FISHERIES SCIENCE 2008; **74**: 128–136

Identification of overwintering vegetative cells of the bivalve-killing dinoflagellate *Heterocapsa circularisquama* in Uranouchi Inlet, Kochi Prefecture, Japan

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ABSTRACT: Red tides of *Heterocapsa circularisquama* have led to serious damage of bivalve aquacultures in western coastal areas of Japan. To understand the whole picture regarding the ecology of this species, it is essential to clarify its overwintering mechanisms. In this study, the population dynamics of *H. circularisquama* were investigated from February 2004 to November 2005, and overwintering cells were identified for the first time in water columns of Uranouchi Inlet, Kochi Prefecture, Japan. *Heterocapsa circularisquama* cells were detected by the indirect fluorescent antibody technique using monoclonal antibodies that specifically recognize and react to this species. Vegetative cells were almost always detected from the first observation in February 2004 to November 2005 with temperatures of 10.5–30.6°C. During the period from winter to spring, this species survived in areas with a temperature higher than 10°C. The overwintering cells of *H. circularisquama* were isolated in March 2004, and identification was made via observation of the morphology and body scales of the cultured cells. These overwintering cells were identified as *H. circularisquama* and reacted to the monoclonal antibody. These results indicate that *H. circularisquama* can overwinter and survive throughout the year in a vegetative cell state in Uranouchi Inlet.

KEY WORDS: **body scale, *Heterocapsa circularisquama*, indirect fluorescent antibody technique, overwintering, population dynamics, temperature, temporary cyst, vegetative cell.**

INTRODUCTION

Heterocapsa circularisquama is one of the most noxious dinoflagellates, especially for bivalves, and its red tides have recurrently caused huge damage to bivalve aquacultures in western coastal areas of Japan.[1,2] This alga was discovered for the first time in Uranouchi Inlet, Kochi Prefecture, Japan, in 1988 and its blooms have expanded since then throughout the coastal waters of western Japan.[3–5] To clarify the formation mechanisms of the red tide occurrences of this species, it is essential to grasp the entire population dynamics.

In Ago Bay, Mie Prefecture, Japan, it has been reported that *H. circularisquama* is usually first detected in late spring, forms a red tide during summer, and decreases to a cell density less than the detection level (<1 cell/L) in early winter.[6,7] However, the overwintering mechanisms have remained unclear. No resting cysts have been found from the sediments of coastal seas. Furthermore, swimming vegetative cells have not yet been detected in water columns from winter to late spring.[6,7] Therefore, it is very important to clarify

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Received 19 February 2007. Accepted 9 August 2007.

doi:10.1111/j.1444-2906.2007.01502.x

the overwintering mechanisms of this species to understand the initial stage of red tide occurrence, and also for the prediction of red tide occurrences in the future.

Recently, antibodies have been developed to specifically identify *H. circularisquama*.[8] The indirect fluorescent antibody technique (IFAT) using monoclonal antibodies is useful for the monitoring of *H. circularisquama* in sea water at a low cell density.[7] This method allows for the detection of both vegetative cells and temporary cysts of *H. circularisquama*.[7]

Uranouchi Inlet is located in a warm water area in south-west Japan. The water temperature rises up to 30°C or higher during summer, and usually does not fall below 10°C, even during winter. According to the growth characteristics of *H. circularisquama*, with growth at 15°C, survival at 12.5°C and death at 10°C,[9,10] it is presumable that this species can exist in a vegetative cell state throughout the year in the inlet. In this study, the population dynamics of *H. circularisquama* were investigated over the seasons of a year, and overwintering cells were identified for the first time in water columns of the inlet. Overwintering cells of *H. circularisquama* were isolated in late winter, clonal cultures were established, and identification was made using the observation of body scales.[11,12] We report the temporal fluctuations of *H. circularisquama* in Uranouchi Inlet, including the winter season, and discuss the overwintering mechanisms.

