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Effect of salinity on interspecific competition between the dinoflagellate *Alexandrium catenella* and the raphidophyte *Heterosigma akashiwo*

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ABSTRACT: We investigated the effect of salinity on interspecific competition between the dinoflagellate *Alexandrium catenella* and the raphidophyte *Heterosigma akashiwo* using bi-algal cultures under several culture conditions. When initial cell densities of *A. catenella* and *H. akashiwo* were both 1×10^2 cells ml^{-1} at salinity 30, the growth of *H. akashiwo* was strongly suppressed by *A. catenella*, and no intact *H. akashiwo* cells were observed. In contrast, the growth of *A. catenella* was suppressed when initial cell densities of *A. catenella* and *H. akashiwo* were 1×10^2 and 1×10^4 cells ml^{-1}, respectively. Interestingly, the growth of *A. catenella* was notably suppressed in bi-algal cultures at salinity 20 when initial cell densities of *A. catenella* and *H. akashiwo* were both 1×10^2 cells ml^{-1}. In addition, growth of *H. akashiwo* in bi-algal cultures with *A. catenella* under cell-contact conditions was significantly inhibited, whereas growth of *H. akashiwo* was not inhibited by *A. catenella* in non-contact culture conditions. Furthermore, live-cell-mediated hemolytic activity of *A. catenella* was detected, but no hemolytic activity was detected in *A. catenella* culture filtrate. In contrast, growth of *A. catenella* was significantly inhibited by 50 to 100 µg ml^{-1} of a crude extract, including allelochemicals, from *H. akashiwo* culture filtrate. Therefore, salinity and interspecific interactions via allelopathy and/or cell contact might be important factors determining the growth dynamics of *A. catenella* and *H. akashiwo* in the field.

KEY WORDS: Allelopathy · Cell contact · Hemolytic activity · Growth inhibition · *Heterosigma akashiwo* · *Alexandrium catenella* · Bi-algal culture

INTRODUCTION

Harmful algal blooms (HABs) are an increasingly serious environmental problem for aquaculture, fisheries, and public health in many coastal areas throughout the world (Anderson 1997). The toxic dinoflagellate *Alexandrium catenella* (Whedon & Kofoid, 1985) Balech inhabits coastal areas in many parts of the world (Hallegraeff 1993, Imai et al. 2006) and causes paralytic shellfish poisoning (PSP) via consumption of marine bivalves such as the Pacific oyster *Crassostrea gigas* (Lassus et al. 2007) and short-necked clam *Ruditapes philippinarum* (syn. *Tapes japonica*) (Samsur et al. 2006). In Japan, Iwasaki (1992) reported that optimal temperature for growth of *A. catenella* was between 10 and 15°C, and optimal salinity for growth of this species was 32. In addition, Laabir et al. (2011) reported that *A. catenella* can survive at salinities as low as 10 and grow at salinities up to 40, and can grow at water temperatures between 15 and 30°C.

The raphidophyte *Heterosigma akashiwo* (Hada, 1987) Hada ex Hara et Chihara is also a HAB species, and large-scale blooms of this species have occurred

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all over the world (Honjo 1993, Smayda 1998). *Heterosigma akashiwo* can grow between 10 and 30°C and grows well at up to 15°C (Smayda 1998). In addition, *H. akashiwo* can survive and grow at salinities from 2 to 50 (Tomas 1978, Zhang et al. 2006, Martínez et al. 2010). Thus *A. catenella* and *H. akashiwo* have the potential to grow at the same time and location in nature.

In Tokuyama Bay, Yamaguchi Prefecture, Japan, a bloom of *A. catenella* in 2004 was followed by a massive bloom of *H. akashiwo* (Baba et al. 2006). Interspecific competition between *A. catenella* and *H. akashiwo* via allelopathy and macronutrients was suspected because hydrographic conditions during the *H. akashiwo* bloom appeared suitable for the growth of *A. catenella* (Baba et al. 2006). However, the change from *A. catenella* to *H. akashiwo* occurred in early May when salinity decreased to under 20 (Baba et al. 2006). Therefore, we hypothesize that interspecific competition between *A. catenella* and *H. akashiwo* may involve both biological factors (i.e. allelopathy and cell contact) and environmental factors (i.e. salinity).

Bloom formation by phytoplankton can be affected by interspecific interactions caused by allelopathy and/or cell contact. Allelopathy refers to any direct or indirect inhibitory or stimulatory effect of one plant including microalgae on another through the production of chemical secretions (Maestrini & Bonin 1981, Rice 1984). Both *in situ* and *in vitro* experiments have suggested that allelopathy plays a key role in the growth dynamics of red-tide blooms through inhibitory effects (Cembella 2003, Gross 2003, Legrand et al. 2003, Granéli & Hansen 2006, Yang et al. 2010, Poulson-Ellestad et al. 2014). Furthermore, cells of the dinoflagellate *Gyrodinium instriatum* Freudenthal & Lee, 1963 become immotile immediately after contact with cells of the dinoflagellate *Heterocapsa circularisquama*; some immotile *G. instriatum* cells lyse, whereas others later recover their motility (Uchida et al. 1995). In another study used as the basis for a mathematical model, *H. circularisquama* cells formed temporary cysts after 59 cell-to-cell contacts with the dinoflagellate *Gymnodinium mikimotoi* Miyake et Kominami, 1935 ex Oda (=*Karenia mikimotoi*), whereas *K. mikimotoi* was killed after 19 contacts with *H. circularisquama* (Uchida et al. 1999). These studies suggest that both cell contact and allelopathy play key roles in the growth dynamics of phytoplankton through inhibitory effects.

