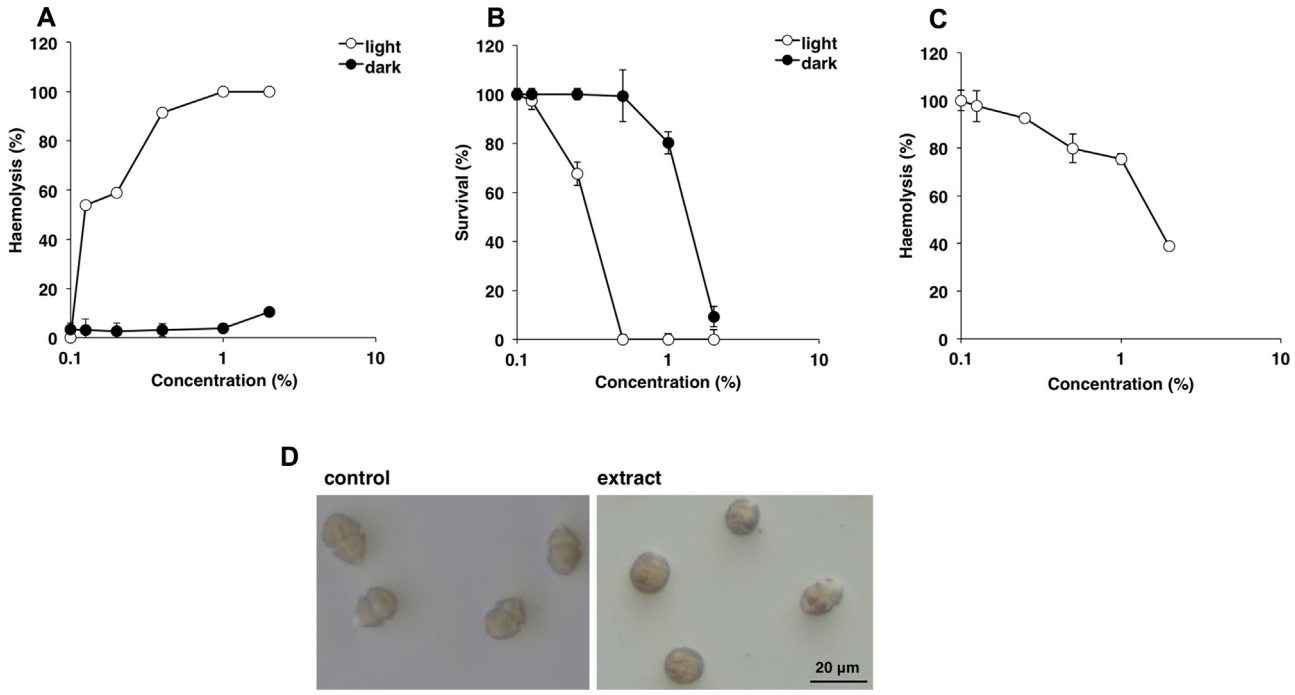

Fig. 5. Bioactivities of ethanol extract of *Heterocapsa circularisquama*. (A) Haemolytic activity of the ethanol extract. The ethanol extract at indicated final concentrations was mixed with rabbit erythrocytes and incubated at 26 °C for 1 h either in the light (○) or in the dark (●), and then the haemolysis was measured as described in the text. Each point represents the mean of triplicate measurements. Each bar represents standard deviation. (B) Toxic effect of the ethanol extract on *H. circularisquama*. The indicated final concentrations of the ethanol extract were added to *H. circularisquama* (final 1×10^4 cells mL^{-1}) and incubated at 26 °C for 24 h in the light (○) or in the dark (●). Then the numbers of viable *H. circularisquama* cells were counted as described in the text. Each point represents the mean of triplicate measurements. Each bar represents standard deviation. (C) Effects of the ethanol extract on live *H. circularisquama* cell (final 2×10^5 cells mL^{-1})-mediated haemolytic activities on rabbit erythrocytes in the dark. The indicated final concentrations of the ethanol extract were mixed with *H. circularisquama* and with rabbit erythrocytes and then incubated in 96-well plates in SWM-3 medium at 26 °C for 5 h in the dark. Then the haemolysis was measured as described in the text. Each point represents the mean of triplicate measurements. Each bar represents standard deviation. (D) Morphological changes of *H. circularisquama*. Micrographs of *H. circularisquama* cells after 5 h incubation without (control) or with the ethanol extract (final 2%) (extract) in the dark.

free culture supernatant and ruptured cell suspension were used immediately after their preparation for the haemolytic assay and the rotifer toxicity test.

