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Allelopathic interactions between the bacillariophyte *Skeletonema costatum* and the raphidophyte *Heterosigma akashiwo*

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ABSTRACT: We investigated growth interactions between *Skeletonema costatum* (Greville) Cleve and *Heterosigma akashiwo* (Hada) Hada ex Hara et Chihara using bi-algal cultures under axenic conditions. When inoculated at high cell densities, growth of both species was coincidentally suppressed. In other combinations of inoculation density, the species first reaching stationary phase substantially reduced maximum cell densities of the other species. When cultured together under conditions without cell contact, growth of *S. costatum* and *H. akashiwo* were both suppressed. Furthermore, despite re-enrichment with nutrients, filtrates from dense cultures of *S. costatum* and *H. akashiwo* reciprocally reduced their maximum cell densities. In additional experiments, growth of *Chaetoceros muelleri* was also suppressed with filtrates from the above cultures, but growth of *Prorocentrum minimum* was not. Therefore, growth interactions between these species strongly suggest the involvement of allelopathic substances secreted by both species. Finally, growth and interaction of *S. costatum* and *H. akashiwo* in bi-algal cultures were simulated using a mathematical model. This model indicated that *S. costatum* and *H. akashiwo* steadily approach a stable equilibrium point of about 3.4×10^5 cells ml^{-1} and 4.8×10^5 cells ml^{-1}, respectively, when the 2 species coexist.

KEY WORDS: Allelopathy · Bacillariophyceae · *Skeletonema costatum* · Raphidophyceae · *Heterosigma akashiwo* · Bi-algal culture · Growth inhibition

INTRODUCTION

Molisch (1937) introduced the term allelopathy to refer to biochemical interactions between all types of plants, including microorganisms. Rice (1974) defined allelopathy as any direct or indirect harmful effect by one plant, including microorganisms, on another through production of chemical compounds that escape into the environment. Later, Rice (1984) recanted his earlier definition because apparently most allelopathic compounds have both inhibitory and stimulatory effects on growth. Despite recent studies and reviews of the allelopathic interactions among marine and freshwater phytoplankton (Cembella 2003, Legrand et al. 2003), no satisfactory explanations have been provided to explain why certain phytoplankton species are able to completely dominate a phytoplankton community. Thus, we need to improve our understanding of the inhibitory and stimulatory interactions among many phytoplankton species in order to elucidate the role of allelopathy in algal succession and the outbreak mechanisms of algal blooms.

The diatom *Skeletonema costatum* (Greville) Cleve and the flagellate *Olisthodiscus luteus* Carter form alternating blooms in Narragansett Bay (Rhode Island, USA; Pratt 1966). In the fishing port of Hakozaki (Hakata Bay, Fukuoka, Japan), Honjo et al. (1978) found that several kinds of centric diatom disappeared temporarily during a *Heterosigma* sp. red tide. Note that *Heterosigma akashiwo* (Hada) Hada ex Hara et Chihara has been called *H. inlandica* Hada in Japan and has been confused with *O. luteus* in many countries (Honjo 1993). Hara & Chihara (1987) reported that *H. inlandica* was synonymous with *H. akashiwo*, and

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Honjo (1994) and Smayda (1998) indicated that most pelagic blooms attributed to *O. luteus* were almost certainly those of *H. akashiwo*. Therefore, we will treat *O. luteus* described by Pratt (1966) and Honjo & Tabata (1985) as *H. akashiwo*.

In *in situ* and *in vitro* experiments, high concentrations of *Olisthodiscus luteus* inhibit the growth of *Skeletonema costatum*, while lower concentrations stimulate the growth of *S. costatum* (Pratt 1966). Similarly, Honjo et al. (1978) reported that *Heterosigma akashiwo* and *S. costatum* alternated in forming red tide blooms in the fishing port of Hakozaki. They found that filtrate from dense cultures of *H. akashiwo*, re-enriched with nutrients, suppressed the growth of *S. costatum.* Furthermore, Honjo & Tabata (1985) reported an apparent and reciprocal codominance between *O. luteus* and diatoms in a 70 m^3 outdoor tank with flowing coastal water.

Unfortunately, none of the *in vitro* experiments mentioned above used axenic strains. In this study, we used axenic strains of *Heterosigma akashiwo* and *Skeletonema costatum.* First, we conducted bi-algal culture experiments with several combinations of initial cell densities of the 2 species. Second, we examined allelopathic interactions between *H. akashiwo* and *S. costatum* by way of both growth experiments using culture filtrates and of bi-algal culture experiments under non-contact conditions. Finally, we simulated the growth of *H. akashiwo* and *S. costatum* in bi-algal cultures using a mathematical model and growth parameters derived from this study to quantify the relationships.

MATERIALS AND METHODS

Algal species and culture conditions. Axenic strains of *Skeletonema costatum* (NIES-324) and *Heterosigma akashiwo* (NIES-10) were obtained from the National Institute for Environmental Studies (NIES, Japan). An axenic strain of *Chaetoceros muelleri* Lemmermann (CCMP 1316) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, USA). *Prorocentrum minimum* (Pavillard) Schiller cells were isolated from the fishing port of Hakozaki, Fukuoka, Japan, in 1996 and were repeatedly washed using capillary pipettes. The above strains were tested for bacterial contamination using the fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) staining method (Porter & Feig 1980); all were verified as axenic.

Cultures were maintained in 200 ml flasks containing 100 ml of modified sea water medium (SWM-3) (Table 1) from the original recipe (Chen et al. 1969) with a salinity of 25 at 25°C under 228 (±5) µmol m^{-2} s^{-1} of cool-white fluorescent illumination on a 12:12 h light:dark cycle. The modified SWM-3 medium was autoclaved (121°C, 15 min) and contained a pH buffer to avoid pH effects. Irradiance in the incubator was measured with a Quantum Scalar Laboratory Irradiance Sensor (QSL-2100/2101; Biospherical Instruments).