In this study, we conducted bi-algal culture experiments using several combinations of initial cell densities of *A. catenella* and *H. akashiwo* at 2 different salinities. In addition, we examined interspecific interactions between *A. catenella* and *H. akashiwo* through bi-algal culture experiments under conditions without cell contact. To determine the mode of action of the growth inhibitory effect, we investigated the hemolytic activity of *A. catenella* and *H. akashiwo* toward horse erythrocytes under continuous light and dark conditions. Furthermore, to clarify whether an allelochemical reportedly produced by *H. akashiwo* (Yamasaki et al. 2009) was involved in interspecific interactions of these species, we examined the effects of crude extracts from culture filtrates of *H. akashiwo* on the growth of *A. catenella*.

MATERIALS AND METHODS

Algal species and culture conditions

Non-axenic strains of *Alexandrium catenella* and *Heterosigma akashiwo* were isolated from Tokuyama Bay, Yamaguchi, Japan, in 1997, and the fishing port of Udagou, Yamaguchi, Japan, in 1998, respectively. An axenic strain of *H. akashiwo* (strain NIES-10) was obtained from the National Institute for Environmental Studies (NIES) Japan. Cultures were maintained in an incubator in 100-ml flasks containing 50 ml of T-1 medium (Baba 2010) with salinity 30 at 20°C under 200 (±5 [SD]) µmol photons m^{-2} s^{-1} of cool-white fluorescent illumination on a 12:12 h light:dark cycle. To minimize bacterial effects on phytoplankton growth, benzyl penicillin (100 µg ml^{-1}) and streptomycin (100 µg ml^{-1}) were added to non-axenic cultures of *A. catenella*. The T-1 medium was autoclaved at 121°C for 15 min; it included a buffer to avoid pH effects. Irradiance in the incubator was measured with a Quantum Scalar Laboratory Irradiance Sensor (QSL-2100/2101; Biospherical Instruments, San Diego, CA, USA).

Growth experiments under different salinities

A 40 µl sample of *A. catenella* or *H. akashiwo* cell suspension at an initial cell density of 1×10^2 cells ml^{-1} was added to 3960 µl of T-1 medium with salinity 20, 25, or 30 in a glass test tube. Triplicate growth experiments were performed at 20°C for each test condition. After the start of each incubation, the *in vivo* fluorescence of each tube was measured every 2 d (Trilogy Laboratory Fluorometer; Turner Designs). Growth rates (divisions d^{-1}) were calcu-

lated for each tube from 3 consecutive data points using the method of Brand et al. (1981), and the maximum growth rates during the entire incubation period were determined.

Bi-algal culture experiments

Bi-algal culture experiments were conducted at 20°C in 100-ml flasks containing 50 ml of medium at salinity 20 or 30. For the medium with salinity 30, *A. catenella* cells in early stationary phase (4–5 × 10^4 cells ml^{-1}) were inoculated at a final cell density of 10^2 cells ml^{-1} into cultures of *H. akashiwo* (stock cultures: 50–60 × 10^4 cells ml^{-1}) with cell densities of 10^2 and 10^4 cells ml^{-1}. For the medium with salinity 20, *A. catenella* cells in early stationary phase (4–5 × 10^4 cells ml^{-1}) were inoculated at a final cell density of 10^2 cells ml^{-1} into cultures of *H. akashiwo* (stock cultures: 50–60 × 10^4 cells ml^{-1}) with cell densities of 10^2 cells ml^{-1}. As controls, *A. catenella* was cultured individually at cell densities of 10^2 cells ml^{-1}, and *H. akashiwo* was cultured at a cell density of 10^2 and 10^4 cells ml^{-1}. Three replicate flasks were used for each treatment. All flasks were gently mixed by hand twice a day and randomly rearranged to minimize the effects of light or temperature gradients in the incubator. *Alexandrium catenella* and *H. akashiwo* cells were counted microscopically in 1-ml subsamples collected at 2 d intervals. When cell densities exceeded 2 × 10^4 cells ml^{-1}, subsamples were diluted by a factor of 10–50 with fresh T-1 medium before counting.

The data were analyzed by the *t*-test. The *p*-values were adjusted by the Benjamini-Hochberg method. We used the statistical software R version 3.3.3 for Mac (R Core Team 2017).