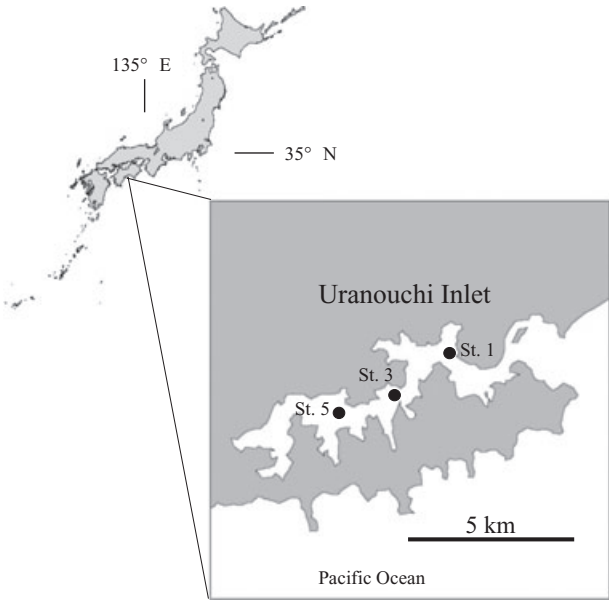

Fig. 1 Location of the sampling stations (●) in Uranouchi Inlet.

MATERIALS AND METHODS

Monitoring by the indirect fluorescent antibody technique using monoclonal antibodies

Sampling stations were located in Uranouchi Inlet, Kochi Prefecture, Japan (Fig. 1). Sample collections were made once or twice per month at three stations (Sts 1, 3 and 5) from 3 February 2004 to 16 November 2005. One-liter seawater samples were collected from depths of 0 m and 2 m at each station, and immediately fixed with formaldehyde at a final concentration of 0.37%. Simultaneously, measurements were conducted for water temperature and salinity at each sampling. Cell densities of *H. circularisquama* in the samples were determined by IFAT using monoclonal antibodies that specifically react to this species.[7] For the IFAT, treatments of water samples and staining procedures were made as reported by Shiraishi *et al.*[7] and are briefly described as follows. Sudan Black B-stained Nuclepore polycarbonate filters (3.0-μm pore size) were set on a filtration device. Fixed samples were collected on the filters by filtration and washed twice with phosphate-buffered saline without calcium chloride and magnesium chloride [PBS(–)]. The samples were incubated for 10 min at room temperature (20–25°C) with primary monoclonal antibodies against *H. circularisquama*. After washing twice again with 5 mL of PBS(–), the samples were incubated for 10 min at 20–25°C after the addition of fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. The room temperature was sufficient for the procedures of staining and detecting *H. circularisquama* cells.[7] After washing twice with 5 mL of PBS(–), the filters were mounted on glass slides with non-fluorescence immersion oil and covered with a cover glass, and the cells were observed with an epifluorescence microscope (ECLIPSE TE300, Nikon, Tokyo, Japan) under blue-light excitation (B-3A filter: excitation wavelength 420–490 nm, absorption wavelength < 520 nm) and the same excitation with blocking of chlorophyll-a autofluorescence (B-2E/C filter: excitation wavelength 465–495 nm, absorption wavelengths <515 nm and >555 nm). In the present study, the detection and enumeration of cells were feasible without the blocking process. The IFAT observations were made in triplicate for each sample. When no *H. circularisquama* cells were detected with ordinary triplicate observations, one liter of

the whole seawater sample was observed and enumerated.

Isolation and identification of overwintering cells

One seawater sample was collected from a depth of 2 m at St. 5 on 3 March 2004 to isolate overwintering vegetative cells of *H. circularisquama* in Uranouchi Inlet (Fig. 1). Clonal cultures were established using the micropipette washing method. The cultured strains were maintained at 20°C under an illumination of 180 μmol photons/m^2/s with a 14-h light:10-h dark photo-cycle in modified SWM-3 medium.[13,14]

To identify the cultured cells of each strain, observations were made regarding the morphological characteristics of the cells and the fine structure of the body scales. The body scale is a cell covering situated immediately above the plasma membrane and is recognized as a species-specific criterion in the genus *Heterocapsa* because their structures vary considerably from each other.[11,12] For the observation of the shape and the measurement of the size of the cultured cells, the cells were fixed with formaldehyde at a final concentration of 0.37%. Observations and measurements ($n = 100$) of the fixed cells were carried out with an optical microscope (Nikon, ECLIPSE TE300). For the observation of body scales using a transmission electron microscope (TEM), whole-mounts were prepared as follows. A drop of cell suspension was placed on a polyvinyl formvar-coated grid. The cells were fixed with 2% osmium tetroxide for 1 min and subsequently dried and rinsed three times with distilled water. Then, the cells were stained with 1% uranyl acetate for 1.5 min. The cells were again rinsed three times with distilled water. The stained whole-mount preparations were examined using a transmission electron microscope (JEOL JEM-1010, Japan Electronics, Tokyo, Japan). The cells were identified according to the taxonomy of the species.[11,12]

Reactivity was examined for the isolated strains of *H. circularisquama* using the monoclonal antibodies. The vegetative cells of the cultured strains were fixed with formaldehyde at a final concentration of 0.37% and stored at 5°C until IFAT treatment. The IFAT and observations were carried out as described by Shiraishi *et al.*[7] The ratios of positively reacting cells were calculated using the positive cell number out of the total cell number observed. Observation and enumeration were carried out in triplicate for each strain.