Bi-algal culture experiments. Bi-algal culture experiments were conducted in 100 ml flasks containing 50 ml of medium. *Skeletonema costatum* cells in stationary phase (10 to 12×10^5 cells ml^{-1}) were inoculated at a final cell density of 10^2 or 10^4 cells ml^{-1} in all combinations into cultures of *Heterosigma akashiwo* (stock cultures: 5 to 6×10^5 cells ml^{-1}) with cell densities of 10^2 and 10^4 cells ml^{-1}. As controls, both *S. costatum* and *H. akashiwo* were cultured individually at cell densities of 10^2 and 10^4 cells ml^{-1} (Table 2). 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. *S. costatum* and *H. akashiwo* cells were counted microscopically in 1000 µl subsamples collected at 2 d intervals. When cell densities exceeded 20 000 cells ml^{-1}, subsamples were diluted 10 to 50× with fresh modified SWM-3 medium before counting. During the experiments, pH of each subsample collected for counting was measured using a pH-meter (B-212; Horiba).

Table 1. Components of modified SWM-3 medium

Component	Quantity
Modified SWM-3 medium	
Seawater	1000 ml
$NaNO_3$	170 mg
$NaH_2PO_4 \cdot 2H_2O$	15.6 mg
Na_2EDTA	11.16 mg
Fe-EDTA	0.84 mg
$NaSiO_3 \cdot 9H_2O$	56.8 mg
P1-metal mixture solution[1]	10 ml
P2-metal mixture solution[2]	5 ml
Vitamin mixture solution[3]	5 ml
Tris (hydroxymethyl) aminomethane	400 mg
[1]P1-metal mixture solution	
Distilled water	1000 ml
EDTA	1 g
$MnCl_2 \cdot 4H_2O$	0.69 g
$CoCl_2 \cdot 6H_2O$	2.38 mg
$ZnCl_2$	54.8 mg
H_2SeO_3	173 µg
[2]P2-metal mixture solution	
Distilled water	1000 ml
H_3BO_3	6.18 g
[3]Vitamin mixture solution	
Distilled water	500 ml
Vitamin B_{12}	100 µg
Vitamin B_1	1 g
Biotin	100 µg

Macronutrient analyses. At the beginning of bi-algal experiments, 1 ml of each culture was passed through a 0.22 µm syringe filter (SLGV 025 LS; Millipore) and frozen at −80°C until analysis. At the end of the bi-algal experiments, 20 ml of each culture was gravity-filtered through a 5.0 µm pore size membrane filter (SMWP04700; Millipore) on a 47 mm polysulfone holder (KP-47H; Advantec). Filtrates were then passed through 0.22 µm syringe filters and frozen at −80°C until analysis of macronutrients. Nitrogen (NO_2^- + NO_3^-) and phosphorus (PO_4^{3-}) were measured using an autoanalyzer (TRACCS 800; Bran + Luebbe) after samples were diluted 2 to 80× with Milli-Q water.

Bi-algal culture experiments under non-contact conditions. For these experiments, we used 6-well plates with BD Falcon cell culture insert plates (BD35-3091; Becton-Dickinson) (Fig. 1). This insert plate has a membrane filter (pore size 3.0 µm) on the bottom. *Skeletonema costatum* cells in stationary phase (10 to 12 × 10^5 cells ml^{-1}) were suspended into modified SWM-3 medium at densities of 10^2 and 10^4 cells ml^{-1}, and 5 ml was inoculated into the outer chambers of the well plates. *Heterosigma akashiwo* cells in stationary phase (5 to 6 × 10^5 cells ml^{-1}) were then suspended at a density of 10^2 cells ml^{-1} in modified SWM-3 medium, and 3 ml of the cell suspension was inoculated into each inner chamber. Similarly, *H. akashiwo* cells in stationary phase (5 to 6 × 10^5 cells ml^{-1}) were diluted to densities of 10^2 and 10^4 cells ml^{-1} with modified SWM-3 medium, and 5 ml was added into the outer chambers of the well plates. *Skeletonema costatum* cells in stationary phase (10 to 12 × 10^5 cells ml^{-1}) were then diluted to a density of 10^2 cells ml^{-1} with modified SWM-3 medium, and 3 ml of the cell suspension was added to the inner chambers of the well plates. Three replicate wells were used for each treatment. On Day 8 of the incubation, the cells in each of the five 10 µl subsamples from each well were counted microscopically. If necessary, subsamples were diluted 10 to 50 times with fresh modified SWM-3 medium before counting.

Table 2. *Skeletonema costatum* and *Heterosigma akashiwo*. Initial cell densities for bi-algal culture experiments

H. akashiwo (cells ml^{-1})	*S. costatum* (cells ml^{-1})		
	0	10^2	10^4
0	–	See Fig. 2A,B	See Fig. 2C,D
10^2	See Fig. 2A,C	See Figs. 2A & 6A	See Figs. 2C & 6C
10^4	See Fig. 2B,D	See Figs. 2B & 6B	See Figs. 2D & 6D

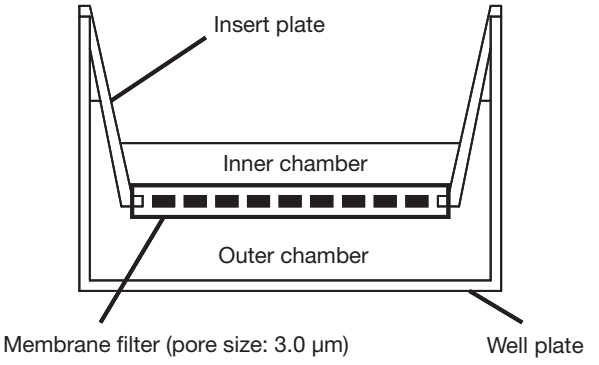

Fig. 1. Cross section of well plate with a BD Falcon cell culture insert

Effect of filtrates from *Skeletonema costatum* and *Heterosigma akashiwo* cultures on phytoplankton growth. For each species, 80 ml samples from 5 replicate flasks were withdrawn by glass pipette after 1, 3, 5, 9 and 17 d (*S. costatum*) and 1, 5, 7, 11 and 17 d (*H. akashiwo*), and passed through a 5.0 µm pore size membrane filter on a 47 mm polysulfone holder under gravity filtration. Nutrients were added to the same concentrations as the modified SWM-3 medium in 80 ml of each filtrate. As a control, an equal amount of Milli-Q water was added to 80 ml of fresh modified SWM-3 medium. These filtrates were passed through 0.22 µm syringe filters. Filtrates from both species were labeled in sequence of their growth as Phases I to V (see Fig. 4A,B). In addition, the pH of each filtrate was measured using a pH-meter.