Bi-algal culture under non-contact conditions

For this experiment, we used 6-well plates (BD BioCoat; Becton Dickinson) with cell culture inserts (24 mm Transwell with 3.0-µm pore polycarbonate membrane insert; Becton Dickinson). This insert plate has a membrane filter (pore size 3.0 µm) on the bottom. *Alexandrium catenella* cells in early stationary phase (4–5 × 10^4 cells ml^{-1}) were suspended in T-1 medium (salinity 30) at densities of 10^3 cells ml^{-1}, and 12 ml of this suspension was inoculated into the outer chambers of the well plates. *Heterosigma akashiwo* cells in early stationary phase (50–60 × 10^4 cells ml^{-1}) were then suspended at a density of 10^2 cells ml^{-1} in T-1 medium, and 3 ml of this suspension was inoculated into each inner chamber. As mono-algal controls, 12 ml of *A. catenella* suspension (10^3 cells ml^{-1}) was added into each outer chamber, and 3 ml of T-1 medium was added into each inner chamber. Similarly, 12 ml of T-1 medium was added into each outer chamber, and 3 ml of *H. akashiwo* (10^2 cells ml^{-1}) was added into each inner chamber as mono-algal controls. As the bi-algal control, both *A. catenella* (10^3 cells ml^{-1}) and *H. akashiwo* (10^2 cells ml^{-1}) were cultured in the same well without cell-culture inserts. Three replicate wells were used for each treatment and were cultured at 20°C. On Day 2 of the incubation, the cells in each of five 10 µl subsamples from each well were counted microscopically.

The data were analyzed by using the Mann-Whitney *U* test. The analysis was performed using SPSS version 19.0 for Windows (SPSS Inc.). A significance level of $p < 0.05$ was used for the test.

Hemolytic assay

Horse blood cells were obtained from Nippon Bio-Test Laboratories and were used within 7 d of receipt. These erythrocytes were washed 3 times with phosphate-buffered saline (PBS) and adjusted to a final concentration of 4% (v/v) in PBS. Triplicate 70 µl aliquots of serial 2-fold dilutions of intact cell suspension or cell-free culture supernatant of *A. catenella* or *H. akashiwo* were added to round-bottom 96-well plates (Becton Dickinson). A 70 µl aliquot of the 4% (v/v) erythrocyte suspension was added each well, and the well plates were gently shaken. After incubation for 5 h at 20°C under 200 (±5) µmol photons m^{-2} s^{-1} of cool-white fluorescent illumination or continuous dark, the plates were centrifuged at 360 × *g* for 10 min at 4°C. Aliquots (100 µl) of supernatant were withdrawn from the wells and transferred to flat-bottom 96-well plates (Becton Dickinson). Released hemoglobin was measured using absorbance at 480 nm in a microplate reader (Multiskan GO; Thermo Fisher Scientific). Negative (zero hemolysis) and positive (100% hemolysis) controls were included using erythrocytes suspended in T-1 medium alone or in T-1 medium containing 1% (v/v) Triton X-100, respectively.

For the hemolytic kinetic analysis of *A. catenella* (cell density: 4.4 × 10^4 cells ml^{-1}) toward horse erythrocytes, triplicate 2-ml aliquots of *A. catenella* cell suspension in T-1 medium were added to disposable culture tubes. The same volume of 4% (v/v) ery-

throcyte suspension in PBS was added to each tube, and the tubes were gently shaken. After incubation for 0, 15, 30, 45, 60, 120, 180, 240, and 300 min, 150 µl aliquots of the assay mixtures were withdrawn and centrifuged at 360 × *g* for 10 min at 4°C. Aliquots (100 µl) of the resulting supernatant were transferred to flat-bottom 96-well plates, and the released hemoglobin was measured using absorbance at 480 nm in a microplate reader (Thermo Fisher Scientific).

Allelopathic effect of *H. akashiwo* on *A. catenella* growth

To check for possible extracellular compounds with growth-inhibitory effects, we prepared extracts from filtrates of *H. akashiwo* culture. For this experiment, an axenic strain of *H. akashiwo* (NIES-10) was used to prevent contamination with substances produced by bacteria. Cells of *H. akashiwo* were inoculated at a density of 100 cells ml^{-1} into two 200 ml glass flasks containing 100 ml of T-1 medium. After 14 d (cell density: 5–6 × 10^5 cells ml^{-1}), 100 ml samples of culture from each flask were combined to give a total of 200 ml. The combined sample was gravity-filtered through a 5.0 µm pore-size membrane filter (Merckmillipore) and then through a 0.45 µm pore-size membrane filter (Merckmillipore). The filtrate was dialyzed against deionized water for 3 d at 4°C using dialysis membranes with a 3500 Da molecular weight cut-off (Spectrum Laboratories). After dialysis, the inner solution was frozen at −80°C and lyophilized (FDU-1200; Tokyo Rikakikai).

A bioassay with the crude extract was conducted at 20°C using 48-well plates (Corning, New York, NY, USA). Fifty microliters of *A. catenella* cell suspension (initial cell density: 10^2 cells ml^{-1}) were added to 950 µl of each test solution of crude extract dissolved in T-1 medium. Final concentrations of the crude extract in bioassays were 12.5, 25, 50, and 100 µg ml^{-1}. Each bioassay was conducted in triplicate. For controls, 50 µl of *A. catenella* cell suspension was added to well plates containing 950 µl of T-1 medium without any of the crude extract obtained from *H. akashiwo* culture. After 7 d of incubation, the cells in each of five 10 µl subsamples from each well were counted microscopically.

The data were analyzed by 1-way analysis of variance (ANOVA) and then tested using Dunnett's post hoc test. The analysis was performed using SPSS version 19.0 for Windows (SPSS Inc.). A significance level of $p < 0.05$ was used for the test.

