ABSTRACT

We investigated growth interactions between the raphidophytes *Chattonella antiqua* (Hada) Ono and *Heterosigma akashiwo* (Hada Hada ex Hara et Chihara) using bi-algal cultures under axenic conditions. The growth of *C. antiqua* and *H. akashiwo* each tended to be strongly suppressed when the other species reached early stationary phase. A mathematical model was used to simulate the growth interactions of *C. antiqua* and *H. akashiwo* in bi-algal cultures. The model showed that *C. antiqua* outcompetes *H. akashiwo* over time in bi-algal cultures under all experimental conditions. Furthermore, despite re-enrichment with nutrients, the filtrate from dense cultures of *C. antiqua* reduced the maximum growth rate of *H. akashiwo* and filtrate from dense cultures of *H. akashiwo* reduced both the maximum growth rate and maximum yield of *C. antiqua*.

INTRODUCTION

Physicochemical factors, such as water temperature, salinity, light intensity and nutrients, strongly affect species succession in phytoplankton (Tilman et al. 1982; Erga and Heimdal 1984; Karentz and Smayda 1984; Karentz and Smayda 1998; Diehl et al. 2002). On the other hand, the role of interspecific interactions in algal succession also has been emphasized in many reviews (Smayda 1998; Granéli
and Hansen 2006; Nabivailo and Titlyanov 2006; Granéli et al. 2008), and evidence that growth interactions influence algal succession in the field has been presented in some studies that combined field observations with laboratory experiments (Keating 1977, 1978; Sukenik et al. 2002; Fistarol et al. 2003; Yamasaki et al. 2010).

The results of many studies suggest that the growth interactions between phytoplankton species involve allelopathy, which is defined as any direct or indirect inhibitory or stimulatory effect of one plant on another through the production of chemical secretions (Maestrini and Bonin 1981; Rice 1984). Some researchers have hypothesized that allelopathy plays a role in species succession (Keating 1977) and the formation of harmful algal blooms (Smayda 1997, 1998). Cell contact is also thought to be one of the important factors affecting growth interactions of phytoplankton (Uchida et al. 1995, 1999; Uchida 2001; Yamasaki et al. 2007a). These factors in association with other physicochemical factors, such as limiting nutrients and higher light intensities, can affect phytoplankton growth and algal succession (Uchida 2001; Sukenik et al. 2002; Granéli and Hansen 2006; Granéli et al. 2008).

From 1994 to 2004 in Isahaya Bay, Ariake Sea, Japan, blooms of Chattonella antiqua (Hada) Ono generally followed blooms of Heterosigma akashiwo (Hada) Hada ex Harata et Chihara (Yamatogi et al. 2006). Mikhail (2007) reported that during a massive red-tide bloom of C. antiqua in waters near Alexandria, Egypt, cells of a Heterosigma species did not exceed 150 cells l⁻¹. Thus, it is possible that interactions between H. akashiwo and C. antiqua affect their growth in nature.

The raphidophyte C. antiqua is a harmful algal bloom (HAB) species that has caused serious damage to cultured fish in many coastal areas throughout the world (Peperzak 2002; Imai et al. 2006; Yamatogi et al. 2006; Mikhail 2007). In Japan, the maximum fishery damage that has been reported was a loss of 7.1 billion yen (about US $60 million) from the death of 14.2 million yellowtails caused by C. antiqua in Harima-Nada in 1972 (Imai et al. 2006). The physiology and ecology of C. antiqua have been intensively studied (Nakamura and Watanabe 1983a, b; Nakamura et al. 1990; Imai et al. 1991; Watanabe et al. 1995; Amano et al. 1998; Peperzak 2003). Heterosigma akashiwo is another harmful raphidophyte species that has caused red tides in subpolar to subtropical eutrophic coastal.
GROWTH INTERACTIONS BETWEEN RAPHIDOPHYTES *Chattonella antiqua* AND *Heterosigma akashiwo* wers (Honjo 1993; Smayda 1998). This species has been called *H. inlandica* Hada in Japan and has been confused with *Olisthodiscus luteus* in many countries. In Japan, it has been reported that *H. akashiwo* killed cultured fish worth 1,090 million yen in Kagoshima Bay in 1995 and 237 million yen in Nomi Bay in 1997 (Nagasaki et al. 1999). The physiological and ecological characteristics of *H. akashiwo* have been reviewed by Honjo (1993) and Smayda (1998).