The effects of these filtrates from *Skeletonema costatum* and *Heterosigma akashiwo* cultures on growth of *S. costatum*, *H. akashiwo*, *Chaetoceros muelleri,* and *Prorocentrum minimum* were examined in 48-well plates. For each species, cells from cultures in stationary phase were diluted to a density of 10^4 cells ml^{-1} with modified SWM-3 medium. Then 10 µl of each cell suspension was inoculated into 990 µl of re-enriched filtrate of *S. costatum* and *H. akashiwo* (final cell density: 10^2 cells ml^{-1}), with 6 replicate wells for each treatment. After incubation (*S. costatum*: 5 d; *H. akashiwo*: 8 d; *C. muelleri*: 6 d; *P. minimum*: 8 d), the cells in each of the five 10 µl subsamples from each well were counted microscopically. If necessary, subsamples were diluted 10 to 50 times with fresh modified SWM-3 medium before counting.

Effect of pH on phytoplankton growth rate. The growth rates of 4 phytoplankters were measured at different pH values between 7.8 and 9.1. The pH in modified SWM-3 medium was adjusted by the addition of 1 N HCl or NaOH. Growth was determined by daily measurement (*Skeletonema costatum*: 8 d; *Heterosigma akashiwo*: 7 d; *Chaetoceros muelleri*: 5 to 6 d;

Prorocentrum minimum: 8 to 9 d) of *in vivo* fluorescence of cultures using a fluorometer (10-AU-005-CE; Turner Designs). Three replicate polystyrene tubes were used for each treatment.

Growth simulation in bi-algal cultures. To model the behavior of bi-algal cultures of *Skeletonema costatum* and *Heterosigma akashiwo*, we adopted the growth simulation of Uchida et al. (1999) using the following equations

$$dx\,dt^{-1} = r_x x[(1 - x)\,K_x^{-1}] - Axy = r_x x\,[1 - (x + ay)K_x^{-1}] \quad (1)$$

$$dy\,dt^{-1} = r_y y[(1 - y)K_y^{-1}] - Bxy = r_y y[1 - (bx + y)K_y^{-1}] \quad (2)$$

Here, x and y are the cell densities of *Skeletonema costatum* and *Heterosigma akashiwo*, respectively. The parameters r_x and K_x are the growth rate and final cell density of *S. costatum*, respectively, and r_y and K_y are the corresponding parameters for *H. akashiwo* when each species is in a mono-algal culture. A is a measure of the degree of inhibition of *S. costatum* by *H. akashiwo*, and B is that of *H. akashiwo* by *S. costatum*. If we set $A = ar_xK_x^{-1}$ and $B = br_yK_y^{-1}$, then Eqs. (1) & (2) become the same as the formulae for the growth of 2 populations competing with each other for limited resources (Iwasa 1998). Parameters a and b are non-dimensional and are a measure of the degree of inhibition by the other species compared with self-interference. When each species is cultured in a 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 this study. Next, the parameters a and b were calculated directly from Eqs. (1) & (2) using the bi-algal culture data. Precise estimates of a and b were carried out using the Marquardt method (Marquardt 1963), with the most appropriate values of a and b determined when the difference between the squared values of observed cell density and theoretical cell density became minimum.

RESULTS

Bi-algal culture experiments

When initial cell densities of both *Skeletonema costatum* and *Heterosigma akashiwo* were 10^2 cells ml^{-1}, the growth of *S. costatum* 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 6 onwards — when *S. costatum* reached stationary phase — after which the average maximum cell density of *H. akashiwo* was about 32% of that in mono-algal cultures (Fig. 2A).

When initial cell densities of *Skeletonema costatum* and *Heterosigma 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 contrast, the growth of *S. costatum* became remarkably suppressed in bi-algal cultures beginning on Day 4 when *H. akashiwo* reached stationary phase, after which the average maximum cell density of *S. costatum* was about 13% of that in mono-algal cultures (Fig. 2B).

When initial cell densities of *Skeletonema costatum* and *Heterosigma akashiwo* were 10^4 cells ml^{-1} and 10^2 cells ml^{-1}, respectively, the growth of *S. costatum* in both bi-algal and mono-algal cultures was virtually the same. In contrast, the growth of *H. akashiwo* was significantly suppressed in bi-algal cultures beginning on Day 4 when

