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The role of interactions between *Prorocentrum minimum* and *Heterosigma akashiwo* in bloom formation

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This manuscript has not been submitted elsewhere in identical or similar form, nor will it be during the first three months after its submission to Hydrobiologia.

Abstract We examined the growth and interactions between the bloom-forming flagellates *Prorocentrum minimum* and *Heterosigma akashiwo* using bi-algal culture experiments. When both species were inoculated at high cell densities, growth of *H. akashiwo* was inhibited by *P. minimum*. In other combinations of inoculation densities, the species first reaching the stationary phase substantially suppressed maximum cell densities of the other species, but the growth inhibition effect of *P. minimum* was stronger than that of *H. akashiwo*. We used a mathematical model to simulate growth and interactions of *P. minimum* and *H. akashiwo* in bi-algal cultures. The model indicated that *P. minimum* always out-competed *H. akashiwo* over time. Additional experiments showed that crude extracts from *P. minimum* and *H. akashiwo* cultures did not affect the growth of either species, but both strongly inhibited the growth of the bloom-forming diatom *Skeletonema costatum*. Further experiments showed that it was unlikely that reactive oxygen species produced by *H. akashiwo* were responsible for the inhibition of *P. minimum* growth.

Introduction

Harmful algal blooms (HABs) are an increasingly serious environmental problem for aquaculture, fisheries and public health in many coastal areas throughout the world (Anderson, 1997). Although there have been various studies on the time–space distribution and eco-physiological characteristics of algal populations (Karentz and Smayda, 1984; Haigh et al., 1992; Smayda, 1998; Diehl et al., 2002; Bruggeman and Kooijman, 2007), it is not always clear how certain phytoplankton species come to dominate a community. Thus, various environmental factors may influence competition between phytoplankton, and allelopathy and cell contact may also be involved (Rice, 1984; Uchida, 2001; Cembella, 2003; Legrand et al., 2003; Granéli and Hansen, 2006).

Three cosmopolitan species are known to inhabit coastal areas around the world: a diatom (*Skeletonema costatum* [Greville] Cleve), a dinophyte (*Prorocentrum minimum* [Pavillard] Schiller) and a raphidophyte (*Heterosigma akashiwo* [Hada] Hada ex Hara et Chihara) (Smayda, 1998; Heil et al., 2005; Shikata et al., 2008). It is also well known that the two flagellates, *P. minimum* (Heil et al., 2005) and *H. akashiwo* (Smayda, 1998), are HAB species, and that *S. costatum* is a major competitor of these flagellates (Pratt, 1966; Honjo et al., 1978; Honjo and Tabata, 1985; Yamasaki et al., 2007a; Tameishi et al., 2009).

Long-term field investigations have suggested that competition for the summer phytoplankton niche in Narragansett Bay (Rhode Island, USA) and other estuaries is usually between a diatom (typically *S. costatum*) and flagellates such as *P. minimum* and *H. akashiwo* (Smayda and Borkman, 2003). Temperature, salinity and nutrients are generally important environmental factors related to phytoplankton growth, and strongly affect the population dynamics of individual species and species succession in the field (e.g. Karentz and Smayda, 1984), but the growth characteristics of these three species are not very different from each other (Eppley et al., 1969; Tomas, 1979; Cembella et al., 1982; Sciandra, 1991; Tarutani and Yamamoto, 1994; Herndon and Cochlan, 2007; Shikata, 2009). This could explain the occurrence of blooms of these species in the same season in the same coastal area, such as Narragansett Bay, although they do not always co-occur in the field. Several studies have suggested that growth and interactions of *H. akashiwo* and *S. costatum* (Pratt, 1966; Honjo et al., 1978; Honjo and Tabata, 1985), and between *P. minimum* and *S. costatum* (Bodeanu and Usurelu, 1979; Silva, 1985; Kondo et al., 1990) may be involved in the mechanism behind alternating blooms of these species.