2.2. Preparation of ethanol extract

Since our previous study showed that light-dependent haemolytic agents in *Heterocapsa circularisquama* can be efficiently extracted into ethanol and maintained in a stable condition (Oda et al., 2001), an ethanol extract was prepared for further analysis. The harvested cells from 300 mL of the cultured *H. circularisquama* in its late exponential growth phase (2×10^8 cells L^{-1}) were resuspended in 3 ml of ethanol and vigorously agitated with sonication at room temperature. After centrifugation at 15,000 × *g* for 10 min at 4 °C, the supernatant was withdrawn and stored at −30 °C until its use as a cell-free ethanol extract.

2.3. Gel-filtration chromatography on a column of Sephadex LH-20

The ethanol extract prepared from 2 L of the *Heterocapsa circularisquama* culture was applied to a column (1 × 10 cm) of Sephadex LH-20 (Pharmacia, Uppsala, Sweden), equilibrated with ethanol, and eluted with ethanol. Since our previous study showed that the ethanol extract contains porphyrin-related compounds with the maximum absorbance at 450 nm as a main ingredient (Miyazaki et al., 2005), the elution was monitored for absorption at 450 nm. Separated fractions were pooled and concentrated by evaporation, and the bioactivities of these samples were examined.

2.4. Measurement of haemolytic activity

Since our previous study has demonstrated that *H. circularisquama* causes haemolysis of rabbit erythrocytes most potently among the erythrocytes from different species tested (Oda et al., 2001), we conducted a haemolytic assay using rabbit erythrocytes in this study as described previously (Oda et al., 2001). Rabbit blood was obtained from Nippon Bio-Test Laboratories (Tokyo, Japan). Erythrocytes were washed three times with phosphate-buffered saline (PBS) and put into samples adjusted to a final concentration of 4% (v/v) in modified SWM-3. Triplicate 50-μL aliquots of serial two-fold dilutions of the intact cell suspension, cell-free culture, ruptured cell suspension, ethanol extract, or other samples in modified SWM-3 were added to round-bottom 96-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA). To the wells containing samples, 50 μL of a 4% (v/v) suspension of erythrocytes in modified SWM-3 were added, after which the well plates were gently shaken. After incubation for 5 h at 26 °C either under illumination from a fluorescent lamp (200 μmol m^{-2} s^{-1}) or in the dark, the plates were centrifuged at 900 × *g* for 10 min at 4 °C. Aliquots (50 μL) of supernatant were withdrawn from the wells and transferred to flat-bottom 96-well plates (Becton-Dickinson). The amount of haemoglobin released was determined by measuring absorbance at 560 nm using a microplate reader (Multiscan GO, Thermo Fisher Scientific Inc., MA, USA). Negative controls (zero haemolysis) and positive controls (100% haemolysis) were included by using erythrocytes suspended in modified SWM-3 alone and in modified SWM-3 containing 1% v/v Triton X-100, respectively.

2.5. Rotifer exposure experiment

The rotifer exposure test was conducted in 48-well plates (Becton-Dickinson) using the method described previously (Zou et al., 2010). Ten rotifers in each well were incubated with varying concentrations of cell suspension, cell-free culture supernatant, or ruptured cell suspension in modified SWM-3 (1 mL well^{-1}) at 26 °C in either light (200 μmol m^{-2} s^{-1}) or dark for the indicated periods of time (0–6 h). Then, the viable individuals, which were defined as those actively swimming in each well, were counted using a stereomicroscope.