RESULTS

Monitoring of *Heterocapsa circularisquama* populations using monoclonal antibodies in Uranouchi Inlet

Figure 2a–d presents photomicrographs of a vegetative cell and a temporary cyst of *H. circularisquama* in a seawater sample collected from a depth of 2 m at St. 3 in Uranouchi Inlet on 18 February 2004 after IFAT treatment. The vegetative cell emitted red autofluorescence of chloroplasts and green FITC fluorescence along the outline of the cell under blue-light excitation (Fig. 2a), and FITC fluorescence was clearly observed under blue-light excitation with the blocking of chlorophyll-a autofluorescence (Fig. 2b). The temporary cyst was also observed in the same way as the vegetative cell (Fig. 2c,d). *Heterocapsa circularisquama* could be detected specifically by the IFAT method using monoclonal antibodies in Uranouchi Inlet.

Figures 3 and 4 indicate seasonal changes in cell densities of *H. circularisquama* and water temperature in Uranouchi Inlet from 3 February 2004 to 16 November 2005. Table 1 shows a summary of the field data in this study. According to monitoring by the IFAT using monoclonal antibodies, *H. circularisquama* cells were detected from the first monitoring on 3 February 2004. The cell densities and temperatures at St. 1 were 2.03×10^2 cells/L and 13.8°C at a 0-m depth, and 3.57×10^2 cells/L and 13.8°C at a 2-m depth. *Heterocapsa circularisquama* cells were also detected at the other two stations ($1.60–2.20 \times 10^2$ cells/L, 10.5–11.0°C). Both vegetative cells and temporary cysts were present in samples collected at the three stations on 3 February 2004 (Table 2). Temporary cysts were detected until 3 March 2004, but not detected thereafter. In 2004, the cell densities of *H. circularisquama* fluctuated at a low level ($<1.26 \times 10^3$ cells/L) from winter to summer. In April 2004, a red tide of *Heterosigma akashiwo* occurred (4.18×10^3 cells/mL using common microscopy). During June and July 2004, a red tide of *Karenia mikimotoi* occurred (1.66×10^4 cells/mL using common microscopy). The cell densities of *H. circularisquama* increased in autumn, reaching a maximum of 3.11×10^4 cells/L at a depth of 2 m at St. 3 on 6 October (water temperature: 26.2°C). The densities gradually decreased from an order of 10^3 cells/L in November to an order of 10^0 cells/L by the following March and April. The low cell densities continued until July. The cell densities of *H. circularisquama* increased in autumn and a red tide formed on 4 October 2005 (maximum cell density of 1.23×10^7 cells/L and water temperature of 26.9°C at a depth of 0 m at St. 5). The red tide