In previous studies to verify the role of growth and interactions of *P. minimum*, *H. akashiwo* and *S. costatum* on their alternating blooms, we used bi-algal cultures of *P. minimum* and *S. costatum* under axenic conditions and showed that under any combination of initial cell densities, *P. minimum* out-competed *S. costatum* over time

(Tameishi et al., 2009). In addition, we reported that allelochemical(s), including a polysaccharide produced by *P. minimum,* significantly inhibited the growth of *S. costatum* (Tameishi et al., 2009). On the other hand, we reported on growth and interactions of *H. akashiwo* and *S. costatum* using bi-algal cultures under the same conditions and found that the growth of either species could be suppressed by the presence of the enriched culture filtrate of the other, depending on cell densities (Yamasaki et al., 2007a). Furthermore, allelopathic polysaccharide–protein complexes (APPCs) produced by *H. akashiwo*, which were detected in field samples at concentrations exceeding their experimentally-determined action threshold, inhibited the growth of *S. costatum* (Yamasaki et al., 2009). However, the growth of *P. minimum* and *H. akashiwo* and their interactions are not well understood.

In the present study, we examined growth of and interaction between *P. minimum* and *H. akashiwo* using bi-algal culture experiments under axenic conditions. We also simulated the growth of the two species in bi-algal cultures using a mathematical model. Furthermore, to clarify whether allelochemicals, reportedly produced by *P. minimum* and *H. akashiwo*, were involved in the growth and interactions of these species, we examined the effects of crude extracts from culture filtrates of both species on the growth of each other and on a third target species, *S. costatum*. *H. akashiwo* is also known to produce reactive oxygen species (ROS) (Oda et al., 1997; Twiner et al., 2001), and several studies have reported that ROS produced by

raphidophytes affect growth and viability of the bacterium *Vibrio alginolyticus* (Miyamoto et al.) Sakazaki deposited as *Oceanomonas alginolytica* Miyamoto et al. (Oda et al., 1997; Kim et al., 1999; Marshall et al., 2005). Therefore, we also examined the effect of ROS produced by *H. akashiwo* on the growth and interactions of *P. minimum* and *H. akashiwo* by using a comparative approach with the raphidophyte *Chattonella antiqua* (Hada) Ono, which is also an ROS producer.

Materials and Methods

Field study

Hakata Bay, Japan, is a small, shallow bay (Shikata et al., 2008; east–west 20 km, north–south 10 km; maximum depth 23 m; tidal range 2 m). The southeastern area of the bay is closed off by a large breakwater. Our investigation was conducted in Hakozaki Fishing Port (33°37′30″N, 130°25′90″E; Shikata et al. 2008; water depth 2.5–4.5 m), which is located inside the breakwater in the southeastern area of the bay.

Seawater sampling was conducted daily or every two days in Hakozaki Fishing Port from May to June 2005. Seawater was sampled from the surface using a plastic bottle (volume 1 liter), and water temperature and salinity were measured using a

dissolved oxygen/temperature/salinity meter (model 85 YSI/Nanotech, Tokyo, Japan). The water samples were used for counting phytoplankton. The sample bottle was gently turned upside down five times before removing a subsample for counting. Vegetative cells of *P. minimum*, *H. akashiwo* and *S. costatum* in triplicate 1-ml subsamples were counted microscopically. Seawater samples were not fixed before counting phytoplankton because counting occurred within 1 h after sampling.