Previous studies have shown that there are some similarities in growth characteristics of *C. antiqua* and *H. akashiwo*. For example, both *C. antiqua* and *H. akashiwo* exhibit diel vertical migration behavior, which allows them to more effectively carry out photosynthesis and utilize nutrients (Yamochi and Abe 1984; Watanabe et al. 1995). In their life cycle, both species form cysts and the germination process is strongly affected by temperature (Imai et al. 1991; Shikata et al. 2007). Furthermore, the strains of *C. antiqua* and *H. akashiwo* in the Ariake Sea are eurythermal and euryhaline, and the optimum water temperatures for the growth of vegetative cells of each species overlap; for example, vegetative cells of both species grow well at water temperatures of 25 °C (Yamatogi et al. 2006).

Although both *C. antiqua* and *H. akashiwo* have high potential to form blooms in summer in the Ariake Sea, Japan, they rarely form blooms simultaneously. Consequently, both environmental factors and growth interactions between these species may play important roles in bloom formation in the sea area. Unfortunately, although there have been several studies of growth interactions between *H. akashiwo* and other phytoplankton (Yamasaki et al. 2007b, 2010) and between *C. antiqua* and *Skeletonema costatum* (Greville) Cleve (Matsuyama et al. 2000, Yamasaki et al. 2010), studies on growth interactions between *C. antiqua* and *H. akashiwo*, to our knowledge, have not been carried out.

In this study, we conducted bi-algal culture experiments under axenic conditions using several combinations of initial cell densities of the two species. Second, we simulated the growth of each species using a mathematical model to quantify the relationships between the growth of *C. antiqua* and *H. akashiwo* in bi-algal cultures. Finally, we investigated the growth inhibitory effects of filtrates prepared from cultures of each species on the growth of the other.

Figure 2: Growth simulation of *Chattonella antiqua* and *Heterosigma akashiwo* in bi-algal cultures for various combinations of initial cell densities. (A) *H. akashiwo* $1 \times 10^2$ cells ml$^{-1}$, *C. antiqua* $1 \times 10^2$ cells ml$^{-1}$; (B) *H. akashiwo* $1 \times 10^2$ cells ml$^{-1}$, *C. antiqua* $2 \times 10^3$ cells ml$^{-1}$; (C) *H. akashiwo* $1 \times 10^4$ cells ml$^{-1}$, *C. antiqua* $1 \times 10^2$ cells ml$^{-1}$. Lines show simulated growth curves and symbols show actual data from bi-algal cultures: (□) *H. akashiwo*, (○) *C. antiqua*. 
RESULTS

Bi-algal culture experiments

At the start of the bi-algal culture experiments, concentrations of nitrogen (NO\textsubscript{2}– + NO\textsubscript{3}–) and phosphorus (PO\textsubscript{4}\textsuperscript{3–}) in modified SWM-3 medium were 1055 μM and 52 μM, respectively. By the end of the bi-algal culture experiments, (NO\textsubscript{2}– + NO\textsubscript{3}–) and PO\textsubscript{4}\textsuperscript{3–} concentrations in all cultures had decreased compared with the initial concentrations (Table 1).

When initial cell densities of *Chattonella antiqua* and *Heterosigma akashiwo* were both 1 × 10\textsuperscript{2} cells ml\textsuperscript{–1} (Fig. 1A), growth of *C. antiqua* was suppressed beginning on day 8 when the cell density of *H. akashiwo* reached early stationary phase at around 2 × 10\textsuperscript{5} cells ml\textsuperscript{–1}. The average maximum cell density of *C. antiqua* in bi-algal culture was about 32% of that in mono-algal culture. In contrast, the growth of *H. akashiwo* in bi-algal culture was suppressed beginning on day 10 when the cell density of *C. antiqua* reached early stationary phase at around 9 × 10\textsuperscript{3} cells ml\textsuperscript{–1}, and the average maximum cell density of *H. akashiwo* in bi-algal culture was about 61% of that in mono-algal culture. The pH values of culture media for experiments with this combination of initial cell densities ranged from 7.98 to 8.62 (Table 1).

