Aquatic Toxicology 179 (2016) 95–102

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

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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ARTICLE INFO

Article history:
Received 25 June 2016
Received in revised form 19 August 2016
Accepted 28 August 2016
Available online 29 August 2016

Keywords:
Heterocapsa circularisquama
Brachionus plicatilis
Antidote
Hemolytic activity
Porphyrin derivative
Mitigation

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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http://dx.doi.org/10.1016/j.aquatox.2016.08.019
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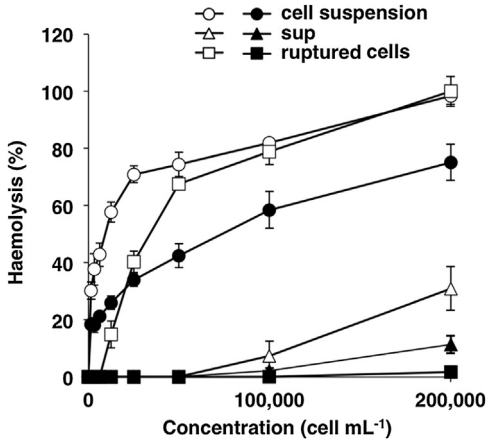

Fig. 1. Haemolytic activities of live cell suspension (○,●), cell-free cultured supernatant (△,▲), and ultrasonically ruptured cells (□,■) of *Heterocapsa circularisquama* on rabbit erythrocytes in the light (○,△,□) and in the dark (●, ▲, ■). Each sample and all erythrocytes were incubated in 96 well-plates 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.

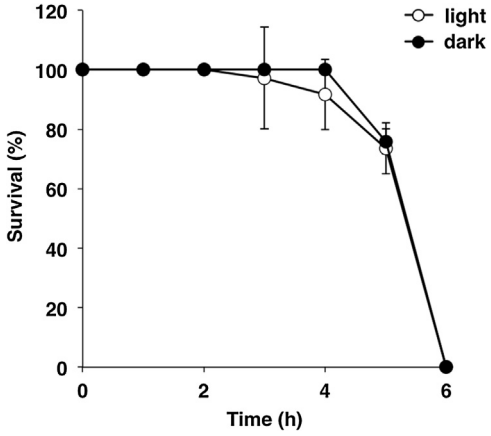

Fig. 2. Toxicity of *Heterocapsa circularisquama* on rotifers in the light (○) and in the dark (●). Rotifers in 48-well plates (10 rotifers well^{-1}) were exposed to *H. circularisquama* (final 1×10^4 cells mL^{-1}) suspended in SWM-3 medium at 26 °C for the indicated periods of time, and then the number 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

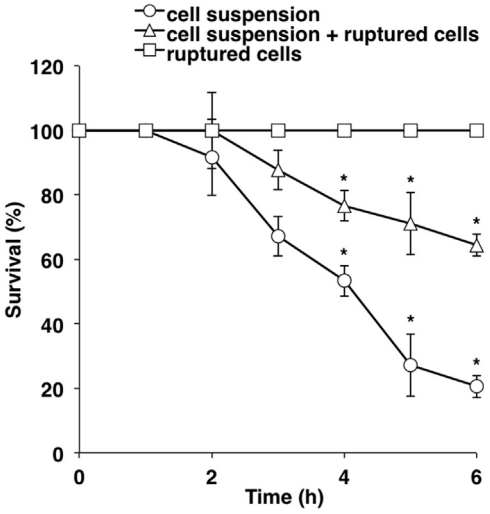

Fig. 3. Effects of ultrasonically ruptured cells of *Heterocapsa circularisquama* on the toxicity of *H. circularisquama* against rotifers in the light. Rotifers in 48-well plates (10 rotifers well^{-1}) were exposed to *H. circularisquama* (final 1×10^4 cells mL^{-1}) suspended in SWM-3 medium in the 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