2.6. Cytotoxicity test

The cells of *Heterocapsa circularisquama* in their late exponential growth phase were used throughout the cytotoxicity experiments. To fifty microliters of SWM-3 containing varying concentrations of ethanol extract prepared from *H. circularisquama* or pheophorbide a solution (1 mg mL^{-1} in ethanol) in 96-well plates, 50 μl of the cell suspension was added. The cells were incubated (final volume of 0.1 mL well^{-1}) in varying concentrations of each test sample in SWM-3 at 26 °C either in light or dark for the indicated periods. Cells with motility were considered viable cells. The number of viable cells in the control, which did not contain a sample, was taken as 100%, and the survival in test groups treated with each sample was calculated as % of that. Ethanol alone as a control did not show any cytotoxic effect on phytoplankton species used in this study up to 4% either in dark or light.

2.7. Statistical analysis

All the experiments were repeated at least three times. Data were expressed as the mean ± standard deviation, and data were analysed with a paired Student's *t*-test to evaluate significant differences. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Haemolytic activity of Heterocapsa circularisquama under various conditions

To gain clues about the roles of haemolytic substances in the toxic effects of *Heterocapsa circularisquama* and their biological significance, we carried out detailed combination experiments with haemolytic and rotifer-exposure experiments in this study. The intact cell suspension of *H. circularisquama* used in this study, which had been cultured for more than 15 years under laboratory conditions, showed a potent haemolytic effect on rabbit erythrocytes in a cell density-dependent manner, suggesting that haemolytic activity is a relatively stable phenotype in this dinoflagellate (Fig. 1). The cell suspension showed higher haemolytic activity in the light than in the dark, whereas the activity of the cell-free culture supernatant was significantly lower than those of the cell suspensions under both lighting conditions. Interestingly, the ultrasonically ruptured cells showed haemolytic activity only in the light, and no significant activity was detected in the dark even up to the highest concentration. Since cell-free culture supernatant showed only a weak activity in slightly light-dependent manner, small amounts of intracellular light-dependent haemolytic substances may be discharged from the cells during the cultivation. These results confirm that *H. circularisquama* has at least two haemolytic substances with differing light dependency: one shows light-independent haemolytic activity mediated by intact cells and another one shows light-dependent activity caused by the substances released from ruptured cells.

3.2. Toxic effect of Heterocapsa circularisquama on rotifers and bioactivities of ruptured cells

The lethal effect of *H. circularisquama* on rotifers was not affected by light conditions, and almost equal toxicities to rotifers were observed both in the light and in the dark (Fig. 2). Furthermore, the ultrasonically ruptured cells showed no toxic effect on rotifers, even in the light (Fig. 3). Hence, it is obvious that intact *H. circularisquama* cells are essential to produce the toxic effect. Most likely, intact cell-mediated light-independent haemolytic activity is mainly involved in the toxicity to rotifers, whereas the light-dependent haemolytic substances detected in ruptured cells may not be related to the toxic effect of *H. circularisquama* on rotifers under the conditions tested at least when they are delivered in dissolved form. Interestingly, exogenously added ruptured cells significantly suppressed the live cell-mediated rotifer toxicity (Fig. 3), suggesting that intracellular substances discharged from ruptured cells contain some sort of suppressors against the rotifer toxicity of *H. circularisquama* itself. To search for such substances, samples of the cell contents (supernatant) and the cell fragment (precipitate) were prepared from the ruptured cells by centrifugation, and their bioactivities were examined. Most of the light-dependent haemolytic activity was observed in the cell content fraction (Fig. 4A). The cell content fraction prepared from *H. circularisquama* also showed a toxic effect on *H. circularisquama* cells themselves, and complete killing of 1×10^4 cells mL^{-1} of live *H. circularisquama* cells was attained in the presence of the cell content prepared from 12.5×10^4 cells mL^{-1} of *H. circularisquama* cells (Fig. 4B). Furthermore, in the presence of the cell content fraction, *H. circularisquama* cell-mediated light-independent haemolytic activity was significantly suppressed in a dose-dependent manner, whereas no such effect was observed in the cell fragment fraction (Fig. 4C). These results suggest that light-dependent haemolytic, cytotoxic, and cell-mediated haemolysis suppressor substances are present as soluble free form in the ruptured cell suspension, which may influence the live cell-mediated toxic behaviour, including the haemolytic activity.

