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Allelopathy of the raphidophyte *Heterosigma akashiwo* against the dinoflagellate *Akashiwo sanguinea* is mediated via allelochemicals and cell contact

Xuchun Qiu[1], Yasuhiro Yamasaki[2], Yohei Shimasaki[1,*], Hiroaki Gunjikake[1], Masato Honda[1], Mayumi Kawaguchi[1], Tadashi Matsubara[3], Sou Nagasoe[4], Takuya Etoh[5], Shigeaki Matsui[5], Tsuneo Honjo[6], Yuji Oshima[1]

[1]Laboratory of Marine Environmental Science, Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan

[2]National Fisheries University, 2-7-1 Nagata-Honmachi, Shimonoseki 759-6595, Japan

[3]Saga Prefectural Ariake Fisheries Research and Development Center, 2753-2 Nagata, Ashikari-cho, Ogi, Saga 849-0313, Japan

[4]Miyazu Station, National Center for Stock Enhancement, Fisheries Research Agency, 1721 Oda Shukuno, Miyazu, Kyoto 626-0052, Japan

[5]Fukuoka Fisheries and Marine Technology Research Center, 1141-1 Imazu, Nishi-ku, Fukuoka 819-0165, Japan

[6]Seto Inland Sea Regional Research Center, Kagawa University, Saiwaichou 1-1, Takamatsu, Kagawa 760-8521, Japan

ABSTRACT: Growth interactions between the raphidophyte *Heterosigma akashiwo* and the dinoflagellate *Akashiwo sanguinea* were examined by using bi-algal culture experiments under axenic conditions. There was a lethal effect of *H. akashiwo* (initial cell density: 1×10^2 or 1×10^4 cells ml^{-1}) on *A. sanguinea* at an initial cell density of 1×10^2 cells ml^{-1}. Growth of both species was suppressed concurrently when initial cell densities of *H. akashiwo* and *A. sanguinea* were 1×10^2 and 2×10^3 cells ml^{-1}, respectively. A mathematical model was used to simulate growth and interactions in bi-algal cultures. The model predicted that *H. akashiwo* would outcompete *A. sanguinea* over time. A cell-free enriched filtrate prepared from dense culture of *H. akashiwo* reduced both maximum growth rate and maximum yield of *A. sanguinea*. A filtrate of *A. sanguinea* prepared in the same manner only reduced maximum yield of *H. akashiwo* but did not affect its maximum growth rate. Growth of *A. sanguinea* in bi-algal cultures under contact conditions was significantly lower than that in mono-algal culture and in bi-algal cultures under non-contact conditions. Morphologically abnormal *A. sanguinea* cells occurred at high frequency only when *A. sanguinea* was cultured together with *H. akashiwo* under direct cell contact condition. Moreover, growth inhibition and formation of morphologically abnormal cells of *A. sanguinea* were induced in a concentration-dependent manner by allelochemicals (including allelochemical polysaccharide–protein complexes) produced by *H. akashiwo*. These results suggest that growth inhibition effects, via allelochemicals and direct cell contact by *Heterosigma akashiwo*, influence bloom formation of *Akashiwo sanguinea* in the field.

KEY WORDS: *Heterosigma akashiwo* · *Akashiwo sanguinea* · Allelopathy · Polysaccharide–protein complex · Cell contact · Growth interaction · Growth simulation

INTRODUCTION

The dinoflagellate *Akashiwo sanguinea* (Hirasaka) G. Hansen et Moestrup (synonyms: *Gymnodinium sanguineum* Hirasaka [Daugbjerg et al., 2000], *G. nelsonii* Martin [Martin, 1929], *G. splendens* Lebour [Lebour, 1925]) is a harmful algal bloom (HAB) species which has been observed in many coastal

*Corresponding author.
Email: simasaki@agr.kyushu-u.ac.jp

waters around the globe (Domingos & Menezes 1998, Gómez & Boicenco 2004, Kim et al. 2004, Lu & Hodgkiss 2004, Rines et al. 2010). *A. sanguinea* has been associated with mortality in abalone larvae and spat, and in seabirds (Botes et al. 2003, Jessup et al. 2009), and is also suspected to cause discoloration of the edible laver 'nori' in the Ariake Sea, Japan (Nakamura & Hirata 2006, Tsutsumi 2006).

Several physiological and ecological studies (Doucette & Harrison 1991, Voltolina 1993, Silva & Faust 1995, Kudela et al. 2008) have attempted to clarify the mechanisms of bloom formation by *Akashiwo sanguinea*. Matsubara et al. (2007) examined growth characteristics of *A. sanguinea* and suggested that this species has the potential to form blooms during early summer in coastal waters of Japan. However, during spring and early summer, which may be suitable for bloom formation of *A. sanguinea*, the phytoplankton community in the Ariake Sea is frequently dominated by alternative blooms of the raphidophyte *Heterosigma akashiwo* (Hada) Hada ex Hara et Chihara and some certain diatoms, whereas *A. sanguinea* rarely bloom although its vegetative cells have been detected at very low cell density (Tezaki et al. 2005, Tsutsumi 2006, Yamatogi et al. 2006). From the results of field observations over 3 yr, the phytoplankton community structure of the Ariake Sea in autumn was characterized by persistent dominance of *A. sanguinea* (Nakamura & Hirata 2006).

In nature, biological, physical or chemical factors are involved in the bloom formation of phytoplankton. Grazing impact (Johansson & Coats 2002, Smayda 2008) and interspecific growth interactions (Poulson et al. 2009) are major factors affecting the population of phytoplankton. Nakamura & Hirata (2006) reported that *Akashiwo sanguinea* has the ability to escape from grazing pressure and light limitation and could dominate in the Ariake Sea. Similarly, it has been suggested that the predation pressure of microbial loop components or macrograzers is not a significant factor in bloom termination of *Heterosigma akashiwo* because of the broad spectrum allelopathy of this species (Smayda 1998). Therefore, the discrepancy between the laboratory and field observations suggests that interspecific interactions with *H. akashiwo* may affect the survival and growth of *A. sanguinea* in the field during spring and early summer.