Algal species and culture conditions

Axenic strains of *H. akashiwo* (NIES-10), *S. costatum* (NIES-324), and *Chattonella antiqua* (NIES-1) were obtained from the National Institute of Environmental Studies (NIES, Japan). *Prorocentrum minimum* cells were isolated from water collected at Hakozaki Fishing Port (34°24′58″N, 130°12′20″W), Fukuoka, Japan, in 1996 and repeatedly washed using capillary pipettes. Thereafter, these strains were tested for bacterial contamination using the fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) staining method (Porter and Feig, 1980) and all were verified as axenic. Cultures were maintained in 100-ml flasks containing 50 ml modified SWM-3 medium (Yamasaki et al., 2007a), at a salinity of 25 at 25 °C under a photon flux density of 228 (±5) μmol quanta m^{-2} s^{-1} of cool-white fluorescent illumination on a 12:12 h light:dark cycle. The modified SWM-3 medium (pH 7.8-8.0) was autoclaved

(121 °C, 15 min) and buffered (tris(hydroxymethyl) aminomethane; Wako Pure Chemical Industries, Ltd., Osaka, Japan) to prevent a change of pH during the culture period. Irradiance in the incubator was measured with a Quantum Scalar Laboratory (QSL) irradiance sensor (Biospherical Instruments, San Diego, California, USA).

Bi-algal culture experiments

Bi-algal culture experiments were conducted in 100-ml flasks containing 50 ml of medium. *Prorocentrum minimum* cells in stationary phase (stock culture: 4–5 × 10^5 cells ml^{-1}) were inoculated to a final cell density of 10^2 or 10^4 cells ml^{-1} in all combinations into cultures of *H. akashiwo* (stock culture: 5–6 × 10^5 cells ml^{-1}) with cell densities of 10^2 or 10^4 cells ml^{-1}. The resulting combinations of initial cell densities of *P. minimum* and *H. akashiwo* were, respectively: (1) 10^2 cells ml^{-1} each; (2) 10^2 and 10^4 cells ml^{-1}; (3) 10^4 and 10^2 cells ml^{-1}; and (4) 10^4 cells ml^{-1} each. As controls, both *P. minimum* and *H. akashiwo* were cultured individually at initial cell densities of 10^2 and 10^4 cells ml^{-1}. Three replicate flasks were used for each treatment. All flasks were gently mixed by hand once a day and randomly rearranged to minimize the effects of light or temperature gradients in the incubator. *Prorocentrum minimum* and *H. akashiwo* cells were counted under a microscope in 200- to 1000-µl subsamples collected at 2-d intervals over the total 12-d duration of

the bi-algal culture experiments. When cell densities exceeded 2×10^5 cells ml^{-1}, subsamples were diluted 10× to 20× with fresh modified SWM-3 medium before counting.

To determine whether ROS produced by *H. akashiwo* affected the growth of *S. costatum*, we also conducted bi-algal culture experiments using the ROS-producer *C. antiqua* with *P. minimum*, and *C. antiqua* with *S. costatum*. Culture conditions for these experiments were the same as for the bi-algal experiments involving *P. minimum* and *H. akashiwo*, except that initial cell densities were equal, at 10^2 cells ml^{-1}.

Macronutrient analysis

Samples for macronutrient analysis were collected at the beginning of the bi-algal experiments by passing 1 ml of each culture through a 0.22-µm syringe filter (Millipore, Billerica, Massachusetts, USA) and freezing the filtrate 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 (Millipore) on a 47-mm polysulfone holder (Advantec, Tokyo, Japan). Filtrates were then passed through 0.22-µm syringe filters and frozen at –80 °C until analysis for macronutrients. Nitrogen (NO_2^- + NO_3^-) and phosphorus (PO_4^{3-}) were measured with an

autoanalyzer (TRACCS 800; Bran + Luebbe, Hamburg, Germany) after thawed samples were diluted 40× to 90× with Milli-Q water (Millipore).