When initial cell densities of *C. antiqua* and *H. akashiwo* were 2 × 10\textsuperscript{3} cells ml\textsuperscript{–1} and 1 × 10\textsuperscript{2} cells ml\textsuperscript{–1}, respectively (Fig. 1B), the growth of *C. antiqua* in bi-algal culture was almost the same as that in mono-algal culture. In contrast, the growth of *H. akashiwo* was suppressed beginning on day 2 and had almost stopped by day 6 when the cell density of *C. antiqua* reached early stationary phase at around 4 × 10\textsuperscript{4} cells ml\textsuperscript{–1}. In addition, a decrease in cell density...
of *H. akashiwo* was observed from day 12 onward. The average maximum cell density of *H. akashiwo* in bi-algal culture was only about 2% of that in mono-algal culture. The pH values of culture media in experiments with this combination of initial cell densities ranged from 7.82 to 8.50 (Table 1).

When initial cell densities of *C. antiqua* and *H. akashiwo* were $1 \times 10^2$ cells ml$^{-1}$ and $1 \times 10^4$ cells ml$^{-1}$, respectively (Fig. 1C), a drastic growth suppression of *C. antiqua* was observed beginning on day 6 when *H. akashiwo* reached early stationary phase at $2 \times 10^5$ cells ml$^{-1}$. The average maximum cell density of *C. antiqua* was only about 2% of that in mono-algal culture. In contrast, growth of *H. akashiwo* was virtually the same in this bi-algal culture and in mono-algal culture. The pH value of culture media with this combination of initial cell densities ranged from 7.95 to 8.63 (Table 1).

In the present study, no morphologically abnormal cells of either *C. antiqua* or *H. akashiwo* were observed.

**Growth simulation of bi-algal cultures**

The values of all parameters used in the growth simulations are shown in Table 2, and the growth patterns of *C. antiqua* and *H. akashiwo* predicted using these values are shown in Figure 2 (A–C). In addition, the parameter values in Table 2 were used to calculate isolines (where $dx/dt = 0$ and $dy/dt = 0$) and trajectories of populations of two species under various initial cell densities (Fig. 3). All of the simulated trajectories pass through three stages: (1) cell densities of both *C. antiqua* and *H. akashiwo* increase (Fig. 3, area I); (2) cell densities of *C. antiqua* increase but those of *H. akashiwo* decrease (Fig. 3, area II); and (3) cell densities of both *C. antiqua* and

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*Allelopathic interactions between Chattonella antiqua and Heterosigma akashiwo. (A) Effect of filtrate prepared from C. antiqua culture on the growth of H. akashiwo; (B) effect of filtrate prepared from H. akashiwo culture on the growth of C. antiqua. Data are means ± SD of triplicate measurements. White bars indicate maximum growth rates and secondary y-axes indicate maximum yield of the two species as measured by in vivo chlorophyll fluorescence (filled circles). ‘Modified SWM-3 (100%)’ is the basic medium used for culture as controls; ‘Modified SWM-3 (200%)’ is the same medium with double the nutrient concentrations and is used to check for possible effects from re-enriching the culture filtrates by adding nutrients at the original concentrations (see text). Asterisks above bars and filled circles indicate values significantly different (P < 0.01) from controls (cultures in fresh modified SWM-3).*
H. akashiwo decrease (Fig. 3, area III). This model indicates that C. antiqua outcompetes H. akashiwo in bi-algal culture over time under all experimental conditions because the estimates of parameters show $K_y < bK_x$ and $aK_y < K_x$ (Iwasa 1998).

**Effect of filtrates prepared from cultures on phytoplankton growth**

In the experiments testing the effects of culture filtrates on C. antiqua and H. akashiwo, the two species were cultured alone in fresh modified SWM-3 medium as a control. The two species were also cultured alone in modified SWM-3 medium with double (200%) nutrient concentrations to evaluate possible nutrient inhibition of phytoplankton growth in re-enriched filtrates because the final nutrient concentrations in re-enriched filtrates were expected to be 100–200% of fresh modified SWM-3 medium. The maximum growth rate and maximum yield of the two species cultured in the modified SWM-3 medium with 200% nutrient concentrations were almost the same as those cultured in fresh modified SWM-3 medium (Fig. 4A and B), which indicates that the nutrient concentrations in re-enriched filtrates did not have inhibitory effects in the present experiment.