RESULTS

Effect of salinity on growth

Alexandrium catenella grew at all salinities tested (Fig. 1A): maximum growth rate was obtained at salinity 30 and decreased with decreasing salinity (Table 1). *Heterosigma akashiwo* also grew at all

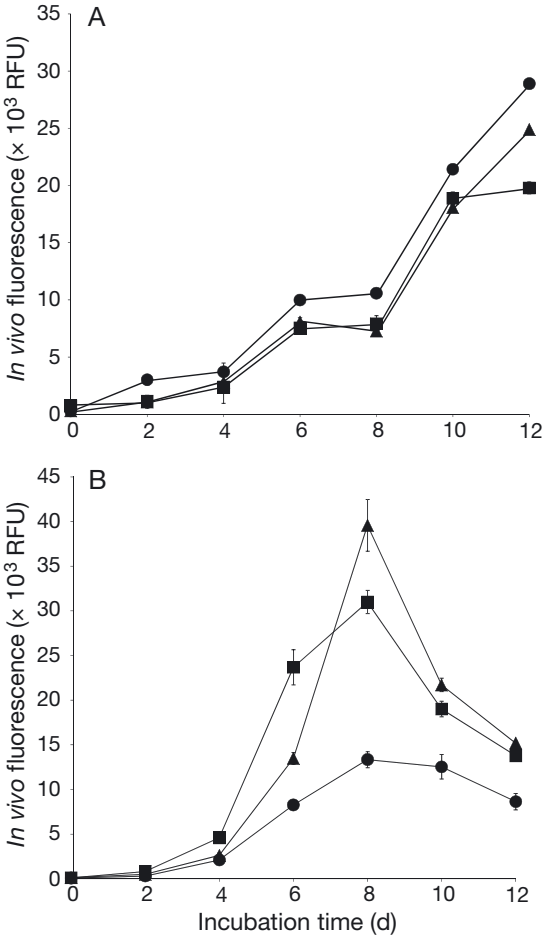

Fig. 1. Growth of *Alexandrium catenella* and *Heterosigma akashiwo* at 3 salinities. (A) *A. catenella* at salinity 20 (■), 25 (▲), and 30 (●). (B) *H. akashiwo* at salinity 20 (■), 25 (▲), and 30 (●). Data are means ± SD of triplicate measurements. RFU: relative fluorescence unit

Table 1. Maximum growth rates of *Alexandrium catenella* and *Heterosigma akashiwo* cultured at 3 salinities. Data are means ± SD (n = 3)

Species	Growth rate (divisions d^{-1})		
	Salinity 20	Salinity 25	Salinity 30
A. catenella	1.29 ± 0.04	1.66 ± 0.11	1.88 ± 0.13
H. akashiwo	2.62 ± 0.19	2.37 ± 0.07	2.19 ± 0.27

salinities tested (Fig. 1B), but unlike *A. catenella* maximum growth rates were seen at salinities 20 and 25 (Table 1), with much lower growth at salinity 30 (Table 1).

Growth of bi-algal cultures under various experimental conditions

In T-1 medium at salinity 30, the growth of *A. catenella* was virtually the same in both bi-algal and mono-algal cultures (Fig. 2A). In contrast, the growth of *H. akashiwo* was gradually suppressed from Day 2 onwards, and no intact *H. akashiwo* cells were observed by Day 14.

In T-1 medium at salinity 20, the presence of *A. catenella* induced a slight decrease in growth rate of *H. akashiwo*, even if both bi-algal and mono-algal cultures still reached the same cell yield (Fig. 2B). In contrast, the growth of *A. catenella* was notably suppressed in bi-algal cultures from Day 4 onwards, when *H. akashiwo* reached exponential growth phase, after which the average maximum cell density of *A. catenella* was about 5% of that in mono-algal cultures (Day 14; Fig. 2B).

When initial cell densities of *A. catenella* and *H. akashiwo* were 10^2 cells ml^{-1} and 10^4 cells ml^{-1}, respectively, the growth of *H. akashiwo* was virtually the same in both bi-algal and mono-algal cultures in T-1 medium at salinity 30. In contrast, the growth of *A. catenella* was substantially suppressed in bi-algal cultures from Day 2 onwards, when *H. akashiwo* reached exponential growth phase, after which the average maximum cell density of *A. catenella* was about 0.6% of that in mono-algal cultures (Day 8; Fig. 3).