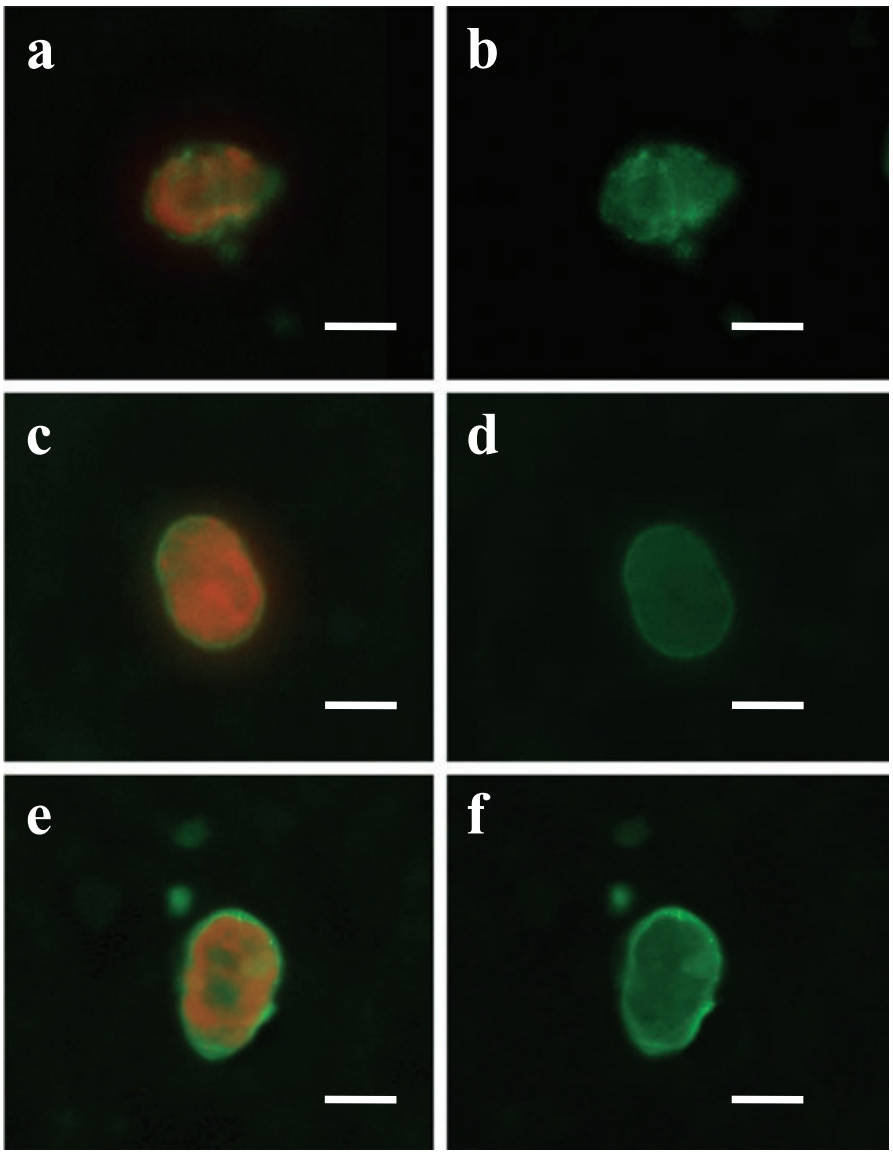

Fig. 2 Photomicrographs of *Heterocapsa circularisquama* cells observed under blue-light excitation after treatment using the indirect fluorescent antibody technique. (a) Vegetative cell in a seawater sample collected from a depth of 2 m at St. 3 in Uranouchi Inlet on 18 February, (b) the same cell as (a) with the blocking of chlorophyll-a autofluorescence, (c) temporary cyst in the same sample as (a), (d) the same cell as (c) with the blocking of chlorophyll-a autofluorescence, (e) vegetative cell of cultured strain U0403A and (f) the same cell as (e) with the blocking of chlorophyll-a autofluorescence. Scale bar, 10 μm.

Table 1 Ranges of water temperature, salinity and cell densities of *Heterocapsa circularisquama* monitored over the study period from 3 February 2004 to 16 November 2005 in Uranouchi Inlet

Station	Water temperature (°C)	Salinity (psu)	Cell density (cells/L)
St. 1	13.0–30.4	6.85–32.9	n.d.–2.10×10^6
St. 3	11.0–30.6	11.6–32.8	n.d.–1.28×10^6
St. 5	10.5–30.3	10.2–32.6	n.d.–1.23×10^7
Total	10.5–30.6	6.85–32.9	n.d.–1.23×10^7

n.d., not detected.