The re-enriched filtrate prepared from C. antiqua culture significantly reduced the maximum growth rate of H. akashiwo (71.7% of control, $P < 0.01$; Fig. 4A). The re-enriched filtrate prepared from H. akashiwo culture significantly reduced both the maximum growth rate (70.2% of control, $P < 0.01$) and maximum yield (81.9% of control, $P < 0.01$) of C. antiqua (Fig. 4B).

<table>
<thead>
<tr>
<th>Culture Species</th>
<th>Initial cell density (cells ml⁻¹)</th>
<th>Final macronutrient concentrations (day 16)</th>
<th>The ranges of pH values in media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NO₂⁻ + NO₃⁻ (µM)</td>
<td>PO₄³⁻ (µM)</td>
</tr>
<tr>
<td><strong>Mono-algal</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. antiqua</td>
<td>1×10²</td>
<td>11.2±1.46</td>
<td>3.22±2.33</td>
</tr>
<tr>
<td></td>
<td>2×10³</td>
<td>7.98±1.14</td>
<td>1.34±0.28</td>
</tr>
<tr>
<td>H. akashiwo</td>
<td>1×10²</td>
<td>5.54±0.89</td>
<td>1.76±0.09</td>
</tr>
<tr>
<td></td>
<td>1×10⁴</td>
<td>5.86±1.31</td>
<td>2.51±0.09</td>
</tr>
<tr>
<td><strong>Bi-algal</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. antiqua</td>
<td>1×10²</td>
<td>9.14±4.45</td>
<td>6.05±0.30</td>
</tr>
<tr>
<td>H. akashiwo</td>
<td>1×10²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. antiqua</td>
<td>2×10³</td>
<td>8.43±0.37</td>
<td>1.35±0.05</td>
</tr>
<tr>
<td>H. akashiwo</td>
<td>1×10²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. antiqua</td>
<td>1×10²</td>
<td>5.80±0.53</td>
<td>2.12±0.16</td>
</tr>
<tr>
<td>H. akashiwo</td>
<td>1×10⁴</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Initial cell densities, final macronutrient concentrations and pH ranges in culture media during bi-algal culture experiments with Chattonella antiqua and Heterosigma akashiwo. Data are means ± SD of triplicate measurements. The initial macronutrient concentrations were 1055 µM for NO₂⁻ + NO₃⁻ and 52 µM for PO₄³⁻.
DISCUSSION

Our results show that Chattonella antiqua and Heterosigma akashiwo each interfered with the growth of the other, and the initial cell density combination affected the growth inhibitory effects (Fig. 1A–C). The dependence of growth inhibitory effects on the combination of initial cell densities was also observed in some previous studies (Uchida et al. 1999; Yamasaki et al. 2007b; Tameishi et al. 2009).

Furthermore, when bi-algal cultures included one species with lower initial cell density and the other species with higher initial cell density, the growth of the former tended to be strongly suppressed when the latter reached early stationary phase (Fig. 1B & C). These results may be used to explain the field observations reported by Yamatogi et al. (2006) and Mikhail (2007).

In a field study from 1994 to 2004 in Isahaya Bay, Ariake Sea, Japan (Yamatogi et al. 2006), H. akashiwo mainly formed blooms from late April to early June when the cell density of C. antiqua was low or below the detection limit, and C. antiqua blooms occurred mainly in late July to early September from 1994 to 2002. In addition, blooms of H. akashiwo also occurred in August of 2003 and 2004, after which dense blooms of C. antiqua were observed. It seems that blooms of H. akashiwo and C. antiqua occurred alternately in Ariake Sea, Japan. Mikhail (2007) reported that during a massive red-tide bloom of C. antiqua in waters off Alexandria, Egypt, few cells of a Heterosigma species appeared and their density did not exceed 150 cells l\(^{-1}\). These phenomena, when considered along with the results of the present study, suggest that growth interactions between C. antiqua and H. akashiwo as well as other environmental factors are involved in the succession of these species in the field.