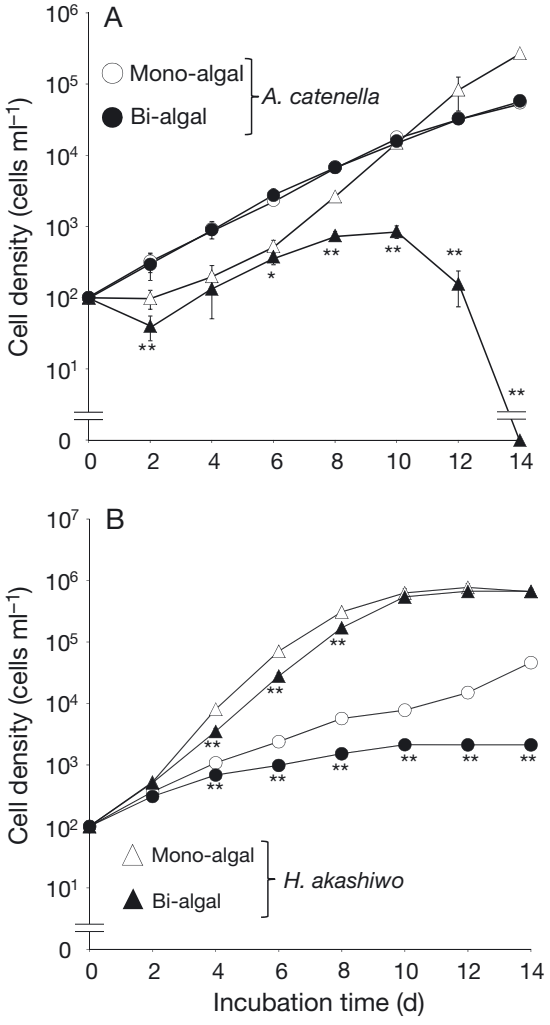

Fig. 2. Growth of *Alexandrium catenella* when cultured alone (○) or together with *Heterosigma akashiwo* (●), and of *Heterosigma akashiwo* when cultured alone (△) or together with *A. catenella* (▲) at salinity (A) 30 or (B) 20. Initial cell densities of *A. catenella* and *H. akashiwo* were 10^2 cells ml^{-1}. Data are means ± SD (cells ml^{-1}) of triplicate measurements. Asterisks indicate significant differences from cell densities in mono-algal cultures (*$p < 0.05$, **$p < 0.01$)

Growth of bi-algal cultures under non-contact conditions

Cell density of *H. akashiwo* (initial cell density: 10^2 cells ml^{-1}) in bi-algal cultures with *A. catenella*

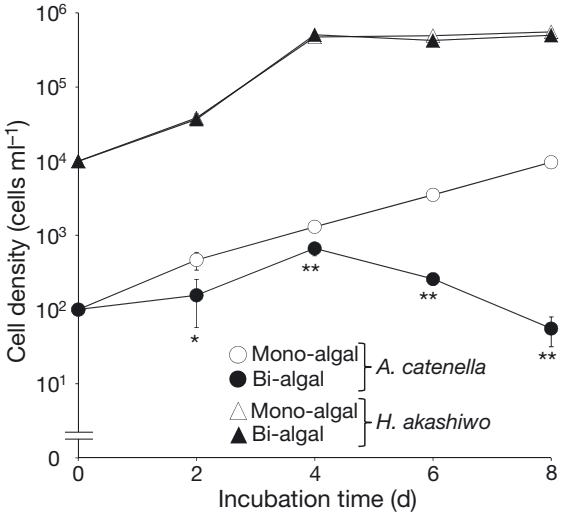

Fig. 3. Growth of *Alexandrium catenella* when cultured alone (○) or together with *Heterosigma akashiwo* (●), and of *Heterosigma akashiwo* when cultured alone (△) or together with *A. catenella* (▲) at salinity 30. Initial cell densities of *A. catenella* and *H. akashiwo* were 10^2 and 10^4 cells ml^{-1}, respectively. Data are means ± SD (cells ml^{-1}) of triplicate measurements. Asterisks indicate significant differences from cell densities in mono-algal cultures (*$p < 0.05$, **$p < 0.01$)

(initial cell density: 10^3 cells ml^{-1}) under contact conditions decreased significantly (about 35% of the mono-algal control, Mann-Whitney *U* test, $p < 0.05$; Fig. 4). However, cell density of *H. akashiwo* did not decrease under non-contact conditions with *A. catenella*.

In contrast, cell density of *A. catenella* (initial cell density: 10^3 cells ml^{-1}) in bi-algal cultures with *H. akashiwo* (initial cell density: 10^2 cells ml^{-1}) did not decrease under either contact or non-contact conditions (Fig. 4).

Hemolytic activity

Intact cell suspensions of *A. catenella* showed hemolytic activity in a cell density-dependent manner under both continuous light and dark conditions (Fig. 5). However, the culture filtrate of *A. catenella* showed no hemolytic activity under either continuous light or dark conditions (Fig. 5).

Time-course results revealed that horse erythrocytes were highly sensitive to *A. catenella*, and hemolysis began immediately after exposure (Fig. 6). Intact cell suspensions of *A. catenella* showed hemolytic activity at 70% of the positive (100% hemolysis) control after 15 min of incubation, and extremely high hemolysis (90% of the control) after only 30 min (Fig. 6).

In contrast, *H. akashiwo* showed no significant hemolytic activity up to the highest cell density (2.5×10^5 cells ml^{-1}) under either continuous light or dark

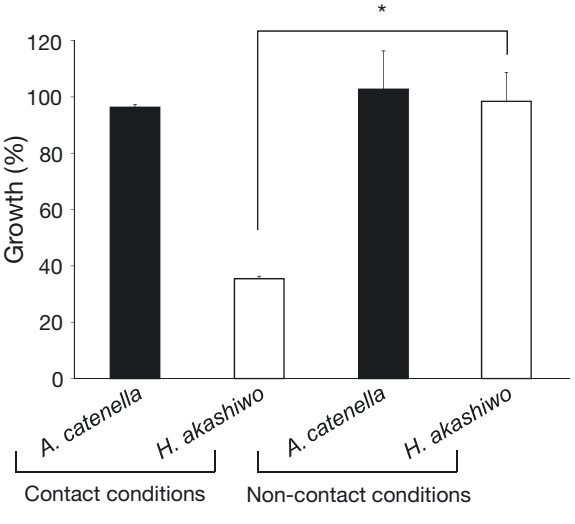

Fig. 4. Percent growth of *Alexandrium catenella* and *Heterosigma akashiwo* after 2 d under contact and non-contact conditions. Data are means ± SD of triplicate measurements. *Significant difference between contact and non-contact conditions ($p < 0.05$)