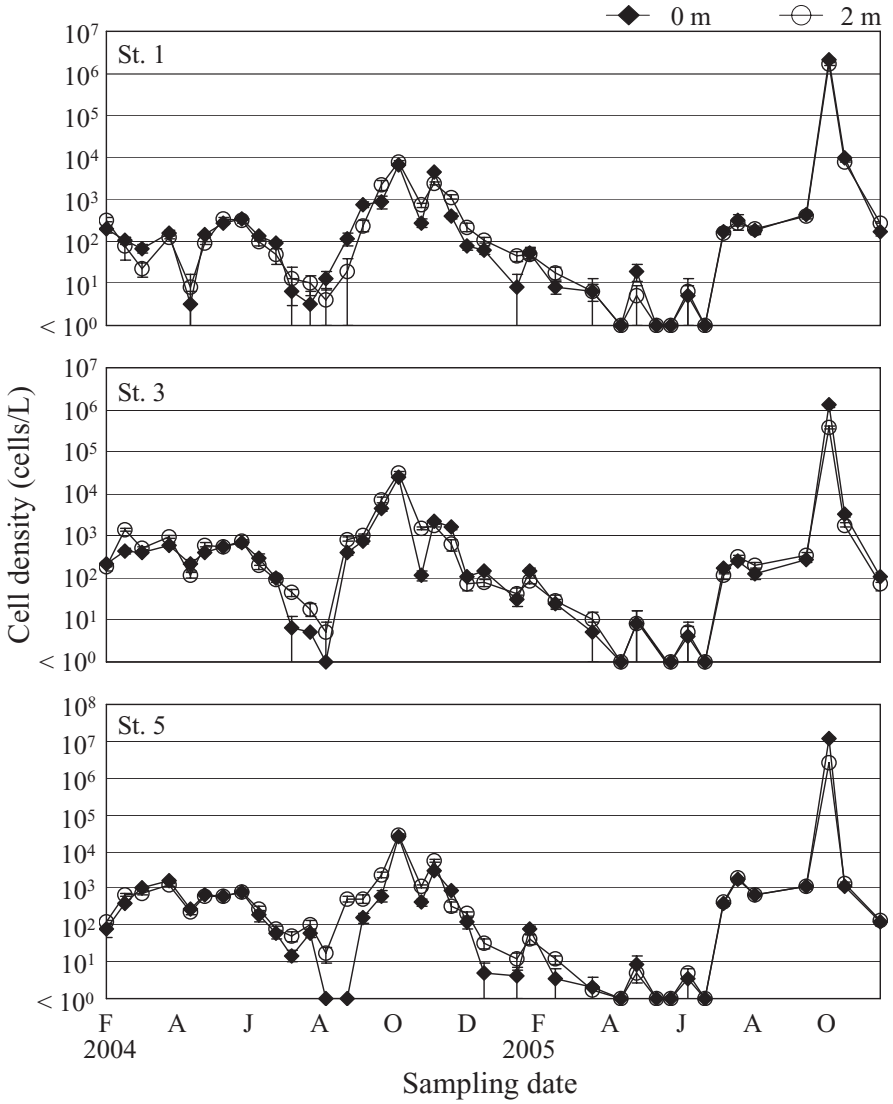

Fig. 3 Seasonal changes of *Heterocapsa circularisquama* cell densities in Uranouchi Inlet from 3 February 2004 to 16 November 2005. Error bars, ± 1 standard deviation.

disappeared within 1 week and the cell densities dropped to less than 2.73×10^2 cells/L at each point in November. In April and July 2005, red tides of *H. akashiwo* (7.68×10^3 cells/mL and 2.82×10^4 cells/mL, respectively) occurred and the cell densities of *H. circularisquama* showed low values over these periods. Vegetative cells of *H. circularisquama* were almost always detectable throughout the study period.

Over the study period in Uranouchi Inlet, water temperatures rose to over 30°C during the summer season, and were higher than 25°C, even in October (Fig. 4). The water temperatures never dropped down to 10°C or below in winter. The water temperature at St. 5, the inner part of the inlet, was comparatively lower than that of the other two stations (closer to the mouth of the inlet) in winter (Table 1). Figure 5 shows the seasonal changes in salinity in Uranouchi Inlet. Salinities occasionally declined (sometimes to 20 psu or less), but were generally stable within the range of 24.1–32.8 psu throughout the year.

Identification of overwintering cells

Two clonal cultures of the strains (U0403A and U0403B) of overwintering cells were successfully established after isolation from water samples collected from a depth of 2 m at St. 5 in Uranouchi Inlet on 3 March 2004. Light microscopic observation revealed that the cells possessed conical epitheca and hemispherical hypotheca, which were almost equal in length (Fig. 6a). The range of cell sizes was 20.0–28.2 μm ($\bar{x} = 24.1$ μm, $n = 100$) in length and 13.5–19.0 μm ($\bar{x} = 16.1$ μm, $n = 100$) in