The model simulation based on the results of our bi-algal culture experiments predicted that C. antiqua will outcompete H. akashiwo over time in bi-algal cultures under all experimental conditions, although H. akashiwo can grow to densities around \(5 \times 10^5\) cells ml\(^{-1}\) before declining when the cell density of C. antiqua is low. Several previous studies have used this mathematical models to examine the growth interactions between algae. Uchida et al. (1999) reported that the inhibition of Gymnodinium mikimotoi Miyake et Kominami ex Oda (=Karenia mikimotoi) by Heterocapsa circularisquama Horiguchi was three times greater than the inhibition of H. circularisquama by K. mikimotoi. Yamasaki et al. (2007b) reported that H. akashiwo and S. costatum could co-exist and steadily approached a stable equilibrium of about \(3.4 \times 10^5\) cells ml\(^{-1}\) and \(4.8 \times 10^3\) cells ml\(^{-1}\), respectively. Tameishi et al. (2009) found that Prorocentrum minimum (Pavillard) Schiller outcompeted S. costatum in all combinations of initial densities of the two species. Thus, the growth interactions among phytoplankton appear to differ depending on the combinations of species. However, the parameters used for these model predictions were based on the results of bi-algal culture experiments that were conducted under artificially controlled and fixed conditions. It is necessary in future studies to determine if variations in environmental factors, such as temperature, light intensity, photoperiod, pH, salinity and nutrient concentrations, affect the degree of inhibition between species.

Re-enriched filtrates prepared from dense cultures of both C. antiqua and H. akashiwo inhibited the growth of the other, indicating that substances secreted or released from these two species (allelopathic substances) are involved in the observed growth interactions between them in bi-algal cultures. The chemical properties of these allelopathic substances were not investigated, but Matsuyama et al. (2000) reported that filtrates of C. antiqua and H. akashiwo cultures had allelopathic effects on the growth of S. costatum and suggested that the allelochemicals in these culture filtrates were polyunsaturated fatty acids. Yamasaki et al. (2009) reported that H. akashiwo produces extracellular, high-molecular-weight allelochemicals (that is, polysaccharide–protein complexes) could inhibit the growth of some
diatoms but not *P. minimum*. These allelochemicals produced by various species may be involved in the growth interactions between phytoplankton species.

In the present study, however, the growth suppression effects of the culture filtrates on the average maximum yield (a decrease to 72–82% of controls; Fig. 4A and B) were relatively weaker than those in the bi-algal culture experiments (a decrease to 2% of controls; Fig. 1B and C). The lower effects of filtrates might result from factors such as instability or the lack of a continuous supply of allelopathic substances by live cells (Tillmann et al. 2007).

Also, previous studies have pointed out that growth interactions could be induced by direct cell contact in *Heterocapsa* sp. (Uchida et al. 1995, 1999) and *Cochlodinium polykrikoides* Margalef (Yamasaki et al. 2007a). The synergistic effects of these factors on growth interactions should be examined in further studies.

Nutrient depletion in bi-algal cultures is also a possible factor resulting in stronger growth suppression effects in bi-algal culture experiments than in those with culture filtrates. Indeed, a drastic decrease of nutrients was observed in the mono-algal and bi-algal culture experiments in the present study (Table 1). A previous study showed that the half saturation constants for nitrate and phosphate uptake for *C. antiqua* were 1.47–2.45 μM and 1.00–1.98 μM, respectively (Nakamura et al. 1988), and the half saturation constants for nitrate and phosphate uptake for *H. akashiwo* were 1.47–2.45 μM and 1.00–1.98 μM, respectively (Tomas 1979). This information suggests that sufficient macronutrients remained in bi-algal cultures for the growth of *C. antiqua*. However, when initial cell densities of *C. antiqua* and *H. akashiwo* were 2 × 10^3 cells ml^–1 and 1 × 10^2 cells ml^–1 respectively, the phosphate concentration by the end of the experiment was thought to be insufficient for the growth of *H. akashiwo*. In fact, a decrease in *H. akashiwo* cell density was observed under this combination of initial cell densities but not in mono-algal culture (Fig. 1B). Note that Einhellig (1995) reported that limited nutrient supplies not only increase the production of allelochemicals but may also intensify their action. Therefore, it is possible that nutrient depletion in bi-algal cultures influenced the intensity of growth suppression and especially intensified growth suppression of *H. akashiwo*.