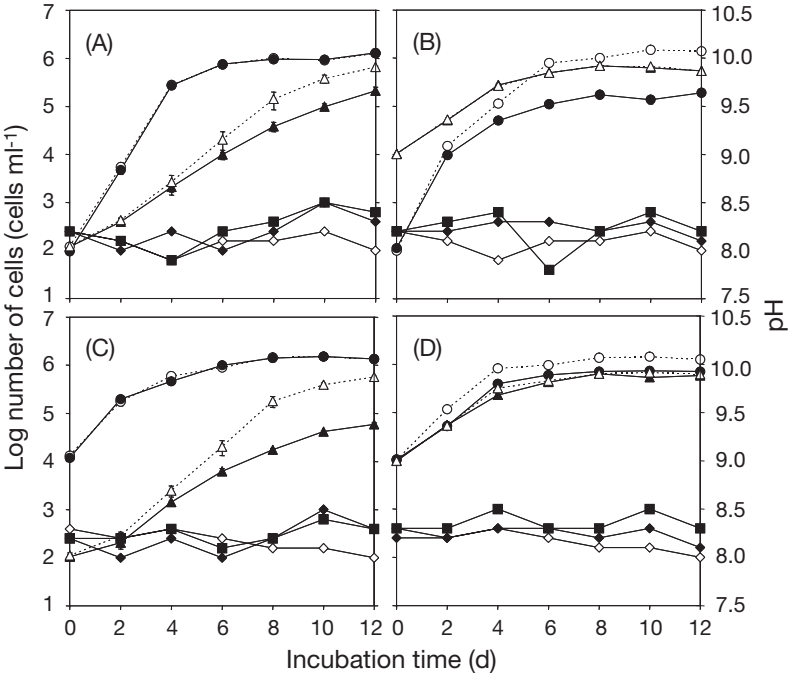

Fig. 2. Growth of *Skeletonema costatum* when cultured alone (○) or together with *Heterosigma akashiwo* (●), and of *H. akashiwo* when cultured alone (△) or together with *S. costatum* (▲). Initial cell density of *S. costatum* was 10^2 (A,B) or 10^4 cells ml^{-1} (C,D); that of *H. akashiwo* was 10^2 (A,C) or 10^4 cells ml^{-1} (B,D). Data are mean ± mean absolute deviation (MAD) (cells ml^{-1}) of triplicate measurements; secondary y-axes indicate pH of bi-algal cultures (■), and of *S. costatum* (◇) and *H. akashiwo* (◆) cultured alone. Errors are small and mostly obscured by the symbols

Table 3. *Skeletonema costatum* and *Heterosigma akashiwo*. Initial and final macronutrient concentrations (± MAD) for bi-algal culture experiments with mono-algal controls

Culture	Species	Initial cell density (cells ml^{-1})	$NO_2^- + NO_3^-$ (µM) Day 0	$NO_2^- + NO_3^-$ (µM) Day 12	PO_4^{3-} (µM) Day 0	PO_4^{3-} (µM) Day 12
Mono-algal	*S. costatum*	10^2	1095 ± 58.01	250 ± 24.10	50 ± 0.89	0.627 ± 0.12
		10^4	1166 ± 5.03	277 ± 4.82	51 ± 0.80	0.650 ± 0.01
	H. akashiwo	10^2	1159 ± 11.95	180 ± 52.30	52 ± 0.30	0.500 ± 0.14
		10^4	1143 ± 10.26	0.369 ± 0.23	49 ± 0.32	0.308 ± 0.08
Bi-algal	*S. costatum*	10^2	1121 ± 15.06	106 ± 45.90	52 ± 1.21	0.415 ± 0.09
	H. akashiwo	10^2				
	S. costatum	10^2	1153 ± 8.48	0.395 ± 0.10	53 ± 1.05	0.294 ± 0.12
	H. akashiwo	10^4				
	S. costatum	10^4	1138 ± 19.15	93.8 ± 8.97	50 ± 1.69	0.923 ± 0.18
	H. akashiwo	10^2				
	S. costatum	10^4	1136 ± 34.56	0.174 ± 0.14	49 ± 2.31	0.300 ± 0.03
	H. akashiwo	10^4				

S. costatum cells reached stationary phase, after which the average maximum cell density of *H. akashiwo* was about 9% of that in mono-algal cultures (Fig. 2C).

When initial cell densities of *Skeletonema costatum* and *Heterosigma akashiwo* were both 10^4 cells ml^{-1}, the growth of *H. akashiwo* became weakly suppressed. The growth of *S. costatum* became slightly suppressed beginning on Day 2, after which the average maximum cell density of *S. costatum* was about 50% of that in mono-algal cultures (Fig. 2D).

Macronutrient analysis on samples from all cultures indicated that nitrogen ($NO_2^- + NO_3^-$) and phosphorus (PO_4^{3-}) concentrations significantly decreased in cultures with initial *Heterosigma akashiwo* cell densities of 10^4 cells ml^{-1} compared with those with initial cell densities of 10^2 cells ml^{-1} (Table 3).

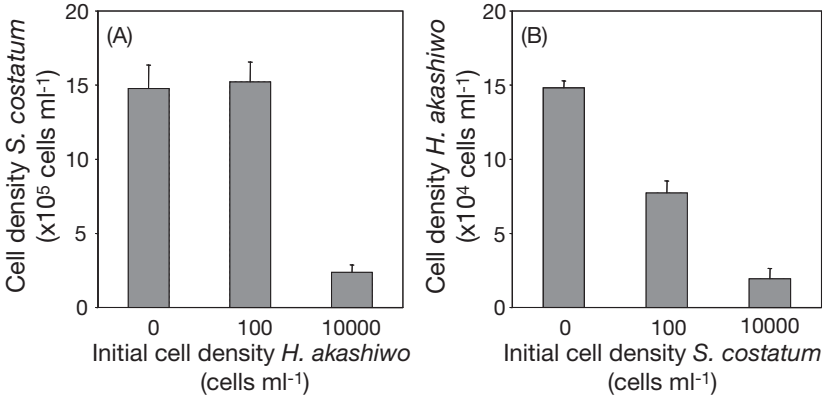

Fig. 3. *Skeletonema costatum* and *Heterosigma akashiwo*. Bi-algal cultures under non-contact conditions. (A) Effects of *H. akashiwo* (initial cell density: 10^2 and 10^4 cells ml^{-1}) on growth of *S. costatum* (initial cell density: 10^2 cells ml^{-1}). (B) Effects of *S. costatum* (initial cell density: 10^2 and 10^4 cells ml^{-1}) on growth of *H. akashiwo* (initial cell density: 10^2 cells ml^{-1}). Data are mean ± MAD (cells ml^{-1}) of triplicate measurements