Interspecific interactions caused by allelopathy or cell contact have been discussed as a phenomenon affecting phytoplankton bloom formation. Legrand et al. (2003) and Granéli & Hansen (2006) reviewed allelopathy in phytoplankton, and Uchida (2001) reported the role of cell contact in some dinoflagellate species. More recently, Prince et al. (2008) reported that the dinoflagellate *Karenia brevis* exudes allelochemicals during blooms, and may achieve nearly monospecific blooms by growth suppression or death through lowering the photosynthetic efficiency and increasing the membrane permeability of their competitors. In addition, Paul et al. (2009) investigated the role of allelopathic interactions between diatom species, and indicated that chemical cross-talk between the species might lead to physiological responses and changed cell abundances. Furthermore, Tang & Gobler (2010) raised the possibility that toxicity to fish and shellfish larvae, and allelopathic effects by the dinoflagellate *Cochlodinium polykrikoides* were caused by the same agents such as reactive oxygen species (ROS)-like chemicals.

As for other species also, these interspecific interactions have been reported to affect bloom formation by *Akashiwo sanguinea* (Kayser 1979, Yamasaki et al. 2007a, Matsubara et al. 2008). In spring and summer, allelopathy of centric diatom blooms is an important factor that inhibits the growth of *A. sanguinea* (Matsubara et al. 2008). Recently, Yamasaki et al. (2009) reported that allelopathic polysaccharide–protein complexes (APPCs) produced by *Heterosigma akashiwo* inhibited the growth of the diatom *Skeletonema costatum*, as the APPCs concentrations in the water exceeded the threshold of allelopathic potential. Thus, it is reasonable to propose that the allelopathic effect of *H. akashiwo* blooms during spring and early summer plays an important role in bloom formation of *A. sanguinea*.

In this study, we conducted bi-algal culture experiments under axenic conditions using several initial cell density combinations of *Heterosigma akashiwo* and *Akashiwo sanguinea*. We also simulated the growth of either species in bi-algal cultures using a mathematical model to quantify growth relationships between them. In addition, we examined allelopathic interactions between *H. akashiwo* and *A. sanguinea* by way of growth experiments using culture filtrates. Furthermore, we examined effects of direct cell contact with *H. akashiwo* on the growth and cell morphology of *A. sanguinea* through bi-algal culture experiments under non-contact and contact conditions. Finally, we tested inhibitory effects of allelochemicals fractions (including APPCs) produced by *H. akashiwo* on *A. sanguinea* growth.

MATERIALS AND METHODS

Algal species and culture conditions

Axenic strains of *Heterosigma akashiwo* (NIES-10) and *Skeletonema* sp. (NIES-324) were obtained from the National Institute of Environmental Studies (NIES, Japan). *Akashiwo sanguinea* used in this study was isolated in November 2002 from Hakozaki Harbor in Hakada Bay, Japan (Matsubara et al. 2007). Both strains were verified as axenic using 4′,6-diamidino-2-phenylindole (DAPI) staining for testing on bacterial contamination (Porter & Feig 1980). Axenic cultures were maintained in 70 ml sterile flasks (Nunc, Thermo Fisher Scientific) containing 20 ml of modified sea water medium (modified SWM-3, Yamasaki et al. 2007b) at a salinity of 30. The modified SWM-3 medium contained 0.04% (w/v) of tris(hydroxymethyl)-aminomethane (Wako Pure Chemical Industries) to buffer pH during the experiments, and the medium was autoclaved before use (121°C, 15 min). All flasks were kept in an incubator (FLI-160, Tokyo Rikakikai) at 25°C under 250 ± 8 µmol photons m^{-2} s^{-1} of cool-white fluorescent illumination at a 12:12 h light:dark cycle. Irradiance in the incubator was measured using a Quantum Scalar Laboratory Irradiance Sensor (QSL-2101, Biospherical Instruments).

Bi-algal culture experiments

Bi-algal culture experiments were conducted in 70 ml sterile flasks (Nunc) containing 20 ml of the modified SWM-3 medium. *Heterosigma akashiwo* and *Akashiwo sanguinea* cells in early stationary phase were inoculated at cell densities of 1×10^2, 2×10^3 and 1×10^4 cells ml^{-1} (see Table 1). In addition, either species was inoculated at the same initial cell densities in mono-algal culture as controls. For each treatment, 3 replicate flasks were prepared. All flasks were gently mixed by hand twice a day and randomly rearranged to minimize the effects of light or temperature heterogeneity in the incubator. Every other day, the pH of each culture medium was measured (B-212 pH meter), and 100 µl subsamples were taken for counting the cells under a light microscope. When cell densities exceeded 2×10^4 cells ml^{-1}, subsamples were diluted by a factor of 10 to 20 with fresh modified SWM-3 medium before counting.

Macronutrient analysis

At the end of the bi-algal experiments, 10 ml of each culture was gravity-filtered through glass microfibre filters (GF/C, Whatman). Filtrates were then passed through 0.22 µm syringe filters and frozen at −80°C until analysis. Nitrogen (NO_2^- + NO_3^-) and phosphorus (PO_4^{3-}) were measured using an auto analyzer (TRACCS 800, Bran & Luebbe) after samples were diluted 10× with Milli-Q water (Millipore).

Simulation of growth in bi-algal cultures

To model the cell growth in bi-algal cultures of *Heterosigma akashiwo* and *Akashiwo sanguinea*, we adopted the growth simulation of Uchida et al. (1999). The following equations were used for the simulation:

$$dx/dt = r_x x(1 - x/K_x) - Axy = r_x x[1 - (x + ay)/K_x] \quad (1)$$

$$dy/dt = r_y y(1 - y/K_y) - Bxy = r_y y[1 - (bx + y)/K_y] \quad (2)$$

Here, x and y are the cell densities of the 2 species in bi-algal culture, r_x and K_x are the growth rate and carrying capacity of *A. sanguinea* in mono-algal culture, respectively, and r_y and K_y are the corresponding parameters for *H. akashiwo*. Parameter A measures the degree of inhibition of *A. sanguinea* by *H. akashiwo*, and B measures the inhibition of *H. akashiwo* by *A. sanguinea*. If we set $A = ar_x/K_x$ and $B = br_y/K_y$, Eqs. (1) & (2) become the same as the formula for the growth of populations competing with each other for limited resources (Iwasa 1998). Parameters a and b are nondimensional and measure the degree of inhibition by the other species compared to self-interference. When each species is cultured in mono-algal culture, we can set $a = b = 0$.