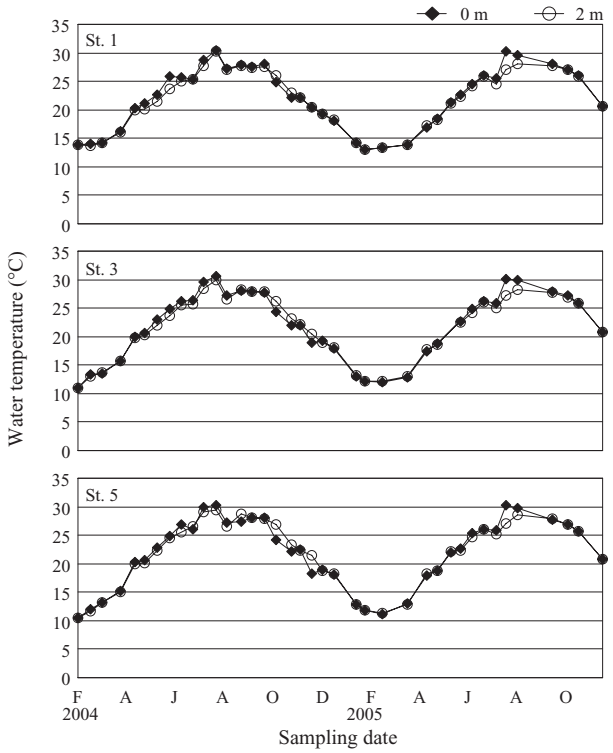

Fig. 4 Seasonal changes of water temperature in Uranouchi Inlet from 3 February 2004 to 16 November 2005.

width. The shape and size of the cells were identical to *H. circularisquama*.[11] There was no morphological difference between the two strains.

Figure 6b and c show electron micrographs of the body scales of the cells observed with TEM. Both mature (Fig. 6b) and immature (Fig. 6c) scales were observed in the same sample. The basal plate of the scales consisted of fine reticulation and six radiate ridges, and was more or less circular in outline. The diameter was approximately 400 nm with no central hole at the center of the basal plate. On mature scales a long upright emerged from the center of the plate and six uprights from the rim were connected with the next arm. These characteristics of body scales were identical to those of *H. circularisquama*.[11,12]

Figure 2e and f present photomicrographs of a vegetative cell from the cultured strain U0403A after IFAT treatment. The cell emitted red autofluorescence of chloroplasts and green FITC fluorescence of the outline of the cell under blue-light excitation (Fig. 2e), and FITC fluorescence was clearly observed under blue-light excitation with the blocking of chlorophyll-a autofluorescence (Fig. 2f). All cells observed positively reacted to the antibodies in both strains.

DISCUSSION

The monoclonal antibodies recognized both vegetative cells and temporary cysts of *H. circularisquama* in field samples from Uranouchi Inlet. The antibodies were capable of detecting cells at a very low cell density (lowest density was 1.67 cells/L in this study). This detection sensitivity was the same as the study in Ago Bay, Mie Prefecture.[7] The monoclonal antibodies recognized not only *H. circularisquama*, but also *Alexandrium catenella* and *Alexandrium tamarense*.[7] In Uranouchi Inlet, *A. catenella* ordinarily exists and cultures have been established.[15] However, *Alexandrium* spp. were not detected using the IFAT method under epifluorescence microscopy in this study. Therefore, it was considered that *Alexandrium* spp. were either absent or present at lower cell densities than the detection limit during the study period. In any case, the existence of *Alexandrium* spp. cells presents no problem for detecting *H. circularisquama* because *Alexandium* spp. are larger in size and more rounded in shape than *H. circularisquama* under microscopic observation.[7]

Heterocapsa circularisquama cells were almost continuously detected during the study period in Uranouchi Inlet (Fig. 3). Over the period from winter to spring, this species could survive in areas with a temperature higher than 10°C (Fig. 4). It can be concluded that *H. circularisquama* overwintered as vegetative cells in Uranouchi Inlet. In Uranouchi Inlet, a red tide of *H. circularisquama* was detected in autumn on 4 October 2005 during the monitoring period. The water temperature was higher than 25°C during the red tide and in the optimum range for the growth of the species, even in October.[9] Red tides of other flagellates (*H. akashiwo* and *K. mikimotoi*) occurred in the spring and summer of 2004 and 2005, and *H. circularisquama* was detected at lower cell densities (an order of 10^3 cells/L or lower). Thus, it is considered that the growth of *H. circularisquama* was inhibited by competition with other species in summer, and *H. circularisquama* successfully grew after the competitive species disappeared in autumn.

The salinity was generally suitable for the growth of *H. circularisquama* and remained over 25 psu throughout the year (Fig. 5). The water temperature was in the range of 13.0–30.4°C at St. 1 (Table 1), and *H. circularisquama* can survive at a temperature of 12.5°C.[10] Consequently, Uranouchi Inlet is a suitable embayment area for *H. circularisquama* to grow and survive as vegetative cells throughout the year and to form red tides.

Temporary cysts of *H. circularisquama* were detected in February 2004 and the densities were

Table 2 Cell densities of vegetative cells and temporary cysts of *Heterocapsa circularisquama* in seawater samples collected on 3 February 2004 in Uranouchi Inlet

Station	Depth (m)	Water temperature (°C)	Vegetative cells (cells/L)	Temporary cysts (cells/L)
St. 1	0	13.8	197	6.67
	2	13.8	330	26.7
St. 3	0	11.0	217	3.33
	2	11.0	187	n.d.
St. 5	0	10.5	76.7	83.3
	2	10.5	123	76.7

n.d., not detected.