3.3. Bioactivities of ethanol extract of Heterocapsa circularisquama

Previous study found that light-dependent haemolytic activity was efficiently extracted into alcohol (Sato et al., 2002), and that the resulting alcohol extract contains at least three haemolytic compounds with similar chemical features (Miyazaki et al., 2005). Among these compounds, a highly purified one named H2-a was the most potent haemolytic as well as cytotoxic agent (Miyazaki et al., 2005). Chemical structural analysis indicated that H2-a is a porphyrin derivative with a structure similar to pyropheophorbide *a* methyl ester (PME), a well-known light-dependent haemolytic agent (Miyazaki et al., 2005). These findings suggest that porphyrin derivatives may be responsible for the bioactivities found in the ruptured *H. circularisquama* cells. To ascertain this point, an ethanol extract was prepared from *H. circularisquama* cells, and the bioactivities of the samples were then examined. Although the ethanol extract was used without further purification in this study, which is consistent with methods of previous studies (Miyazaki et al., 2005; Sato et al., 2002), the ethanol extract showed potent cytotoxicity on *H. circularisquama* in a light-enhanced manner in addition to light-dependent haemolytic activity (Fig. 5A and B). In the presence of the ethanol extract, *H. circularisquama* cell-mediated haemolytic activity in the dark was also suppressed in a concentration-dependent manner (Fig. 5C). The ethanol extract induced morphological changes of *H. circularisquama* cells, leading to an increase in the round cell population during the 5 h haemolytic assay, even in the dark, though no significant decrease in total cell number of *H. circularisquama* was observed during the

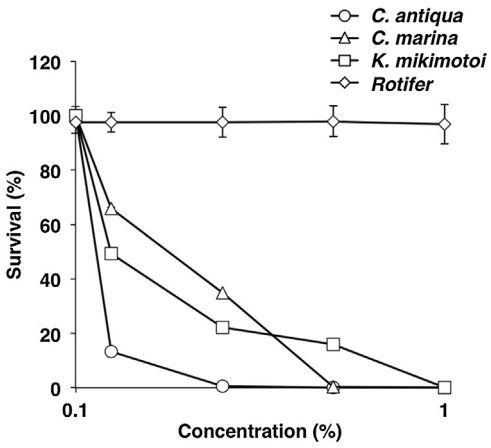

Fig. 6. Toxic effect of the ethanol-extract of *H. circularisquama* on *Chattonella antiqua* (○), *C. marina*, (△), *Karenia mikimotoi* (□), and the rotifer (◊). *C. antiqua* (1×10^4 cells mL^{-1}), *C. marina*, (1×10^4 cells mL^{-1}), *K. mikimotoi* (1×10^4 cells mL^{-1}) in 96-well plates, or rotifers (20 individual mL^{-1}) in 48-well plates were incubated with the indicated final concentrations of the ethanol extract at 26 °C for 24 h in the light, and then the viability of each cell or rotifer was determined as described in the text.

assay (Fig. 5D). Our previous fluorescence microscopic observation of HeLa cells treated with highly purified H2-a showed that H2-a tends to accumulate in the plasma membrane without further penetration into the cytoplasm (Kim et al., 2008). Probably, porphyrin derivatives like the H2-a in the ethanol extract also selectively attack the cell-surface architecture of *H. circularisquama* cells, where they may inactivate haemolytic agents located on the cell surface in the dark. Upon activation on illumination, these porphyrin derivatives may cause further severe membrane damage, leading to cell lysis.