The logistic parameters (r_x, r_y, K_x, K_y) were estimated by Eqs. (1) & (2) using the mono-algal culture data from bi-algal cultures when *Akashiwo sanguinea* and *Heterosigma akashiwo* cells were inoculated to a initial density of 1×10^2 cells ml^{-1}, respectively (n = 3). Next, the parameters a and b were calculated directly from Eqs. (1) & (2) using the data from bi-algal culture experiments. Precise estimation of a and b was carried out using the Marquardt method (Marquardt 1963), with the most appropriate values of a and b determined by when the difference between the squared values of observed cell density and theoretical cell density reached a minimum. Then, the parameters A and B were calculated from the equations $A = ar_x/K_x$ and $B = br_y/K_y$, respectively.

Effect of culture filtrates on growth of each species

As soon as cell densities (cells ml^{-1}) reached 5×10^5 for *Heterosigma akashiwo*, or 1.5×10^4 for *Akashiwo sanguinea*, these cultures were passed through glass microfibre filters (GF/C). To compensate nutrients consumed by algal growth in filtrates, the same amount of nutrients as original modified SWM-3 medium were added to either filtrate (re-enriched) and the final nutrient concentrations of filtrates were expected to be between 100 to 200% of the original modified SWM-3 medium. Thus, both species were cultured alone in modified SWM-3 medium with nutrient concentration elevated to 200% of the original medium concentration (re-enriched medium) to evaluate any nutrient inhibitory effect in re-enriched filtrates. As a control, both species were cultured alone in modified SWM-3 medium (original medium). The pH of each filtrate was adjusted to 7.8–8.0 with 2 N HCl; subsequently, all media (filtrates and controls) were passed through 0.22 µm syringe filters (Millipore).

For the growth experiment in culture filtrates, *Heterosigma akashiwo* and *Akashiwo sanguinea* were cultured separately in 8 ml sterile culture tubes (Evergreen Scientific) containing 5 ml of a prepared test media. Initial cell density of either species was 1×10^2 cells ml^{-1}, with 4 replicate tubes for each treatment. Relative cell abundances were measured daily by in vivo fluorescence (10-AU-005-CE fluorometer, Turner Designs), and growth rates during the exponential growth phase were calculated after Guillard (1973).

Bi-algal culture experiments under non-contact and contact conditions

The experimental design of separating 2 algae cultures by a dialysis membrane has been used to differentiate between allelopathy due to allelochemical molecules and cell contact effect (e.g. Yamasaki et al. 2007a,b, Tang & Gobler 2010), and to monitor the physiological response of the interactions between 2 diatom species approached with metabolic profiling (Paul et al. 2009). For our experiment, we used plates with 6 wells and inserted cell culture plates (BD35-3091, Becton-Dickinson), as described in Yamasaki et al. (2007b). The insert plates have a membrane filter (pore size 3.0 µm) on the bottom. *Akashiwo sanguinea* cells in early stationary phase were suspended in modified SWM-3 medium at a density of 267 cells ml^{-1}, and 3 ml of cell suspension were inoculated into each inner chamber (final density 1×10^2 cells ml^{-1}). *Heterosigma akashiwo* cells in early stationary phase were suspended in modified SWM-3 medium at 2 cell densities (160 and 1.6×10^4 cells ml^{-1}), and 5 ml were inoculated into the outer chambers of the well plates (final densities 1×10^2 and 1×10^4 cells ml^{-1}). For comparison, *H. akashiwo* and *A. sanguinea* were cultured together in the well plates without the insert plates in the same combinations just described, allowing direct cell contact. As a control, *A. sanguinea* (final cell density 1×10^2 cells ml^{-1}) was cultured in fresh modified SWM-3 medium. For each treatment, 3 replicate wells were used. The morphology of *A. sanguinea* cells was observed 4 times per day using an inverted microscope. On Day 8 after incubation, the numbers of morphologically normal and abnormal cells of *A. sanguinea* in each well were counted microscopically (three 100 µl subsamples per well).

Growth inhibitory effect of allelochemicals produced by *Heterosigma akashiwo*

The fraction containing allelochemicals was prepared following Yamasaki et al. (2009). *H. akashiwo* was inoculated at a density of 1×10^2 cells ml^{-1} into 100 ml glass flasks (n = 3) containing 50 ml of modified SWM-3 medium. After 20 d, 50 ml samples from each of the 3 culture flasks (density 5.6×10^5 cells ml^{-1}) were combined to make up a total volume of 150 ml, which was gravity filtered through a membrane filter (5.0 µm pore size, Millipore). The filtrate was passed through another membrane filter (0.45 µm pore size, Millipore). Then, the filtrate was dialysed against deionized water for 3 d at 4°C using dialysis membranes with a 3500 Da molecular weight cut-off (Spectrum Laboratories). After the dialysis, the inner solution was frozen at −80°C and lyophilized with a Free Zone 2.5 freeze-drying apparatus (Labconco). A portion of the lyophilized allelochemicals obtained from filtrates of *H. akashiwo* cultures was dissolved in 10 ml of modified SWM-3 medium (final concentration 200 µg ml^{-1}), and passed through a 0.22 µm syringe filter (Millipore). To confirm whether the sample contained APPCs or not, its inhibitory effect was tested on *Skeletonema* sp. (Fig. 1A). In addition, the sample was analysed by SDS-PAGE on 5% gels according to Laemmli (1970). After electrophoresis, the extremely large molecular sizes (i.e. APPCs) of the allelochemicals, in addition to other minor bands, were visualized by silver staining the SDS-PAGE gel (Fig. 1B). Thus, it was consid-