Some previous studies indicate that elevated pH inhibits the growth of phytoplankton in mixed cultures and drives phytoplankton succession in the field (Schmidt & Hansen 2001; Hansen 2002; Lundholm et al. 2005). The growth rate of *H. akashiwo* is reportedly not affected by pH values in the range from 7.8 to 8.5 (Schmidt & Hansen 2001; Yamasaki et al. 2007b). However, the growth of *C. antiqua* could be affected when the pH is
above 8.4 (Nakamura and Watanabe 1983b). Thus, in the present study when initial cell densities of C. antiqua and H. akashiwo in bi-algal culture were $1 \times 10^2$ cells ml$^{-1}$ and $1 \times 10^4$ cells ml$^{-1}$, respectively (Fig. 1C), the elevated pH values in the culture media (7.95–8.63; Table 1) may have intensified the growth inhibitory effects, especially for the growth of C. antiqua. Nevertheless, our results successfully demonstrate that allelopathic effects, associated with other environmental factors such as nutrient depletion and pH, were involved in the growth suppression observed in our bi-algal culture experiments.

As a further consideration, H. akashiwo reportedly suffers growth suppression effects from some diatoms in field surveys and laboratory experiments (Yamasaki et al. 2007b, 2010). In contrast, sometimes C. antiqua and C. marina were observed to form red tides with diatoms simultaneously at the same location (Mikhail 2007; Mastubara et al. 2009), which suggests that in this case, the growth of C. antiqua was not suppressed by diatoms. Indeed, Yamasaki et al. (2010) observed in a bi-algal culture experiment that the growth of C. antiqua was not suppressed by the diatom S. costatum. Based on this information and the results of the present study, we suspect that C. antiqua will grow well and form red tides in mid-summer with little or no growth suppression from diatoms or other flagellates. However, H. akashiwo should rarely form mid-summer blooms because of the synergistic effects of C. antiqua and diatoms.

The present study demonstrated growth interactions between C. antiqua and H. akashiwo and suggests that these interactions contribute to species succession in the field. In addition, although the results suggest the involvement of allelopathy in the growth interactions between the two species, the effects of any allelopathic substances in culture filtrates were relatively weak. Therefore, further investigation into the stability of allelochemicals and the synergistic effects of biological and physicochemical factors is needed to clarify the mechanisms of phytoplankton succession.

MATERIALS AND METHODS

Algal species and culture conditions

Clonal strains of Chattonella antiqua (NIES-1) and Heterosigma akashiwo (NIES-10) were obtained from the National Institute of Environmental Studies (NIES, Japan). The two strains were verified as axenic using the fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) staining method for testing for bacterial contamination (Porter and Feig 1980). Axenic cultures of the two species were maintained in an incubator (FLI-160, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) in 70-ml sterilized flasks (Nunc brand, Thermo Fisher Scientific Inc., Suwanee, GA, USA) containing 20 ml of modified sea water medium (modified SWM-3, Yamasaki et al. 2007b) at 25 °C and a salinity of 30 under 250 ± 8 μmol photons m$^{-2}$ s$^{-1}$ of cool-white fluorescent illumination on a 12-h:12-h light:dark cycle. The modified SWM-3 medium contained 0.04% (w/v) of the buffer tris(hydroxymethyl)aminomethane (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to prevent changes to pH during the experiments, and the medium was autoclaved (121°C, 15 min) before use. Irradiance in the incubator was measured using a Quantum Scalar Laboratory Irradiance Sensor (QSL-2101, Biospherical Instruments, San Diego, CA, USA).

Bi-algal culture experiments

Bi-algal culture experiments were conducted in 70-ml sterilized flasks containing 20 ml of modified SWM-3 medium. Chattonella antiqua and H. akashiwo cells in early stationary phase were inoculated to a density of $1 \times 10^2$, $2 \times 10^3$ or $1 \times 10^4$ cells ml$^{-1}$. The combinations of initial cell densities for the two species are shown in Table 1. Three replicate flasks were prepared for each treatment. Culture conditions were the same as described in the Materials and Methods subsection entitled ‘algal species and culture conditions’. 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. The cells were counted under a light microscope in 200- to 1000-ml subsamples collected at 2-d intervals. When cell densities exceeded $2 \times 10^4$ cells ml$^{-1}$, subsamples were diluted by a factor of 10 to 20 with fresh modified SWM-3 medium before counting. The pH of each culture was measured at 2-d intervals using a pH meter (model B-212, Horiba, Kyoto, Japan).