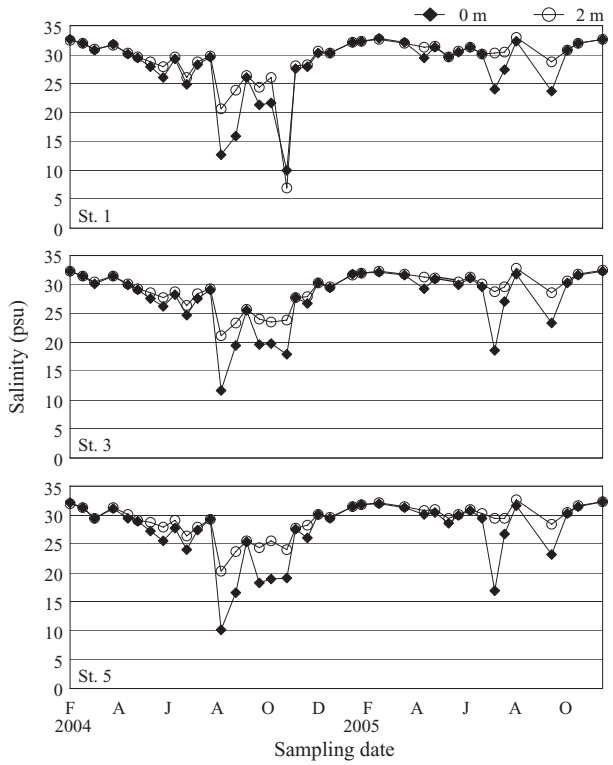

Fig. 5 Seasonal changes of salinity in Uranouchi Inlet from 3 February 2004 to 16 November 2005.

higher at St. 5, located in the inner part of the inlet with lower temperatures (Table 2). The water temperature was 10.5°C at St. 5, which was lower than that of the other stations. Therefore, it is suggested that *H. circularisquama* cells formed temporary cysts because of a decrease in temperature, and the percentages of temporary cysts out of total cells of *H. circularisquama* were higher at St. 5 with a lower temperature than at the other two stations. The temporary cysts presumably recovered to motile cells after the elevation of water temperature. In 2005, the water temperature was higher than 11.2°C, even at St. 5 in winter (higher than 13.0°C at St. 1), and temporary cysts were not detected, but vegetative cells were detected. Thus, it is considered that *H. circularisquama* can overwinter as vegetative cells and can sometimes tolerate lower water temperatures over a limited period by forming temporary cysts in Uranouchi Inlet. In Ago Bay, *H. circularisquama* cells were not generally detected in the winter season and, hence, temporary cysts were not observed.[7] Temporary cysts possibly sank down and survived on the sea bottom. It is, therefore, important to develop a monitoring method of *H. circularisquama* from bottom sediments so that the role of the populations at the sea bottom during periods of low water temperature can be elucidated.

According to the observations of cultured cells established from the sea water in March 2004, the size and shape of the cultured cells were identical to *H. circularisquama*. The body scale structures of the cells were also identical to *H. circularisquama* (Fig. 6). Moreover, the monoclonal antibodies reacted to cells of the cultured strains. These facts clearly indicate that the cells were *H. circularisquama*. Hence, the isolated cells were overwintering cells of *H. circularisquama*.

It is well known that raphidophytes form resting cysts to survive the winter season.[16–20] In contrast, *K. mikimotoi* is known to overwinter in the form of vegetative cells.[21–24] Resting cysts of *H. circularisquama* have never been discovered in the field and there is no report of cyst formation by culture experiments. Based on the discovery of overwintering vegetative cells in Uranouchi Inlet, *H. circularisquama* overwinters as vegetative cells in areas with relatively higher water temperatures during winter.