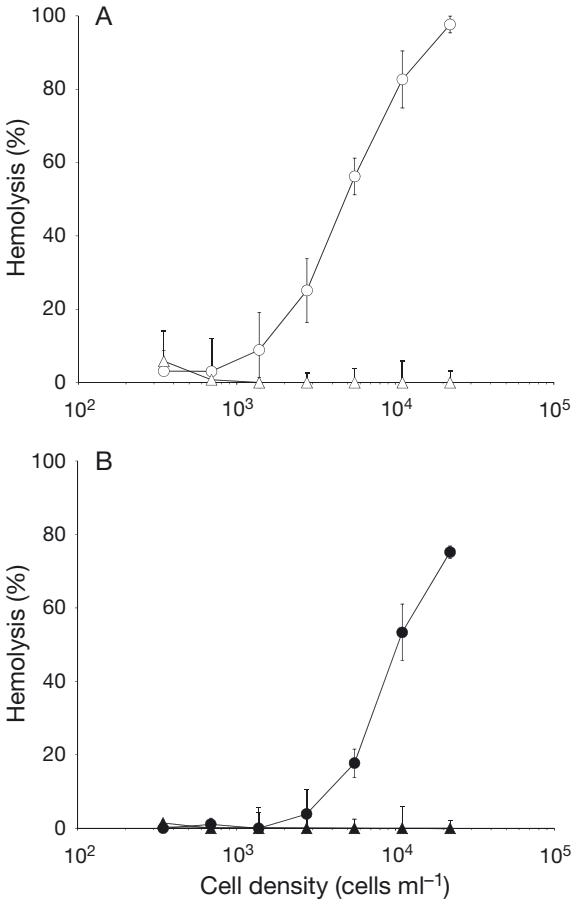

Fig. 5. Hemolytic activity (%) of *Alexandrium catenella* at different cell densities. (A) Intact cell suspension (○) and filtrate (△) of *A. catenella* under continuous light conditions. (B) Intact cell suspension (●) and filtrate (▲) under dark conditions. The extent of hemolysis was measured as described in the text. Data are means ± SD of triplicate measurements

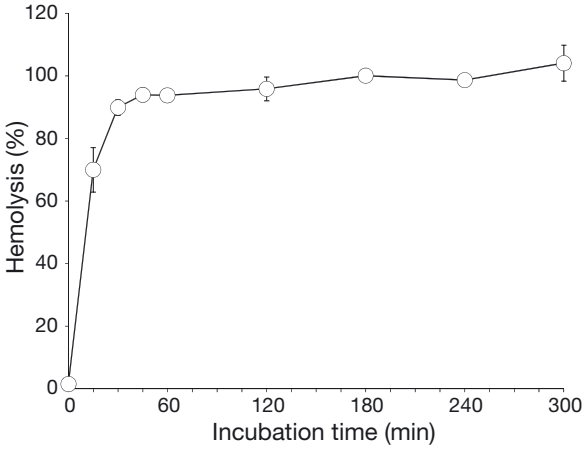

Fig. 6. Time-course of hemolytic activity (%) of *Alexandrium catenella* toward horse erythrocytes. The extent of hemolysis was measured as described in the text. Data are means ± SD of triplicate measurements

conditions (Fig. 7). Similarly, no hemolytic activity was detected in the cell-free supernatant of *H. akashiwo* under either continuous light or dark conditions (Fig. 7).

Effect of *H. akashiwo* extract on *A. catenella* growth

Growth of *A. catenella* was significantly inhibited by 50 to 100 µg ml^{-1} of the crude extract from *H. akashiwo* culture filtrate (Dunnett's post hoc test, $p < 0.05$; Fig. 8). However, *A. catenella* growth was not significantly affected by the crude extract at concentrations from 12.5 to 25 µg ml^{-1} (Fig. 8).