Bi-algal culture experiments under non-contact conditions

Growth of *Skeletonema costatum* (initial cell density: 10^2 cells ml^{-1}) in bi-algal cultures with *Heterosigma akashiwo* (initial cell density: 10^2 or 10^4 cells ml^{-1}) under the non-contact conditions provided by cell culture inserts was substantially inhibited (16% of the control) by *H. akashiwo* when initial cell density was 10^4 cells ml^{-1} (Fig. 3A). In contrast, growth of *H. akashiwo* was not inhibited by *S. costatum* under these conditions. Growth inhibition of *H. akashiwo* (initial cell density: 10^2 cells ml^{-1}) in bi-algal cultures with *S. costatum* (initial cell density: 10^2 or 10^4 cells ml^{-1}) under the same non-contact conditions was dependent on the cell density of *S. costatum* (Fig. 3B). In contrast, growth of *S. costatum* was not inhibited by *H. akashiwo* under these conditions.

Effects of culture filtrates on phytoplankton growth

We observed the effects of filtrates from *Skeletonema costatum* cultures (Phase I to V) on the growth of *Heterosigma akashiwo*, *Chaetoceros muelleri* and *Prorocentrum minimum* cultures (initial cell densities: 10^2 cells ml^{-1}; Fig. 4A). Enriched filtrates from *S. costatum* cultures in Phases III and IV substantially decreased the growth of *H. akashiwo* (Phases III: 48% of control; Phase IV: 18% of control) and *C. muelleri* (Phase III: 43% of control; Phase IV: 28% of control), whereas

filtrates from *S. costatum* cultures in Phase V only slightly decreased the growth of *H. akashiwo* and *C. muelleri* (Fig. 4Ai,Aii). Enriched filtrates from *S. costatum* cultures had little effect on the growth of *P. minimum* (Fig. 4Aiii).

Similarly, we observed the effects of enriched filtrates from *Heterosigma akashiwo* cultures (Phase I to V) on the growth of *Skeletonema costatum*, *Chaetoceros muelleri* and *Prorocentrum minimum* (initial cell density: 10^2 cells ml^{-1}; Fig. 4B). The filtrates from *H. akashiwo* cultures in Phases III and V substantially decreased the growth of *S. costatum* and *C. muelleri* (Fig. 4Bi, Bii). Enriched filtrates from *H. akashiwo* cultures had little effect on the growth of *P. minimum* (Fig. 4Biii).

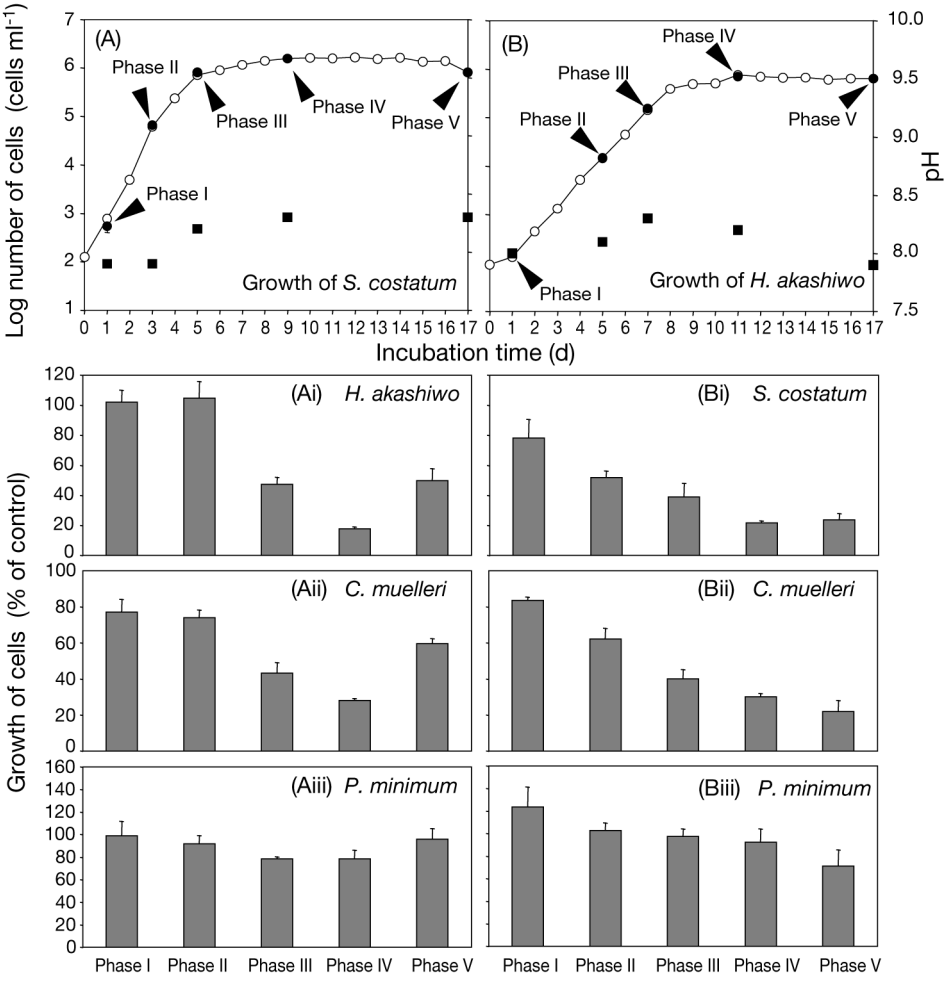

Fig. 4. Effects of culture filtrate on phytoplankton growth. (A) Growth curve of *Skeletonema costatum* showing collection times of filtrate and effects of filtrate on (Ai) *Heterosigma akashiwo*, (Aii) *Chaetoceros muelleri* and (Aiii) *Prorocentrum minimum*. (B) Growth curve of *H. akashiwo* showing collection times of filtrate and effects of filtrate on (Bi) *S. costatum*, (Bii) *C. muelleri* and (Biii) *P. minimum*. Data are mean ± MAD (cells ml^{-1}) of triplicate measurements; secondary *y*-axes (A,B) indicate pH (■)