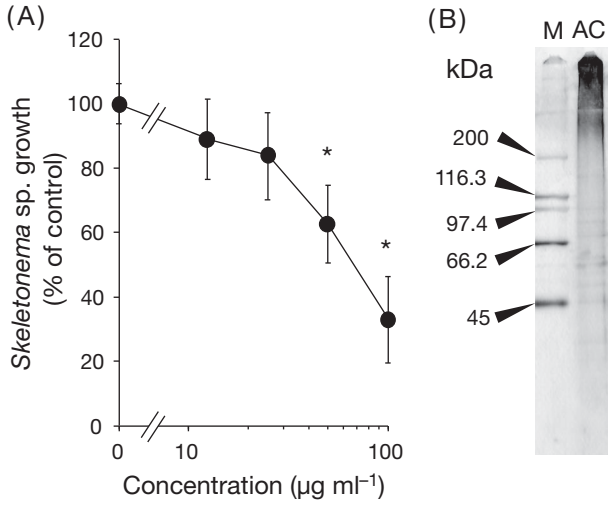

Fig. 1. *Heterosigma akashiwo*. Allelochemicals including allelophobic polysaccharide–protein complexes prepared from culture medium filtrates. (A) Concentration-dependent growth inhibition of *Skeletonema* sp. by *H. akashiwo* allelochemicals. (B) SDS-PAGE of the allelochemicals fraction using 5% polyacrylamide gel. After electrophoresis, the gel was silver stained. Lane M: molecular mass standard markers. Lane AC: the allelochemicals fraction prepared from the culture. *: significant difference between growth of *Skeletonema* sp. exposed to allelo-chemicals and controls ($p < 0.01$)

ered proven that the sample prepared from *H. akashiwo* filtrate contained APPCs (along with other substances).

Akashiwo sanguinea cultures in the late exponential growth phase were diluted to 1×10^3 cells ml^{-1} with modified SWM-3 medium. Of this cell supension, 100 µl were added to 900 µl of each sample solution including allelochemicals of different concentrations to be tested in 48-well plates (initial cell density 1×10^2 cells ml^{-1}). Final concentrations of the allelochemicals fraction in bioassays were 12.5, 25, 50, 100, and 200 µg ml^{-1}. Each bioassay had 3 replicates. As a control, *A. sanguinea* was cultured in modified SWM-3 medium without the addition of any sample solution. After incubation for 4 d, the cells in each of five 10 µl subsamples from each well were counted under the microscope.

Statistical analyses

The experimental data were checked for homogeneity of variance across treatments with Levene's test. When variances were homogeneous, the differences in cell densities among treatments (see Fig. 5A) and proportions of morphologically abnormal cells (see Table 3) of *Akashiwo sanguinea* were tested using 1-way analysis of variance (1-way ANOVA) followed by Tukey test; and the differences in the maximum growth rates and maximum yields between treatments and control (see Fig. 4A,D) were tested using 1-way ANOVA followed by Dunnett's test. When data were heteroscedastic, the differences in cell densities of *A. sanguinea* between treatments and control (see Figs. 1A & 6A) were tested using a non-parametric Mann-Whitney *U*-test. These statistical analyses were conducted by using the Statistical Package for the Social Sciences software (SPSS 13.0; SPSS).

RESULTS

Bi-algal culture experiment

With initial cell densities of 1×10^2 cells ml^{-1} for both *Heterosigma akashiwo* and *Akashiwo sanguinea* (Fig. 2A), the growth of *A. sanguinea* was suppressed from Day 4 onwards. Cell density of *A. sanguinea* started to decline at Day 8 when *H. akashiwo* reached the stationary phase, and *A. sanguinea* had almost disappeared by Day 14 at the end of the experiment. In contrast, the growth of *H. akashiwo* in both bi- and mono-algal cultures was almost identical. The pH of the culture medium at these initial cell densities ranged from 7.82 to 8.72 (Table 1).

When initial cell densities of *Heterosigma akashiwo* and *Akashiwo sanguinea* were 1×10^4 and 1×10^2 cells ml^{-1}, respectively (Fig. 2B), there was no growth of *A. sanguinea* and this species almost disappeared on Day 8 when *H. akashiwo* reached the stationary phase, while the growth of *H. akashiwo* in both bi- and mono-algal cultures was virtually the same. The pH of the culture medium at these initial cell densities ranged from 7.92 to 8.72 (Table 1).

When initial cell densities of *Heterosigma akashiwo* and *Akashiwo sanguinea* were 1×10^2 and 2×10^3 cells ml^{-1}, respectively (Fig. 2C), the growth of *A. sanguinea* was suppressed from Day 8 onwards, after which its average maximum cell density was ~24% of that in mono-algal cultures. On the other hand, the growth rate of *H. akashiwo* was lowered from Day 4 onwards; however, the average cell density of *H. akashiwo* in bi-algal culture increased and reached at same level of mono-algal culture on Day 12. The average maximum cell density of *H. akashiwo* was ~48% of that in mono-algal culture. The pH of culture medium at these initial cell densities ranged from 7.90 to 8.59 (Table 1).