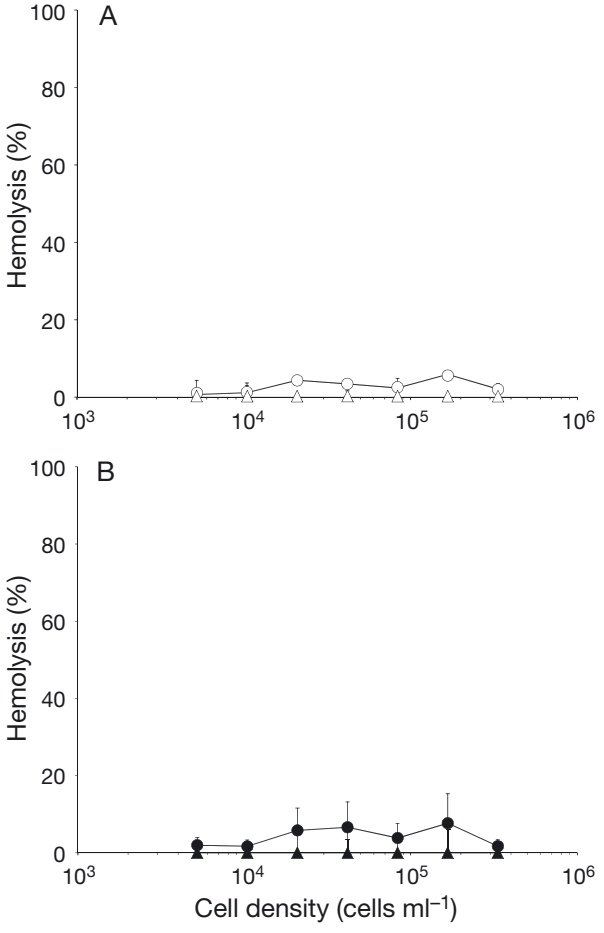

Fig. 7. Hemolytic activity (%) of *Heterosigma akashiwo* at different cell densities. (A) Hemolytic activity of intact cell suspension (○) and filtrate (△) of *H. akashiwo* under continuous light conditions. (B) Hemolytic activity of intact cell suspension (●) and filtrate (▲) of *H. akashiwo* under dark conditions. The extent of hemolysis was measured as described in the text. Data are means ± SD of triplicate measurements

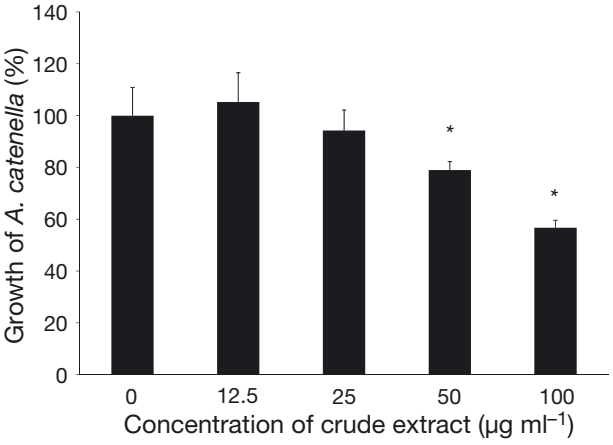

Fig. 8. Effect of a crude extract prepared from *Heterosigma akashiwo* culture filtrate (see text) on the percent growth of *Alexandrium catenella* after 7 d. Data are means ± SD of triplicate measurements. *Significant differences from no-extract control ($p < 0.05$)

DISCUSSION

Interspecific competition

To determine the role of interspecific competition between *Alexandrium catenella* and *Heterosigma akashiwo* in their bloom formation and alteration, we conducted bi-algal culture experiments under several experimental conditions. The species first reaching stationary phase substantially suppressed the maximum cell densities attained by the second species under all experimental conditions (Figs. 2 & 3). At salinity 30, *A. catenella* outcompeted *H. akashiwo* on Day 14 of incubation when the initial cell densities of both species were 10^2 cells ml^{-1} (Fig. 2A). Interestingly, at salinity 20 growth of *A. catenella* was suppressed by *H. akashiwo* when the initial cell densities of both species were 10^2 cells ml^{-1} (Fig. 2B). This may explain observations that *A. catenella* and *H. akashiwo* alternately dominate in the field. Baba et al. (2006) observed that a bloom of *A. catenella* suddenly declined and *H. akashiwo* bloomed in May 2005 when salinity fell below 20. Furthermore, *H. akashiwo* survived well and grew at high rates when the salinity suddenly decreased from 32 to 5 (Shikata et al. 2008). Thus, the observations that *H. akashiwo* grows faster than *A. catenella* at salinity 20 (Fig. 1), and the species first reaching high cell density limits the cell density of the other species (Figs. 2 & 3) may explain why *A. catenella* and *H. akashiwo* alternately dominate in the field.

Fistarol et al. (2005) reported that the diatom *Thalassiosira weissflogii*, grown under nutrient limita-

tion, showed the highest sensitivity to *Prymnesium parvum* filtrate. In addition, several studies suggest that phytoplankton might induce or augment the production of allelochemicals under nutrient limitation (Gross 2003, Legrand et al. 2003). Tillmann et al. (2009) pointed out that quantitative differences of lytic compounds (i.e. allelochemically active compounds) need to be investigated under 'stress' and/or limiting growth conditions such as nutrient limitation. Thus, synergetic effects of stressors may play a strong role in the biological interaction between phytoplankton species. On the other hand, the field observation (Baba et al. 2006) and our results (Fig. 1) suggest that a salinity drop from 30 to 20 during an *A. catenella* bloom will induce a direct decrease of growth rate for *A. catenella* and an increase in growth abilities of *H. akashiwo*. Furthermore, the present study indicated that this succession can be accelerated by the biological interaction. So that means *A. catenella* cells became more sensitive to allelochemicals produced by *H. akashiwo* due to the drop of salinity, and this limitation increased as long as the *H. akashiwo* bloom developed, with the production of more and more allelochemicals.