Table 4. *Skeletonema costatum* and *Heterosigma akashiwo*. Estimated parameters for bi-algal model simulation. *a* and *b* are dimensionless. (For details and definition of *A*, *B*, see 'Growth simulation in bi-algal cultures' in 'Materials and methods')

Species	Carrying capacity (K) (cells ml^{-1})	Growth rate (r) (divisions h^{-1})	Growth rate (r) (divisions d^{-1})	Interaction rate (a or b)	Interaction rate (A or B) (ml cell^{-1} s^{-1})
S. costatum	1144459	0.068	1.63	1.668	2.8×10^{-11}
H. akashiwo	583528	0.042	1	0.302	6.0×10^{-12}

Effect of culture pH on phytoplankton growth rate

The effect of pH on the growth of 4 phytoplankton species was tested over the pH range of 7.8 to 9.1 (Fig. 5). The growth rate of *Skeletonema costatum* declined by 20% at a pH of 8.78, and a similar reduction was observed in the growth rate of *Heterosigma akashiwo* and *Chaetoceros muelleri* at a pH of 8.94. There was no observed effect of pH on the growth rate of *Prorocentrum minimum* over the range tested.

Growth simulation in bi-algal cultures

The values of all parameters of the growth simulation are shown in Table 4. Note that the growth patterns of bi-algal cultures of *Skeletonema costatum* and *Heterosigma akashiwo* predicted using these values are similar to those observed in the culture experiments (Fig. 6).

DISCUSSION

Allelopathy between *Skeletonema costatum* and *Heterosigma akashiwo*

Previous bi-algal culture experiments with *Skeletonema costatum* and *Heterosigma akashiwo* were not conducted under axenic conditions. For example, Imai et al. (1995), Nagasaki et al. (1994a,b) and Tarutani et al. (2000) reported that bacterial attack and viral infection induce bloom termination. To reveal the allelopathic interactions between phytoplankton species, we conducted bi-algal culture experiments using axenic cultures of *S. costatum* and *H. akashiwo*.

The species first reaching stationary phase substantially suppressed the maximum cell densities attained by the second species (Fig. 2A–C). These results may explain observations that *Heterosigma akashiwo* and *Skeletonema costatum* alternately dominate in the field and in laboratory experiments under non-axenic conditions (Pratt 1966, Honjo et al. 1978). Pratt (1966) and Honjo et al. (1978) observed that after the decline of *S. costatum* blooms, *H. akashiwo* multiplied and formed blooms. The fact that the growth rate of *S. costatum* is higher than that of *H. akashiwo* (Fig. 2A) may explain why blooms of *S. costatum* usually precede those of *H. akashiwo* in the field. Furthermore, our laboratory results agree with field observations that during the bloom of one species, the other species disappears (Fig. 2B,C). Interestingly, when the initial cell densities of *S. costatum* and *H. akashiwo* in bi-algal cultures were both 10^4 cells ml^{-1}, growth of both species was slightly suppressed, but the effects of growth inhibition were weaker than in other experimental groups (Fig. 2D). Therefore, allelopathic substances of *S. costatum* and *H. akashiwo* may react antagonistically at certain concentrations.

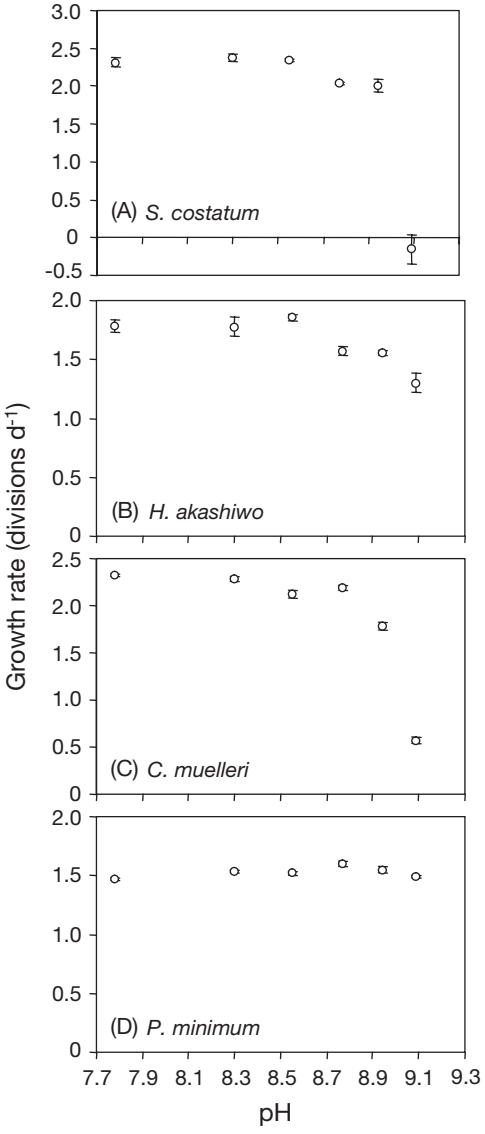

Fig. 5. Growth rates of cultures of (A) *Skeletonema costatum*, (B) *Heterosigma akashiwo*, (C) *Chaetoceros muelleri* and (D) *Prorocentrum minimum* with culture pH values ranging from 7.8 to 9.1. Data are mean ± MAD (cells ml^{-1}) of triplicate measurements

Our experiments with bi-algal cultures under non-contact conditions (Fig. 3) and with re-enriched culture filtrates (Fig. 4) indicated that substances secreted or released by *Skeletonema costatum* and *Heterosigma akashiwo* inhibited the growth of the other species, and that these inhibitions were not caused by light, nutrients or cell contact (Uchida et al. 1995, 1999). These results successfully demonstrate that allelopathy is involved in the growth interactions between