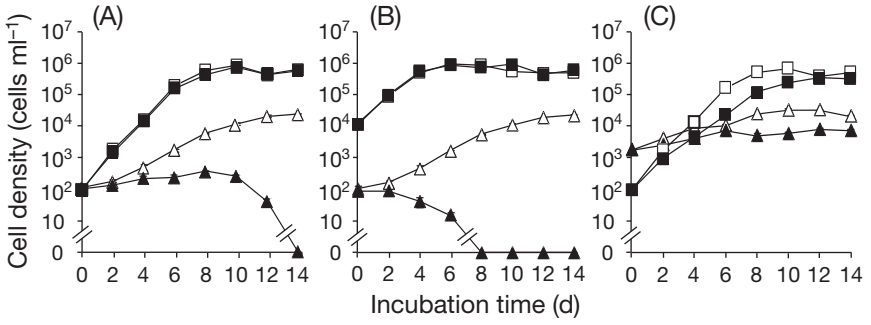

Fig. 2. *Akashiwo sanguinea* and *Heterosigma akashiwo*. Growth of *A. sanguinea* when cultured alone (△) or together with *H. akashiwo* (▲), and of *H. akashiwo* when cultured alone (□) or together with *A. sanguinea* (■). Initial cell densities (cells ml^{-1}) of *A. sanguinea*: 1×10^2 (A,B) or 2×10^3 (C), and of *H. akashiwo*: 1×10^2 (A,C) or 1×10^4 (B). Means ± SD (n = 3). Error bars are small and mostly obscured by the symbols

Table 1. *Heterosigma akashiwo* and *Akashiwo sanguinea*. Initial cell densities, final macronutrient concentrations and pH ranges in culture media during mono- and bi-algal culture experiments with the 2 species. Data are mean ± SD (n = 3). Initial macronutrient concentrations were 1055 µmol l^{-1} for $NO_2^- + NO_3^-$ and 52 µmol l^{-1} for PO_4^{3-}

Species	Initial cell density (cells ml^{-1})	Final macronutrient concentrations (µmol l^{-1}) $NO_2^- + NO_3^-$	PO_4^{3-}	pH range in culture media Lowest	Highest
Mono-algal					
A. sanguinea	1×10^2	4.69 ± 2.06	14.26 ± 1.18	7.82 ± 0.04	8.28 ± 0.01
	2×10^3	2.89 ± 1.65	7.75 ± 0.12	7.82 ± 0.02	8.62 ± 0.01
H. akashiwo	1×10^2	3.21 ± 0.99	0.79 ± 0.14	7.94 ± 0.01	8.51 ± 0.01
	1×10^4	2.75 ± 0.17	0.53 ± 0.14	7.93 ± 0.01	8.64 ± 0.02
Bi-algal					
A. sanguinea	1×10^2	2.67 ± 0.28	1.06 ± 0.02	7.82 ± 0.02	8.72 ± 0.02
H. akashiwo	1×10^2				
A. sanguinea	1×10^2	2.51 ± 0.50	0.77 ± 0.21	7.92 ± 0.01	8.72 ± 0.03
H. akashiwo	1×10^4				
A. sanguinea	2×10^3	2.78 ± 0.67	4.42 ± 1.07	7.90 ± 0.07	8.59 ± 0.06
H. akashiwo	1×10^2				

On Day 14, in all cultures nitrogen ($NO_2^- + NO_3^-$) and phosphorus (PO_4^{3-}) concentrations ranged from 2.5 to 4.7 µmol l^{-1} and from 0.5 to 14.3 µmol l^{-1}, respectively (Table 1).

Growth simulation of bi-algal cultures

The values of all parameters of the growth simulation are shown in Table 2. Importantly, growth patterns of *Heterosigma akashiwo* and *Akashiwo sanguinea* predicted using these values were similar to those observed in bi-algal culture experiments (Fig. 3). The parameter values in Table 2 were also used to calculate isoclines (where $dx/dt = 0$ and $dy/dt = 0$) and population trajectories of the 2 species under various initial cell densities (Fig. 4). All simulated trajectories pass through 2 of the following 3 stages: (1) cell densities of both *H. akashiwo* and *A. sanguinea* increase (Fig. 4, area I); (2) cell densities of *H. akashiwo* increase but those of *A. sanguinea* decrease (Fig. 4, area II), and (3) cell densities of both *H. akashiwo* and *A. sanguinea* decrease (Fig. 4, area III).

Table 2. *Akashiwo sanguinea* and *Heterosigma akashiwo*. Estimated parameters for bi-algal model simulation between the 2 species. Parameters *a* and *b* are dimensionless. For details and definitions of *A* and *B*, see 'Simulation of growth in mixed algal cultures' under 'Materials and methods'

Species	Carrying capacity (K, cells ml^{-1})	Growth rate (r) (divisions h^{-1})	(divisions d^{-1})	Interaction rate a/b	A/B (ml cells^{-1} s^{-1})
A. sanguinea	27 299	0.023	0.547	0.209	4.9×10^{-11}
H. akashiwo	540 009	0.052	1.27	18.8	5.2×10^{-10}

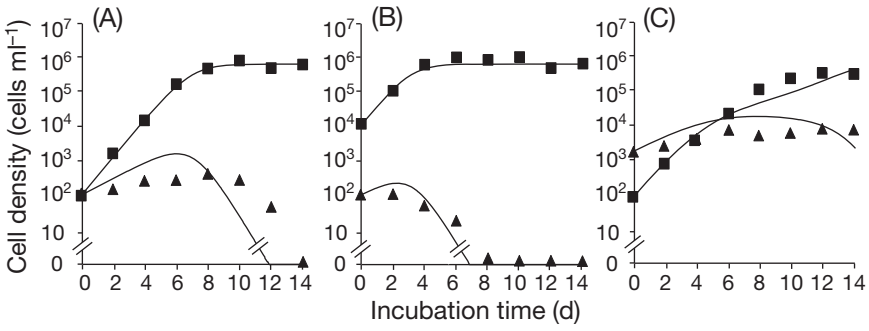

Fig. 3. *Heterosigma akashiwo* (■) and *Akashiwo sanguinea* (▲). Growth simulation in bi-algal cultures under various combinations of initial cell densities (cells ml^{-1}). (A) *A. sanguinea* 1×10^2, *H. akashiwo* 1×10^2. (B) *A. sanguinea* 1×10^2, *H. akashiwo* 1×10^4. (C) *A. sanguinea* 2×10^3, *H. akashiwo* 1×10^2. Symbols: actual data from bi-algal cultures. Lines: simulated growth curves