### Macronutrient analysis

At the end of the bi-algal culture experiments, 20 ml of each culture was passed through a GF/C glass microfibre filter (Whatman International Ltd., Maidstone, UK) on a 47 mm polysulfone holder under gravity filtration. Filtrates were then passed through 0.22-μm syringe filters and frozen at $-80 \degree C$ until analysis. Nitrogen ($\text{NO}_2^-$ and $\text{NO}_3^-$) and phosphorus ($\text{PO}_4^{3-}$) were measured using an auto-analyzer (TRACCS 800, Bran + Luebbe, Hamburg, Germany) after samples were diluted $10^\times$ with Milli-Q water (Millipore, Billerica, MA, USA).

### Simulation of growth in bi-algal cultures

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

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

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

Here, $x$ and $y$ are the respective cell densities of two species in bi-algal culture, $r_x$ and $K_x$ are the growth rate and carrying capacity, of *C. antiqua* 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 *C. antiqua* by *H. akashiwo*, and $B$ measures the inhibition of *H. akashiwo* by *C. antiqua*. If we set $A = ar_xK_x^{-1}$ and $B = br_yK_y^{-1}$, Equations (1) and (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 Equations (1) and (2) using the mono-algal culture data from bi-algal culture experiments (see Fig. 1 and Materials and Methods subsection entitled ‘bi-algal culture experiments’) when *C. antiqua* and *H. akashiwo* cells were inoculated to a final density of $10^5$ cells ml$^{-1}$, respectively ($n = 3$). Next, the parameters $a$ and $b$ were calculated directly from Equations (1) and (2) using the data of bi-algal culture experiments (see Fig. 1 and Materials and Methods subsection entitled ‘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 Equations $A = ar_xK_x^{-1}$ and $B = br_yK_y^{-1}$, respectively.

### Effect of filtrates from cultures of each species on the growth of the other

Culture filtrates for growth tests were prepared from cultures of each species by passing through a GF/C glass microfibre filter (Whatman International Ltd.) on a 47 mm polysulfone holder under gravity filtration when the cell density reached $2.7 \times 10^4$ cells ml$^{-1}$ for *C. antiqua* and $5.0 \times 10^5$ cells ml$^{-1}$ for *H. akashiwo*. To compensate for nutrients consumed by algal growth, the same amounts of nutrients as in fresh modified SWM-3 medium were added to each filtrate (i.e., culture filtrates were re-enriched), and the final nutrient concentrations in filtrates were expected to be 100–200% of those in fresh modified SWM-3 medium. Thus, each species was cultured alone both in modified SWM-3 medium as a control and was cultured in modified SWM-3 medium with...
200% of nutrient concentrations to evaluate possible nutrient inhibitory effects in re-enriched filtrates. The pH of each filtrate was also adjusted to 7.8–8.0 with 2 N HCl, and then filtrates and modified SWM-3 medium (100% and 200%) were passed through 0.22-μm syringe filters (Millipore).

For growth tests using filtrates, each species was cultured in 8-ml sterile culture tubes (Evergreen Scientific, Los Angeles, CA, USA) containing 5 ml of the prepared filtrates. Initial cell densities of each species were 1 × 10^2 cells ml⁻¹, and the culture conditions were the same as described in the Materials and Methods subsection entitled 'algal species and culture conditions'. Control and treatment groups each had four replicates. Growth was measured daily by using an in vivo fluorometer (10-AU-005-CE; Turner Designs, Sunnyvale, CA, USA). Growth rates during the exponential growth phase were calculated using the method of Guillard (1973).

STATISTICAL ANALYSES

The experimental data were checked for assumptions of homogeneity of variance across treatments using Levene’s test. The maximum growth rate and maximum yield in culture filtrate experiments were analyzed by one-way analysis of variance (ANOVA). If the variances were homogeneous, Dunnett’s pairwise multiple comparison t-test was employed to test for differences among growth rates and maximum yields in treatments. When there was no proof of data homoscedasticity, a Mann-Whitney U-test for nonparametric data was used to compare the difference between treatments and controls. These statistical analyses were conducted using the Statistical Package for the Social Sciences software (SPSS 13.0; SPSS, Inc., Chicago, IL, USA). Differences were considered to be significant at P < 0.05.

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