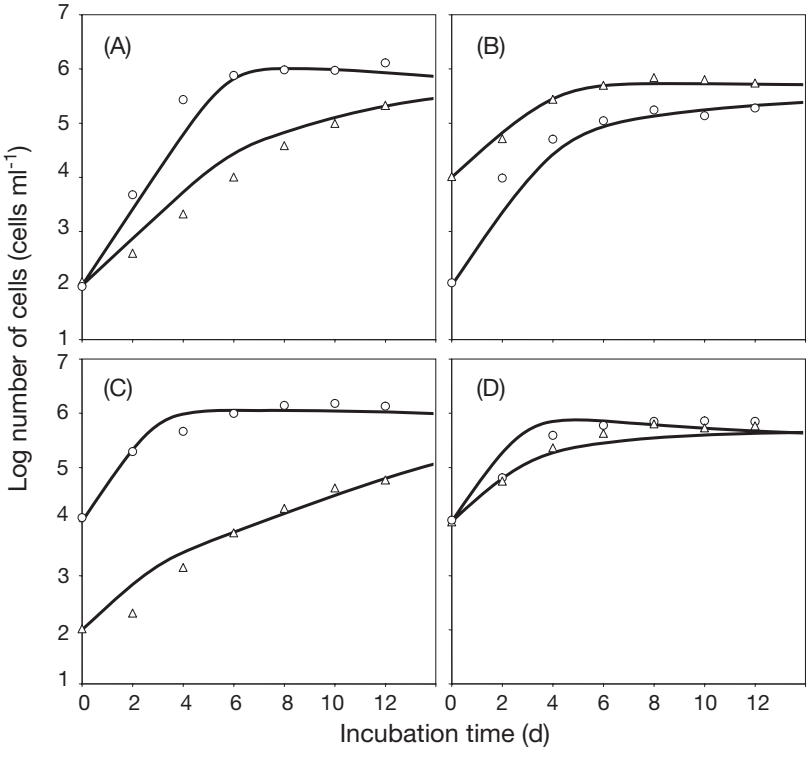

Fig. 6. Growth simulation of *Skeletonema costatum* and *Heterosigma akashiwo* in bi-algal cultures for various combinations of initial cell densities. (A) *S. costatum* 10^2 cells ml^{-1}, *H. akashiwo* 10^2 cells ml^{-1}; (B) *S. costatum* 10^2 cells ml^{-1}, *H. akashiwo* 10^4 cells ml^{-1}; (C) *S. costatum* 10^4 cells ml^{-1}, *H. akashiwo* 10^2 cells ml^{-1}; (D) *S. costatum* 10^4 cells ml^{-1}, *H. akashiwo* 10^4 cells ml^{-1}. Lines show simulated growth curves and symbols show actual data from bi-algal cultures: ○, *S. costatum*; △, *H. akashiwo*

S. costatum and *H. akashiwo*. Additionally, our results agree with Pratt's (1966) results that filtrate from *Olisthodiscus luteus* at a high cell density inhibits the growth of *S. costatum*; however, the inhibitory effects of *S. costatum* on *H. akashiwo* observed in this work were not evident in his results. This difference may result from the effects of bacteria or the use of different strains.

Enriched filtrates from *Skeletonema costatum* and *Heterosigma akashiwo* cultures decreased the growth of *Chaetoceros muelleri*, but had little effect on the growth of *Prorocentrum minimum*, suggesting that effects of allelopathic substances from *S. costatum* and *H. akashiwo* may be species-specific (Fig. 4). Furthermore, the inhibitory effects of enriched filtrates from Phase V *S. costatum* cultures were weaker than those of filtrates from Phases III and IV (Fig. 4A), suggesting that the allelopathic substances are relatively unstable. In contrast, the allelopathic substances of *H. akashiwo* are relatively stable (Fig. 4B). Therefore, allelopathic substances of *S. costatum* and *H. akashiwo* are probably different in chemical nature.

Heil et al. (2005) suggested that *Prorocentrum minimum* blooms were related to the intensity of *Skeletonema costatum* and *Heterosigma akashiwo* blooms. In this study, we found that enriched filtrates from *S. costatum* and *H. akashiwo* cultures had little effect on the growth of *P. minimum* (Figs. 4Aiii, Biii); however, we did not investigate the growth interactions between these species in detail. Future field studies should examine the growth interactions between species that occur with, precede or succeed a target species, for example *P. minimum*.

According to Pratt (1966), the inhibition of *Skeletonema costatum* growth in *Olisthodiscus luteus* filtrates is primarily due to an ectocrine, a tannin-like substance released by *O. luteus*. However, Honjo (1993) suggested that a polysaccharide-protein complex exfoliated from the cell surface or excreted by *Heterosigma akashiwo* acts as an allelopathic substance causing dramatic changes in cell numbers of other phytoplankton species during *H. akashiwo* blooms. Kondo et al. (1990) showed that organic substances excreted by *S. costatum* in culture increased *Prorocentrum minimum* cell yield. In recent years, a negative influence of diatom-derived α-, β-, γ-, and δ-unsaturated aldehydes (PUAs) on the reproductive success of copepods and other invertebrates has been suggested (Ianora et al. 2004, Pohnert 2005, Wichard et al. 2005), and it is necessary to examine the growth effects of *S. costatum*-derived PUAs on phytoplankters. Unfortunately, these allelopathic substances inhibiting the growth of *S. costatum* and *H. akashiwo* have not yet been identified.