We discovered and identified overwintering vegetative cells of *H. circularisquama* in Uranouchi Inlet. However, the overwintering mechanisms of this species are unclear in other embayments and areas. In embayments where the water temperature never drops down to 10.5°C in winter, it is possible for *H. circularisquama* to overwinter as

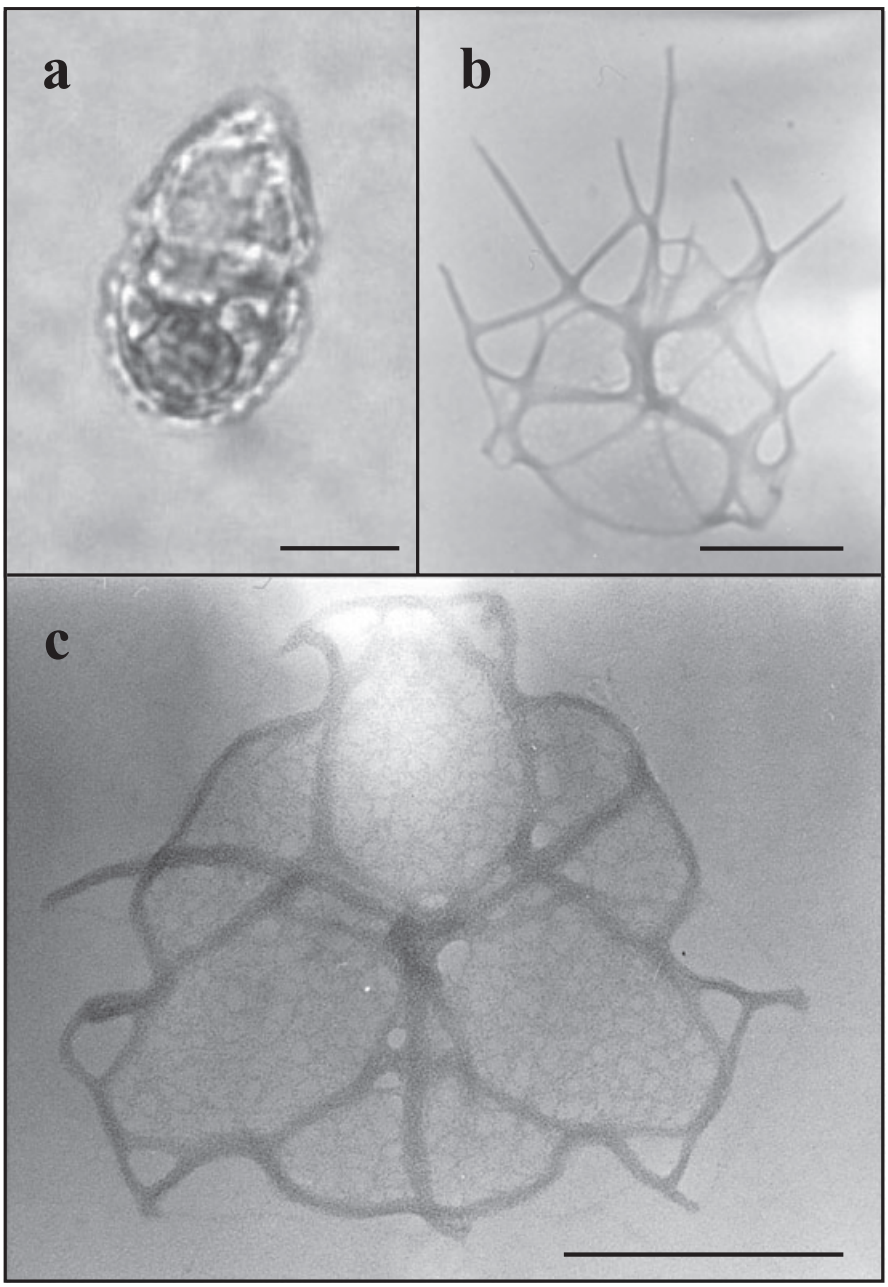

Fig. 6 Photomicrographs of a vegetative cell of a cultured strain observed with optical microscopy and electron micrographs of the body scales of cultured cells observed with transmission electron microscopy. (a) Vegetative cell of cultured strain U0403A, (b) mature body scale and (c) immature body scale. (a) Scale bar, 10 μm, (b,c) scale bar, 200 nm.

vegetative cells, as is the case in Uranouchi Inlet (Table 2). This species might not be able to overwinter in embayments where the water temperature drops down to 10°C or below in winter. In such areas, it is essential for the cells to be imported from other areas for red tide occurrences. In addition, there is a strong possibility that this species is introduced via the transport of bivalves, such as pearl oysters and oysters.[25–27] There is also a marked possibility that *H. circularisquama* will form red tides more frequently in Japanese coastal waters with higher water temperatures because of global warming in the near future. It is essential to carefully monitor this species in coastal areas where there are bivalve aquaculture industries.

ACKNOWLEDGMENTS

We are grateful to Dr H. Nakahara, Professor emeritus of Kyoto University, for useful comments and suggestions during this study. This study was supported in part by a grant from the Fisheries Agency of Japan.

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