Interestingly, the ethanol extract also showed a potent cytotoxic effect on *Chattonella marina*, *C. antiqua*, and *Karenia mikimotoi*, which are highly harmful species that cause mass mortalities of fish and shellfish, in a concentration-dependent manner at a concentration range at which no significant toxicity to rotifers is observed (Fig. 6). Although the compounds in the ethanol extract responsible for the toxicity to these harmful phytoplankton species are not identified yet, our previous studies showed that the ethanol extract contained several porphyrin derivatives with photosensitising haemolytic and cytotoxic activities (Miyazaki et al., 2005). Hence one can speculate that the porphyrin derivatives in the ethanol extract may exhibit toxicity to phytoplankton as well. Further studies to check the susceptibility of other phytoplankton species to the porphyrin derivatives may further describe the potential impact of the porphyrin derivatives against phytoplankton, especially against harmful species.

3.4. Further characterization of ethanol extract of Heterocapsa circularisquama

Three fractions (f-1–f-3) were obtained from the ethanol extract in an analysis using Sephadex LH-20 (Fig. 7A). The fractions eluted later (f-2 and f-3) showed higher haemolytic activity than the fraction eluted first (f-1) (Fig. 7B), even though the optical densities measured at 450 nm of f-1, f-2, and f-3 fractions used for haemolytic assay were 24, 6, and 6, respectively. The bed volume (Vt) of the column (1×10 cm) of Sephadex LH-20 was 7.85 mL, and elution volumes (Ve) of f-1, f-2, and f-3 were estimated to be 14.1, 30, and 42.3 mL, respectively. Theoretically Ve/Vt values of the samples eluted under separable range should be 0.3–1.0. When the value become >1, it is suggested that interaction might occur between the gel and the samples, and elution was delayed. The Ve/Vt of f-1, f-2, and f-3 were calculated to be 1.78, 3.82, and 5.39, respectively, suggesting that elution of f-1, f-2, and f-3 did not necessarily reflect the molecular size. Hydrophobic interaction with Sephadex LH-20 may influence the elution of each fraction, and the potency of such interaction may be of the order of f-3 > f-2 > f-1. The absorption spectra of the latter two fractions were similar to that of H2-a (Kim et al., 2008), and these fractions showed an inhibitory effect on cell-mediated haemolysis by *H. circularisquama* (Fig. 7C). These results suggest that f-2 and f-3 may contain the porphyrin derivatives