Effects of pH on growth of 4 phytoplankton species

Recently, some studies indicated that elevated pH in culture media inhibited the growth of phytoplankters in mixed cultures (Goldman et al. 1981, 1982, Schmidt & Hansen 2001, Lundholm et al. 2005). Therefore, when studying interactions between algae in dense cultures, an elevated pH in the growth media is an important consideration. During our bi-algal culture experiments, the pH ranged from 7.8 to 8.5 (Fig. 2). Moreover, the pH of filtrates from different phases (I to

V) of 2 species ranged from 7.9 to 8.3 (Fig. 4). The growth rates of the 4 phytoplankton species used in this study were not affected by pH values in these ranges (Fig. 5).

Hansen (2002) measured pH in the surface waters of the eutrophic Mariager Fjord, Denmark, over 10 yr (1990 to 1999) and reported profound seasonal variation. He also reported that some species were very sensitive to high pH, while others could grow at their maximum growth rate even at high pH. In comparison, we demonstrated that allelopathy between *Skeletonema costatum* and *Heterosigma akashiwo* exists. Thus, we believe that allelopathy, possibly along with seasonal variations in environmental factors such as pH, is involved in phytoplankton succession and interactions between *S. costatum* and *H. akashiwo* in the field.

Equilibrium point of *Skeletonema costatum* and *Heterosigma akashiwo*

The values of the parameters in Table 4 were used to calculate isoclines (where $dx/dt^{-1} = 0$ or $dy/dt^{-1} = 0$) and trajectories of populations of the 2 species under various initial cell densities (Fig. 7). The equilibrium point (where $dx/dt^{-1} = dy/dt^{-1} = 0$) is reached at x (*Skeletonema costatum*) = 3.4×10^5 cells ml^{-1} and y (*Heterosigma akashiwo*) = 4.8×10^5 cells ml^{-1}. This model indicates that *S. costatum* and *H. akashiwo* steadily approach a stable equilibrium point at which the 2 species can coexist because $K_x > aK_y$ and $bK_x < K_y$ (Iwasa 1998).

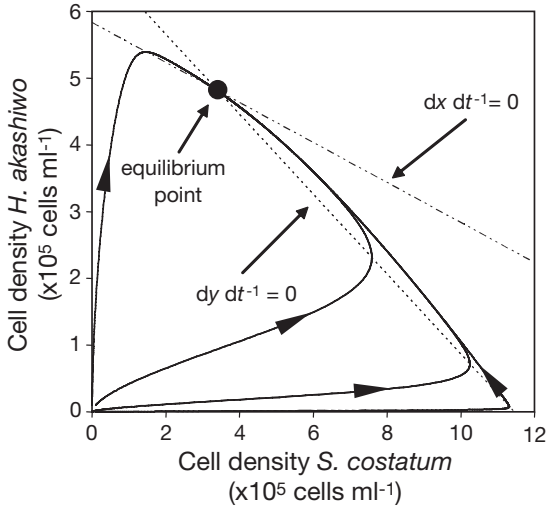

Fig. 7. *Skeletonema costatum* and *Heterosigma akashiwo*. Bi-algal culture simulation isoclines (where $dx/dt^{-1} = 0$ or $dy/dt^{-1} = 0$) and trajectories of *S. costatum* and *H. akashiwo* with various initial cell densities. Regardless of initial cell densities, *S. costatum* and *H. akashiwo* steadily approach a stable equilibrium point at which final cell densities are approx. 3.4×10^5 cells ml^{-1} and 4.8×10^5 cells ml^{-1}, respectively

Uchida et al. (1999) reported that, based on a mathematical model, the inhibition of *Gymnodinium* (=*Karenia*) *mikimotoi* Miyake et Kominami ex Oda by *Heterocapsa circularisquama* Horiguchi is 3× greater than the inhibition of *H. circularisquama* by *G. mikimotoi*. Nagasoe et al. (2006) found that the growth inhibition of *Gyrodinium instriatum* Freudenthal & Lee by *Skeletonema costatum* may require cell contact, but that *G. instriatum* did not affect *S. costatum*. Their equilibrium point was 5×10^3 cells ml^{-1} (*G. instriatum*) and 12×10^5 cells ml^{-1} (*S. costatum*). These results differ from ours in terms of equilibrium point values and in the effects of allelopathy vs. cell contact.

Significance of allelopathy

Allelopathy is of special interest from an evolutionary perspective. Secondary metabolites apparently function as defense (against herbivores, microbes, viruses or competing plants) and signal compounds (to attract pollinating or seed-dispersing animals), and represent adaptive characters that have been subjected to natural selection during evolution (Michael 2003). Allelopathic substances secreted by *Skeletonema costatum* and *Heterosigma akashiwo* may favor the 2 species in competition with other phytoplankton species as well as higher plants. In this study, when one species reached stationary phase in bi-algal cultures, the maximum cell densities of the other species decreased substantially (Fig. 2). This result suggests that allelopathic substances of these 2 species affect each other reciprocally when red tide blooms are formed by only *S. costatum* or *H. akashiwo*. Consequently, allelopathy between *S. costatum* and *H. akashiwo* may be one of the key factors that promotes monospecific bloom formation and allows both species to form alternate red tide blooms in the same ecosystem.

CONCLUSIONS

Our results from laboratory experiments that used axenic strains of *Skeletonema costatum* and *Heterosigma akashiwo* indicated that the growth of either species could be suppressed by the other, depending on cell densities. Thus, allelopathic interaction between *S. costatum* and *H. akashiwo* may play a key role in growth dynamics of red tide blooms through inhibitory effects. Furthermore, simulation using a mathematical model indicated a stable equilibrium point at which *S. costatum* and *H. akashiwo* could

coexist. To our knowledge, there are no previous studies demonstrating that *S. costatum* suppresses the growth of a flagellate species in bi-algal culture by allelopathy. Physical, chemical, and biological factors are all involved in phytoplankton succession and interactions between *S. costatum* and *H. akashiwo* in the field. Hence, it is necessary to purify and identify allelopathic substances that affect the growth of both species, and to verify the role of allelopathy in natural phytoplankton populations.

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