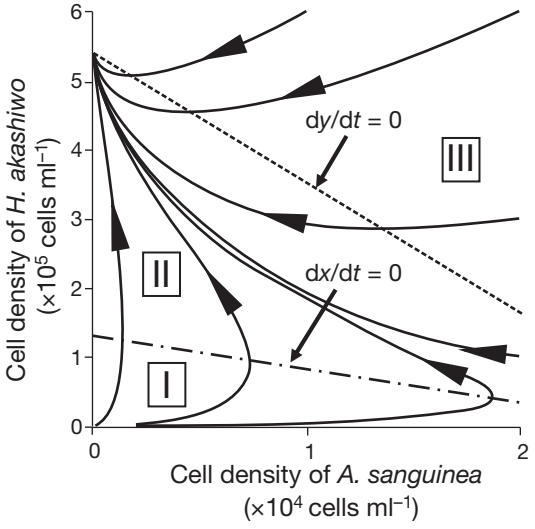

Fig. 4. *Heterosigma akashiwo* and *Akashiwo sanguinea*. Simulation of bi-algal cultures showing isoclines (where $dx/dt = 0$ and $dy/dt = 0$) and trajectories of algal populations cultured under various initial cell densities. Area I: cell densities of both *H. akashiwo* and *A. sanguinea* increase. Area II: cell densities of *H. akashiwo* increase but those of *A. sanguinea* decrease. Area III: cell densities of both *H. akashiwo* and *A. sanguinea* decrease. Regardless of the initial cell densities, *H. akashiwo* outcompetes *A. sanguinea* over time

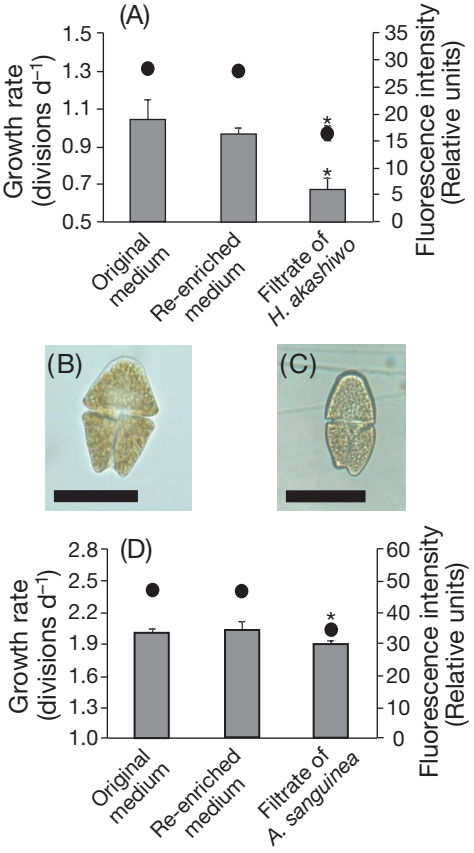

Fig. 5. *Heterosigma akashiwo* and *Akashiwo sanguinea*, allelopathic interactions. (A) *H. akashiwo* culture medium filtrate affects growth of *A. sanguinea*. *A. sanguinea* cell (B) from mono-algal culture (control) and (C) exposed to the re-enriched filtrate from *H. akashiwo* culture. Scale bars = 50 µm. (D) *A. sanguinea* culture filtrate affects growth of *H. akashiwo*. Means ± SD (n = 3). Grey bars: maximum growth rates. ●: maximum yield of the 2 species as measured by *in vivo* chlorophyll fluorescence. *: values significantly different ($p < 0.01$) from controls

Effect of culture filtrates on phytoplankton growth

The filtrate from *Heterosigma akashiwo* cultures significantly decreased the growth of of *Akashiwo sanguinea* (maximum growth rate to 64% and maximum yield to 57% of the control; Dunnett's test, $p < 0.01$) (Fig. 5A). In addition, some *A. sanguinea* cells developed a minor morphological abnormality when they were exposed to filtrate of *H. akashiwo* (compare Fig. 5B,C). The filtrate from *A. sanguinea* cultures decreased the maximum yield of *H. akashiwo* to 73% of the control (Dunnett's test, $p < 0.01$), but did not significantly affect its maximum growth rate (Fig. 5D).

Maximum growth rates and maximum yields of the 2 species cultured in re-enriched media were similar to that of cells cultured in the original modified SWM-3 medium (Fig. 5), demonstrating that nutrient concentrations in re-enriched filtrates were not responsible for observed inhibitory effects.

Bi-algal culture experiments under non-contact and contact conditions

Regardless of differences in initial *Heterosigma akashiwo* cell density, growth of *Akashiwo sanguinea* in bi-algal cultures under contact conditions was significantly lower than that in bi-algal cultures under non-contact conditions and in mono-algal culture (Tukey test, $p < 0.05$, Fig. 6A). When the initial cell density of *H. akashiwo* was 1×10^4 cells ml^{-1}, growth of *A. sanguinea* in bi-algal culture under non-contact condition was significantly lower than in mono-algal culture (Tukey test, $p < 0.01$, Fig. 6A).

During our experiments, most *Akashiwo sanguinea* cells in mono-algal culture and in bi-algal cultures under non-contact conditions maintained the normal cell shape (Fig. 6B,C) as described by Tomas et al. (1997), and the frequency of morphologically abnormal cells was very low during culture period. On Day 8 there was no significant difference in the proportion of morphologically abnormal cells of *A. sanguinea* between mono-algal and bi-algal cultures under non-contact conditions (Table 3). However, in bi-algal cultures under contact conditions, many morphologically abnormal cells of *A. sanguinea* (Fig. 6D) appeared after Day 2 (initial density of *H. akashiwo* 1×10^4 cells ml^{-1}) and 6 (initial density of *H. akashiwo* 1×10^2 cells ml^{-1}), and the proportion of morphologically abnormal cells was significantly higher than that in mono-algal culture (Tukey test, $p < 0.01$, Table 3) and in bi-algal cultures under non-contact conditions (Tukey test, $p < 0.01$, Table 3) on Day 8. There were no morphological changes in the cells of *Heterosigma akashiwo* through the experimental period (data not shown).