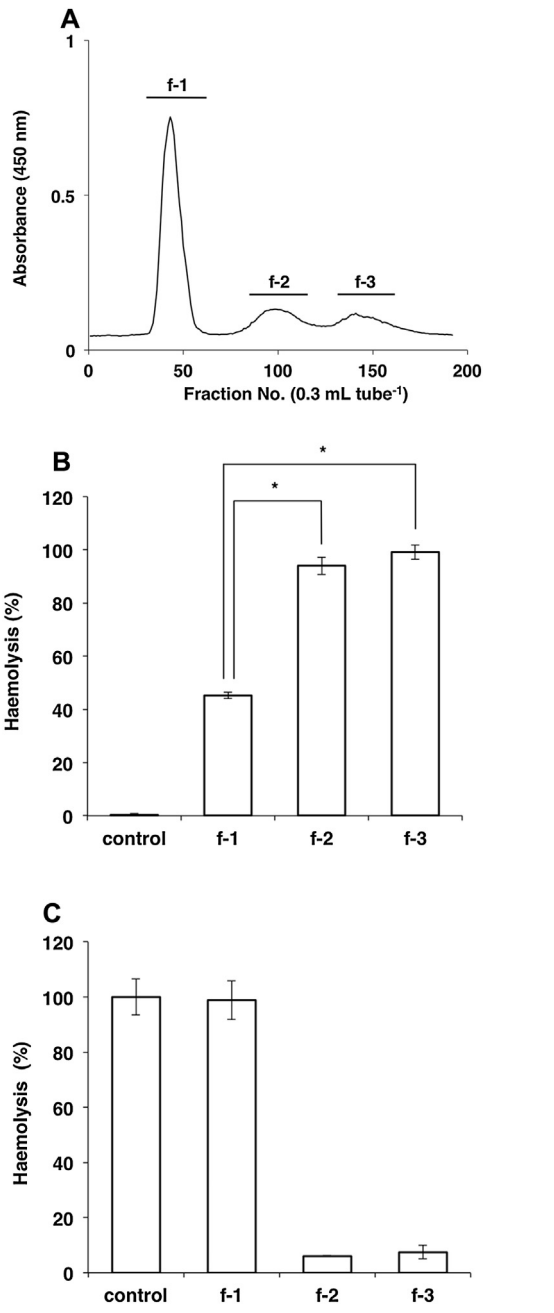

Fig. 7. Analysis of the ethanol extract of *H. circularisquama* on Sephadex LH-20 and bioactivities of separated fractions. (A) Elution profile of the ethanol extract on a column of Sephadex LH-20. (B) Haemolytic activities of f-1–f-3. Each sample (final 2%) was mixed with rabbit erythrocytes and incubated at 26 °C in 96-well plates in SWM-3 medium for 1 h in the light, and then the haemolysis was measured as described in the text. (C) Effects of f-1–f-3 on the live *H. circularisquama* cell (final 2×10^5 cells mL^{-1})-mediated haemolytic activities on rabbit erythrocytes in the dark. Each sample (final 2%) was mixed with *H. circularisquama* together with rabbit erythrocytes, and incubated in 96-well plates in SWM-3 medium at 26 °C for 5 h in the dark. Then the haemolysis was measured as described in the text. Asterisks denote significant differences between f-1 and f-2 or f-1 and f-3. ($p < 0.05$).

responsible for cytotoxicity and the inhibition of cell-mediated haemolysis, as well as light-dependent haemolysis.

Several porphyrin derivatives have been discovered in animal and plant sources (Matroule et al., 1999). For instance, a photosensitising porphyrin derivative was found in a methanol extract of bamboo leaves and was shown to be an inducer of apoptosis in cancer cells (Kim et al., 2003). Pheophorbide *a* was identified as a cytokine receptor antagonist obtained from an extract of the leaves and stems of *Psychotria acuminata* (Rubiaceae), a plant used as a traditional medicine (Arvigo and Balick, 1993). To further confirm the involvement of porphyrin derivatives in the bioactivities of ruptured cells of *H. circularisquama*, as a qualitative analysis, bioactivities of the ethanol extract were compared with ethanol solution of pheophorbide *a* (1 mg mL^{-1}). The ethanol extract at final strength of 0.4% and ethanol solution of pheophorbide *a* at final concentration of 4 μg mL^{-1} caused light-dependent haemolysis with 75.5% and 57.4%, respectively. However, ethanol solution of pheophorbide *a* at final concentration of 4 μg mL^{-1} exhibited no significant toxic effect on *H. circularisquama*, whereas the ethanol extract at final strength of 0.4% showed potent cytotoxicity (Fig. 5B). These results suggest that the action mechanisms of the ethanol extract may be distinct from those of pheophorbide *a*. Our previous study using HeLa cells indicated that the intracellular distribution of H2-a differed significantly from that of PME, and that the LD_{50} of H2-a against HeLa cells was also more than 50 times lower than that of PME (Kim et al., 2008). Although further studies are required to clarify the action mechanisms of *H. circularisquama*-derived porphyrin derivatives, including H2-a, previous structural analysis revealed that H2-a has two aldehyde groups that PME lacks. Aldehyde groups are generally highly reactive, and there are some reports indicating that aldehyde groups are essential for the cytotoxic activity of some toxins (Durant and Karran, 2003; Moon and Pack, 1983; Shelley et al., 2000). Thus, it seems likely that the characteristic action mechanism of H2-a may be partly attributed to functional side groups, such as aldehyde groups, which may explain why the peculiar bioactivities of H2-a differ from those of PME and pheophorbide *a*, even though they have similar porphyrin backbone structures (Miyazaki et al., 2005).