Growth simulation of bi-algal cultures

To model the behavior of the bi-algal cultures, we adopted the growth simulation of Uchida et al. (1999). The following equations were used for the simulation:

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

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

Here, x and y are the cell densities of *H. akashiwo* and *P. minimum*, respectively; r_x and K_x are the growth rate and carrying capacity, respectively, of *H. akashiwo*; and r_y and K_y are the corresponding parameters for *P. minimum* in mono-algal culture. A measures the degree of inhibition of *H. akashiwo* by *P. minimum*, and B the inhibition of *P. minimum* by *H. akashiwo*. When we set $A = ar_xK_x^{-1}$ and $B = br_yK_y^{-1}$, Equations (1) and (2) become the same as the formulae for the growth of populations competing with each other for limited resources (Iwasa, 1998). Parameters a and b are non-dimensional, and measure the degree of inhibition by the other species when

compared to self-interference. When each species is cultured alone, $a = b = 0$. The logistic parameters (r_x, r_y, K_x, K_y) were estimated from Equations (1) and (2) using the data from the mono-algal cultures. Then, the parameters a and b were calculated from Equations (1) and (2) using the bi-algal culture data. Parameters a and b were estimated precisely by the Marquardt method (Marquardt, 1963), with the most appropriate values determined as those that yielded the minimum difference between the squared values of observed cell density and theoretical cell density.

Preparation of crude extracellular compounds

To check for possible extracellular compounds with growth-inhibitory effects, we prepared extracts from filtrates of media used for the monocultures of *P. minimum* and *H. akashiwo*. For *P. minimum* extracts, cells were inoculated into eight 100-ml flasks containing 50 ml modified SWM-3 medium at an initial density of 10^2 cells ml^{-1}. After 17 days (cell density: 4–5 × 10^5 cells ml^{-1}), 50 ml of culture from each of the eight flasks was combined for a total of 400 ml, which was gravity-filtered through a 5.0-μm pore-size membrane filter. This filtrate then was filtered through a 0.22-μm syringe filter and preserved at 4 °C until further processing by ultrafiltration.

Ultrafiltration was performed using a stirred cell (model 8400; Millipore) with a

regenerated cellulose membrane (molecular weight cutoff [MWCO]: 3000 Da; Millipore). Before ultrafiltration, the membrane was pre-washed using 500 ml of 5% (wt:wt) NaCl solution followed by 500 ml of Milli-Q water. Then, the membrane was placed in the ultrafiltration cell and rinsed by passing 100 ml of Milli-Q water through the membrane at working pressure (392 kPa). The 400 ml of *P. minimum* filtrate was concentrated to one-tenth of its original volume using the ultrafiltration membrane at room temperature under N_2 gas at 392 kPa. To remove the remaining low-molecular-weight (LMW) substances from the 40 ml of retentate, 200 ml sterile seawater was added to the 40 ml retentate in the stirred cell and this 240 ml solution was concentrated to a final volume of 40 ml. The retentate obtained was dialyzed against deionized water for 3 d at 4 °C using dialysis membranes with a 3500-Da MWCO (Spectrum Laboratories, Rancho Dominguez, California, USA). After the dialysis, the inner solution was frozen at –80 °C and lyophilized with a Free Zone 2.5 freeze-drying apparatus (Labconco, Kansas City, Missouri, USA).

For *H. akashiwo* extracts, cultures were inoculated at a density of 100 cells ml^{-1} into two 200-ml glass flasks containing 100 ml modified SWM-3 medium. After 20 days (cell density: 5–6 × 10^5 cells ml^{-1}), 100-ml culture samples from each flask were combined to give a total of 200 ml. This was gravity-filtered through a 5.0-μm pore-size membrane filter (Millipore) and then through a 0.45-μm pore-size membrane filter (Millipore). The filtrate was dialysed and lyophilized under the same

conditions as described above for *P. minimum*.

Bioassays using 48-well plates

We performed bioassays to check for growth-inhibitory substances in the crude culture-medium extracts. Bioassays were conducted using 48-well plates (Corning, New York, New York, USA). Ten microliters of each target cell suspension (initial cell density: 10^2 cells ml^{-1}) was added to 990 µl of each solution of crude extract dissolved in modified SWM-3 medium to be tested. Final concentrations of crude extracts in bioassays were 12.5, 25, 50 and 100 µg ml^{-1}. Each bioassay was run in triplicate. For controls, 10 µl of each target species suspension (*P. minimum*, *H. akashiwo*, or *S. costatum*) was added to well plates containing 990 µl of modified SWM-3 medium without any added crude extract. After 5–6 d of incubation, the cells in each of five 10-µl subsamples from each well were counted microscopically. If necessary, the samples were diluted 10× to 20× with modified SWM-3 medium.