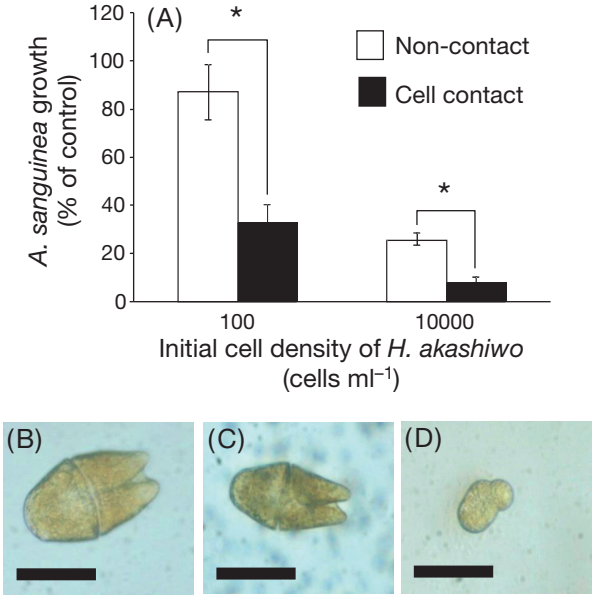

Fig. 6. *Heterosigma akashiwo* and *Akashiwo sanguinea*. Growth inhibitory effects in bi-algal cultures under non-contact and contact conditions. (A) Growth of *A. sanguinea* cultured together with *H. akashiwo* under non-contact and contact conditions. Means ± SD (n = 3). *: significant difference between growth under non-contact and contact conditions ($p < 0.05$). *A. sanguinea* cell (B) from mono-algal culture (control) and cultured together with *H. akashiwo* under (C) non-contact or (D) contact conditions. Scale bars = 50 µm

Growth inhibitory effect of allelochemicals produced by *Heterosigma akashiwo*

To determine whether APPCs produced by *Heterosigma akashiwo* (Yamasaki et al. 2009) affect growth of *Akashiwo sanguinea*, we examined growth inhibitory effects of the allelochemicals fraction prepared from culture filtrate of *H. akashiwo*. The growth of *A. sanguinea* was inhibited by allelochemicals in a concentration-dependent manner (Fig. 7A; Mann-Whitney *U*-test, $p < 0.05$). Furthermore, many morphologically abnormal cells appeared when *A. sanguinea* was exposed to concentrations of allelochemicals >100 µg ml^{-1} (Fig. 7C).

Table 3. *Heterosigma akashiwo* and *Akashiwo sanguinea*. Initial cell densities of the 2 species and proportions of morphologically abnormal cells of *A. sanguinea* on Day 8 for bi-algal culture experiments under non-contact and contact conditions. Data are mean ± SD (n = 3). Different lower case superscripts indicate significantly different means (Tukey test, $p < 0.01$)

Initial cell density (cells ml^{-1})		Proportion of abnormal *A. sanguinea* cells on Day 8 (%)
A. sanguinea	*H. akashiwo*	
Mono-algal culture		
1×10^2	0	3.60 ± 0.92^a
Non-contact		
1×10^2	1×10^2	5.54 ± 1.24^a
1×10^2	1×10^4	4.75 ± 2.32^a
Cell contact		
1×10^2	1×10^2	22.60 ± 5.03^b
1×10^2	1×10^4	73.89 ± 6.74^c

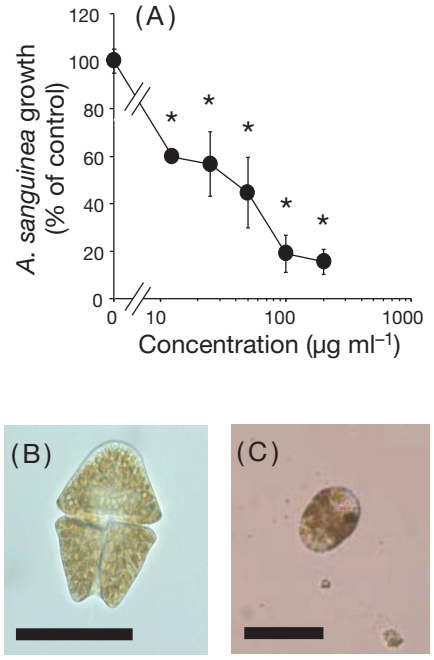

Fig. 7. *Heterosigma akashiwo* and *Akashiwo sanguinea*. Growth inhibitory effects of allelochemicals prepared from *H. akashiwo* culture medium filtrates. (A) Concentration-dependent growth inhibition of *A. sanguinea* by *H. akashiwo* allelochemicals. *: significant growth differences between exposed *A. sanguinea* and controls ($p < 0.01$). *A. sanguinea* cell (B) from mono-algal culture (control) and (C) exposed to allelochemicals. Scale bars = 50 µm

DISCUSSION

The physiological and ecological features of *Heterosigma akashiwo* and *Akashiwo sanguinea* suggest that both species have the potential to bloom during early summer (Honjo 1992, 1993, Smayda 1998, Matsubara et al. 2007). In reality, however, *H. akashiwo* often blooms, while *A. sanguinea* does not dominate in the field during that period. In the laboratory, we observed a strong growth inhibitory effect of *H. akashiwo* on *A. sanguinea* under the absence of various factors affecting phytoplankton growth such as grazing, turbulence, or nutrient limitation. Though further substantial research is required in the field, our results imply that *H. akashiwo* may have survival advantage over *A. sanguinea* through its growth inhibitory effects.

Previous studies demonstrated that nutrient limitation or elevation of pH values in media may affect growth interactions between phytoplankton (Hansen 2002, Fistarol et al. 2005). In the present bi-algal cultures, the concentrations of nitrogen ($NO_2^- + NO_3^-$) and phosphorus (PO_4^{3-}) decreased to <2.78 µmol l^{-1} and <4.42 µmol l^{-1}, respectively, on Day 14, and media pH values ranged from 7.8 to 8.7 over the 14 d incubations (Table 1); that is, macronutrient concentration and pH value in mono-algal and bi-algal cultures were similar during the experiment. However, in bi-algal cultures growth of *Akashiwo sanguinea* was strongly suppressed immediately after the start of the experiments (Fig. 2), while in mono-algal cultures *A. sanguinea* was able to grow well when medium pH value reached ~8.6 (Table 1). Therefore, it seems that neither macronutrient limitation nor pH change in our culture media was a main factor inducing growth inhibitory effects of *Heterosigma akashiwo* on *A. sanguinea*.