Elevated pH in culture media can inhibit the growth of phytoplankton species in mixed cultures (Goldman et al. 1981, 1982, Schmidt and Hansen 2001, Hansen 2002, Lundholm et al. 2005). Therefore, it is important to consider elevated pH in the culture medium when analyzing interspecific interactions between phytoplankton in dense cultures. Competition for nutrients can also affect the growth of phytoplankton in mixed cultures. In our experiments, however, the growth inhibitory effects of both species were observed on Day 2 to Day 4 of incubation when cell densities of both species were less than 10^3 cells ml^{-1} (Fig. 2). It is therefore unlikely that culture pH or nutrient competition were major factors in the observed negative effects.

Inhibitory effects via cell contact

To ascertain whether cell contact might affect the interspecific interaction between *A. catenella* and *H. akashiwo*, we conducted parallel bi-algal culture experiments under contact and non-contact conditions. Growth of *H. akashiwo* in bi-algal cultures with cell contact was strongly inhibited by *A. catenella*, whereas *A. catenella* did not affect the growth of *H. akashiwo* under non-contact conditions after 2 d incubations (Fig. 4). In addition, culture filtrate of *A. catenella* showed no hemolytic activity (Fig. 5). It therefore seems that growth inhibition of *H. akashiwo* is induced by direct contact with *A. catenella* cells, and our findings, as well as those of previous studies (Uchida et al. 1999, Yamasaki et al. 2011), strongly suggest that the interactions through cell contact are a key factor promoting monospecific bloom formation in the field.

Interestingly, there seems to be a correspondence between the growth inhibitory effect of *A. catenella* on *H. akashiwo* and its hemolytic activity. *Alexandrium catenella* induced potent hemolytic activity under both continuous light and dark conditions, whereas filtrate from *A. catenella* culture showed no hemolytic activity (Fig. 5). In addition, hemolysis was induced immediately after exposure (Fig. 6) and is consistent with the almost immediate growth inhibitory effect of *A. catenella* on *H. akashiwo* (Fig. 2; see also Yamasaki et al. 2011). Furthermore, several species of the genus *Alexandrium* have short-term allelopathic effects on heterotrophic, autotrophic, and photoautotrophic protists (Tillmann et al. 2007, 2008). Thus, our results suggest that the live-cell-mediated hemolytic activity may be linked to the growth inhibitory effect of *A. catenella* on *H. akashiwo*. Further studies are required to determine the structure of any hemolysin produced by *A. catenella* and to clarify how hemolytic activity is involved in the growth inhibition caused by *A. catenella*.

Allelopathic effects of *H. akashiwo* on *A. catenella*

To identify the factors affecting the interspecific interaction between *A. catenella* and *H. akashiwo*, hemolytic activity of *H. akashiwo* was investigated and allelochemicals produced by *H. akashiwo* show no hemolytic activity (Fig. 7). We then examined the effects of crude extracts, which for *H. akashiwo* include allelopathic polysaccharide–protein complexes (APPCs) (Yamasaki et al. 2009), on the growth of *A. catenella*. The exposure experiments using crude extracts from *H. akashiwo* cultures suggest that the extracts inhibited the growth of *A. catenella* (Fig. 8). Yamasaki et al. (2009) detected APPCs by dot-blot analysis of *H. akashiwo* cultures under axenic conditions at a density of 10^5 cells ml^{-1}, a density at which *H. akashiwo* can inhibit growth of the diatom *Skeletonema costatum* in the laboratory (Yamasaki et al. 2007). This supports the strong growth inhibitory effect on *A. catenella* that we observed when *H. akashiwo* reached high cell density in bi-algal cultures (Figs. 2 & 3). In our study, however, growth of *A. catenella* was inhibited only at relatively high con-

centrations of crude extract (>50 µg ml^{-1}; Fig. 8). Furthermore, APPCs are present in the glycocalyx (Yokote et al. 1985) on *H. akashiwo* cell surfaces, and their concentration in culture medium is around 40 µg ml^{-1} (Yamasaki et al. 2009). Therefore, cell contact with *H. akashiwo*, which would allow cells of *A. catenella* to contact more APPCs, appears to be a requisite condition for the strong growth inhibitory effects on *A. catenella* observed in our bi-algal culture experiment as well as in previous studies (Qiu et al. 2012, Dunker et al. 2017).

CONCLUSIONS

Our results from laboratory experiments using *Alexandrium catenella* and *Heterosigma akashiwo* indicate that the species first reaching stationary phase substantially suppresses the maximum cell densities attained by the second species. In addition, synergetic effects of stressors may play a strong role in the biological interaction between phytoplankton species because *A. catenella* was not inhibited by *H. akashiwo* under optimal growth conditions (salinity 30), but was negatively impacted when grown under salinity 20. Salinity might therefore be one important determining factor of a winner in terms of the species first reaching stationary phase, in addition to interspecific interactions caused by allelopathy and/or cell contact. Moreover, live-cell-mediated hemolytic activity might explain the growth inhibitory effect of *A. catenella* on *H. akashiwo*, whereas allelochemicals produced by *H. akashiwo* are likely responsible for the growth inhibition of *A. catenella*. Physical, chemical, and biological factors are all involved in phytoplankton succession and in the interspecific interactions between *A. catenella* and *H. akashiwo* in the field. It is therefore necessary to clarify the relationship between interspecific interactions and environmental factors in natural phytoplankton populations.

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