Several methods have been developed to mitigate the deleterious effects of HABs. Direct countermeasures remove HAB species through physical (clays, ultraviolet radiation, ultrasonic or electromagnetic waves), chemical (hydrogen peroxide, copper sulfate, surfactants), or biological control (bacteria, viruses, plankton grazers) approaches (Anderson, 1997; Kim, 2006; Shirota, 1989; Sun et al., 2004). Application of some of these methods can be accompanied by lysis of HAB species, which may result in the discharge of intracellular substances, as was observed in this study. Our results suggest that discharged substances can influence the surrounding organisms, including target HAB species. Although the biological significances of the discharged intracellular substances are uncertain, *H. circularisquama*-derived porphyrin derivatives may facilitate the mitigation effects. Our previous study showed that raphidophycean flagellates *Chattonella marina*, *Heterosigma akashiwo*, *Olisthodiscus luteus*, and *Fibrocapsa japonica* also have light-dependent haemolytic agents, which are highly toxic to HAB species (Kuroda et al., 2005). It has been reported that various strains of *Alexandrium tamarense* produce diverse haemolytic substances which might be responsible for their allelopathic actions (Chen et al., 2015). These findings suggest that the presence of haemolytic substances with various bioactivities is not limited to *H. circularisquama*. Further studies are needed to clarify the biological roles of porphyrin derivatives of *H. circularisquama*, especially in terms of the impact on plankton micro-environments.

In conclusion, our study demonstrated that intracellular light-dependent haemolytic agents in *H. circularisquama*, which may be porphyrin derivatives, could be discharged from ruptured cells and act as an antidote to the effects of *H. circularisquama* cells themselves. Since discharged haemolytic agents show a cytotoxic effect on several HAB species, including *H. circularisquama* itself, such agents might facilitate the mitigation efficiency of the method associated with cell lysis.

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References

Anderson, D.M., 1997. Turning back the harmful red tide. Nature 388, 513–514.
Arvigo, R., Balick, M.J., 1993. Rainforest Remedies: One Hundred Healing Herbs of Belize. Lotus Press, Twin Lakes, WI, pp. p219.
Chen, J., Ye, Q., Gu, H.F., Li, H.Y., Lv, S.H., Liu, J.S., Yang, W.D., 2015. Variability in the allelopathic action of the *Alexandrium tamarense* species complex along the coast of China. Harmful Algae 47, 17–26.
Durant, S., Karran, P., 2003. Vanillins-a novel family of DNA-PK inhibitors. Nucleic Acids Res. 31, 5501–5512.
Habermann, E., Hudel, M., Dauzenroth, M.E., 1989. Palytoxin promotes potassium outflow from erythrocytes, HeLa and bovine adrenomedullary cells through its interaction with Na^+, K^+ −ATPase. Toxicon 27, 419–430.
Horiguchi, T., 1995. *Heterocapsa circularisquama* sp: nov. (Peridiniales. Dinophyceae): a new marine dinoflagellate causing mass mortality of bivalves in Japan. Phycol. Res. 43, 129–136.
Igarashi, T., Aritake, S., Yasumoto, T., 1999. Mechanisms underlying the hemolytic and ichthyotoxin activities of maitotoxin. Nat. Toxicol. 7, 71–79.
Kamiyama, T., 1997. Growth and grazing respones of tintinnid ciliates feeding on the toxic dinoflagellate *Heterocapsa circularisquama*. Mar. Biol. 128, 509–515.
Kamiyama, T., Arima, S., 1997. Lethal effect of the dinoflagellate *Heterocapsa circularisquama* upon the tintinnid ciliate *Favella taraikaensis*. Mar. Ecol. Prog. Ser. 160, 27–33.
Kim, D., Sato, Y., Oda, T., Muramatsu, T., Matsuyama, Y., Honjo, T., 2000. Specific toxic effect of *Heterocapsa circularisquama* dinoflagellate on the rotifer *Brachionus plicatilis*. Biosci. Biotechnol. Biochem. 64, 2719–2722.
Kim, D., Sato, Y., Miyazaki, Y., Oda, T., Muramatsu, T., Matsuyama, Y., Honjo, T., 2002. Comparison of hemolytic activities among strains of *Heterocapsa circularisquama* isolated in various localities in Japan. Biosci. Biotechnol. Biochem. 66, 453–457.
Kim, D., Miyazaki, Y., Nakashima, T., Iwashita, T., Fujita, T., Yamaguchi, K., Choi, K.S., Oda, T., 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, H.G., 2006. Mitigation and controls of HABs. In: Granéli, E., Turner, T.J. (Eds.), Ecology of Harmful Algae. Springer-Verlag, Berlin, Germany, pp. 327–338.
Kim, K.K., Kawano, Y., Yamazaki, Y., 2003. A novel porphyrin photosensitizer from bamboo leaves that induces apoptosis in cancer cell lines. Anticancer Res. 23, 2355–2361.
Kuroda, A., Nakashima, T., Yamaguchi, K., Oda, T., 2005. Isolation and characterization of light-dependent hemolytic cytotoxin from harmful red tide phytoplankton *Chattonella marina*. Comp. Biochem. Physiol. C 141, 297–305.
Matroule, J.Y., Hellin, A.C., Morliere, P., Fabiano, A.S., Santus, R., Merville, M.P., Piette, J., 1999. Role of nuclear factor-kappa B in colon cancer cell apoptosis mediated by aminopyropheophorbide photosensitization. Photochem. Photobiol. 70, 540–548.
Matsuyama, Y., Nagai, K., Mizuguchi, T., Fujiwara, M., Ishimura, M., Yamaguchi, M., Uchida, T., Honjo, T., 1992. Ecological features and mass mortality of pearl oysters during red tide of *Heterocapsa* sp. in Ago Bay in 1992. Nippon Suisan Gakk. 61, 35–41.
Matsuyama, Y., Uchida, T., Nagai, K., Ishimura, M., Nishimura, A., Yamaguchi, M., Honjo, T., 1996. Biological and environmental aspects of noxious dinoflagellate red tides by *Heterocapsa circularisquama* in the west Japan. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, pp. 247–250.
Matsuyama, Y., Uchida, T., Honjo, T., 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., 1999. The toxic effects of *Heterocapsa circularisquama* on bivalve molluscs. Bull. Plankton Soc. Japan 46, 157–160.
Matsuyama, Y., 2012. Impacts of the harmful dinoflagellate *Heretocapsa circularisquama* bloom on shellfish aquaculture in Japan and some experimental studies on invertebrates. Harmful Algae 14, 144–155.