The model obtained from the results of bi-algal cultures indicated that *Heterosigma akashiwo*, which has a much higher growth rate (Table 2), will always outcompete *Akashiwo sanguinea* over time because the estimated parameters show that $K_x < aK_y$ and $bK_x < K_y$ (Iwasa 1998). It can be concluded that *H. akashiwo* will always outcompete *A. sanguinea* because the trajectories of algal populations always converge on the point [(*A. sanguinea*, *H. akashiwo*) = (0, K_y)] (Fig. 4) despite different combinations of their initial cell densities in bi-algal cultures. For example, in a case where initial cell densities of *H. akashiwo* and *A. sanguinea* are 1×10^2 and 2×10^3 cells ml^{-1}, respectively, cell densities of both *H. akashiwo* and *A. sanguinea* increase (Fig. 4, area I). Next, cell densities of *H. akashiwo* increase, but those of *A. sanguinea* decrease (Fig. 4, area II). Finally, cell densities of the 2 species converge on the point [(*A. sanguinea*, *H. akashiwo*) = (0, K_y)] (Fig. 4). Even though parameters used here for model predictions were derived from well-defined culture conditions (e.g. water temperature, light intensity, photoperiod, salinity, and artificially high nutrient levels), the growth inhibitory effect of *H. akashiwo* should be considered as a potentially important mechanism explaining the rarity of spring and early summer blooms of *A. sanguinea* in coastal waters of western Japan (Nakamura & Hirata 2006, Tsutsumi 2006, Matsubara et al. 2008).

Though growth of *Akashiwo sanguinea* was inhibited by *Heterosigma akashiwo* via allelochemicals dissolved in the filtrate (Fig. 5), its effect was weaker than that in bi-algal culture experiments (lethal effects, see Fig. 2). Similar phenomena have been observed in previous studies (e.g. Wang & Tang 2008, Tang & Gobler 2010), and may be explained by lack of continuous supply of the allelochemicals (Fistarol et al. 2005) or effects of direct cell contact (Uchida 2001). Several recent studies, however, throw doubt on the significance of allelopathy in the

field; that is, the relative importance of allelopathy in a species-rich ecological community may not be as high as predicted from laboratory studies (Suikkanen et al. 2005, Poulson et al. 2010). For example, *A. sanguinea* growth was inhibited by an extract of *Karenia brevis* cultures but not by waterborne compounds from *K. brevis* blooms (Prince et al. 2008, Poulson et al. 2010). Similarly, Jonsson et al. (2009) used simple models to analyze whether allelopathic effects may play a significant role under field conditions, and proposed that formation of HABs cannot be explained by allelopathy, whereas they pointed out the necessity to explore the presence of chemically mediated aggressive cell–cell interaction among phytoplankton. When growth inhibitory effects on *A. sanguinea* by *H. akashiwo* under conditions with and without cell contact were examined, effects of allelopathy and direct cell contact with *H. akashiwo* maximize growth inhibitory effects and induced formation of many morphologically abnormal cells in *A. sanguinea* (Fig. 6, Table 3). This suggests that compounds on the cell surface of *H. akashiwo* may play important roles in growth inhibition and formation of abnormal cells in *A. sanguinea*.

The exposure experiments using chemicals including APPCs produced by *Heterosigma akashiwo* suggested that allelopathic effects caused by APPCs strongly inhibited *Akashiwo sanguinea* growth (Fig. 7). Yamasaki et al. (2009) detected APPCs by dot-blot analysis of *H. akashiwo* cultures under axenic conditions at a density of 1×10^5 cells ml^{-1}. At this cell density, *H. akashiwo* can inhibit the growth of *Skeletonema costatum* (Yamasaki et al. 2007b). This supports the observed strong growth inhibitory effect on *A. sanguinea* when *H. akashiwo* reached high cell density in bi-algal cultures (Fig. 2). Furthermore, Yamasaki et al. (2009) reported that the concentration (estimated from dot-blot analysis) of APPCs dissolved in natural red-tide seawater was ~40 µg ml^{-1}, at which *A. sanguinea* growth was significantly inhibited (Fig. 6A). This is indicative of *H. akashiwo* APPCs being one important factor to inhibit *A. sanguinea* growth in the field. Yamasaki et al. (2009) reported that APPCs are present in the glycocalyx (Yokote et al. 1985) on *H. akashiwo* cell surfaces, and that their concentration dissolved in the media was ~40 µg ml^{-1}. In our study, only extremely high concentrations of allelochemicals including APPCs (>100 µg ml^{-1}) could induce the formation of many morphologically abnormal cells of *A. sanguinea* (Fig. 6). Furthermore, only a few morphologically abnormal cells of *A. sanguinea* occurred in bi-algal cultures under non-contact conditions (allows continuous supply of the allelochemicals of *H. akashiwo*). Hence, direct cell contact with *H. akashiwo*, which allows *A. sanguinea* to contact more APPCs, is a requisite condition for the strong growth inhibitory effects on *A. sanguinea* associated with formation of many morphologically abnormal cells.

CONCLUSIONS

Heterosigma akashiwo may have a competitive advantage over *Akashiwo sanguinea* for bloom formation in the field. Both allelopathy and direct cell contact can induce growth inhibition effects of *H. akashiwo* on *A. sanguinea*. Though the molecular mechanism of growth inhibition is still unclear, the APPCs released into the media (i.e. allelopathy) and those existing on the cell surface of *H. akashiwo* (i.e. cell contact effects) may play important roles in the growth inhibition of *A. sanguinea*. Further studies with purified APPCs from *H. akashiwo* will clarify the molecular mechanism of growth inhibition of *A. sanguinea* by *H. akashiwo*, and further experiments in the natural environment (e.g. species-rich ecological community under oligotrophic conditions) should clarify its role in bloom formations in the field.

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