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

Moon, K.H., Pack, M.Y., 1983. Cytotoxicity of cinnamic aldehyde on leukemia L1210 cells. Drug Chem. Toxicol. 6, 521–535.

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

Oda, T., Sato, Y., Kim, D., Muramatsu, T., Matsuyama, Y., Honjo, T., 2001. Hemolytic activity of *Heterocapsa circularisquama* (Dinophyceae) and its possible involvement in shellfish toxicity. J. Phycol. 37, 509–516.

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

Shelley, M.D., Hartley, L., Groundwater, P.W., Fish, R.G., 2000. Structure-activity studies on gossypol in tumor cell lines. Anticancer Drugs 11, 209–216.

Shirota, A., 1989. Red tide problem and counter measures. Int. J. Aquat. Fish Technol. 1, 195–223.

Sun, X.X., Han, K.N., Choi, J.K., Kim, E.K., 2004. Screening of surfactants for harmful algal blooms mitigation. Mar. Pollut. Bull. 48, 937–945.

Yamamoto, C., Tanaka, Y., 1990. Two species of harmful red tide plankton increased in Fukuoka Bay. Bull. Fukuoka Fish. Exp. Stn. 16, 43–44.

Yamasaki, Y., Nagasoe, S., Matsubara, T., Shikata, T., Shimasaki, Y., Oshima, Y., Honjo, T., 2007. Allelopathic interactions between the bacillariophyte *Skeletonema costatum* and the raphidophyte *Heterosigma akashiwo*. Mar. Ecol. Prog. Ser. 339, 83–92.

Zou, Y., Yamasaki, Y., Matsuyama, Y., Yamaguchi, K., Honji, T., Oda, T., 2010. Possible involvement of hemolytic activity in the contact-dependent lethal effects of the dinoflagellate *Karenia mikimotoi* on the rotifer *Brachionus plicatilis*. Harmful Algae 9, 367–373.