Detection of reactive oxygen species (ROS)

To detect ROS, we carried out a chemiluminescence analysis using L-012 (Wako Pure Chemical Co.) as a chemiluminescent probe (Imada et al., 1999; Kadomura et

al., 2006). The probe was dissolved in distilled water and stored at –80 °C until use. The reaction mixtures typically consisted of 80 µl of a flagellate cell suspension (*H. akashiwo*, *P. minimum*, or *C. antiqua*), 10 µl of L-012 (final concentration, 10 µmol l^{-1}), and 10 µl of superoxide dismutase (SOD; Cu, Zn-SOD, Wako Pure Chemical Co.) solution as a radical scavenger (final concentration, 100 U ml^{-1}; "+SOD") or 10 µl of modified SWM-3 medium ("–SOD"). After the addition of L-012 into each flagellate cell suspension in a 1.5 ml-cuvette, the chemiluminescence was recorded immediately with a Bio-Orbit Luminometer (1254-001; Bio-Orbit Oy, Turku, Finland). As a control, we measured the chemiluminescence response in modified SWM-3 medium containing the probe alone, without added flagellate cells. Chemiluminescence analysis was performed at 26 °C and in triplicate.

Results

Daily fluctuations of *P. minimum*, *H. akashiwo* and *S. costatum* cell densities in the field

Field sampling from May to June 2005 at Hakozaki Fishing Port showed that cell densities of *P. minimum*, *H. akashiwo* and *S. costatum* fluctuated from 0 to 4.3×10^4, 6.5×10^4 and 8.4×10^4 cells ml^{-1}, respectively. These species appeared and

alternately bloomed (bloom defined by cell density > 10^3 cells ml^{-1}) from May to June 2005 in Hakozaki Fishing Port, and dense blooms (cell density > 10^4 cells ml^{-1}) were also sometimes observed (Fig. 1). Although the three species could coexist when they coincidentally reached high cell density in late May (>10^4 cells ml^{-1}), when only one species reached a high cell density, the others did not reach high cell densities (Fig. 1). It is unlikely that water temperature or salinity affected *P. minimum*, *H. akashiwo* and *S. costatum* growth because these factors did not fluctuate widely during the field study (data not shown).

Bi-algal culture experiments with *P. minimum* and *H. akashiwo*

When initial cell densities of both *P. minimum* and *H. akashiwo* were 10^2 cells ml^{-1} in bi-algal culture (Fig. 2A), patterns of growth and maximum cell density of *H. akashiwo* were almost the same as in the mono-algal cultures. In contrast, the growth of *P. minimum* was strongly suppressed from day 4 onward, when *H. akashiwo* reached densities of 10^4 cells ml^{-1}, after which the average maximum cell density of *P. minimum* was about 20% of that in mono-algal cultures.

When initial cell densities of *P. minimum* and *H. akashiwo* in bi-algal cultures were 10^2 cells ml^{-1} and 10^4 cells ml^{-1}, respectively (Fig. 2B), *P. minimum* growth was notably suppressed beginning on day 2, when *H. akashiwo* reached late exponential

growth phase, after which the average maximum cell density of *P. minimum* was about 1.4% of that in mono-algal cultures. However, with these initial cell densities, *H. akashiwo* growth was not affected by the presence of *P. minimum* (Fig. 2B).

When initial cell densities of *P. minimum* and *H. akashiwo* were 10^4 cells ml^{-1} and 10^2 cells ml^{-1}, respectively (Fig. 2C), patterns of growth and maximum cell density of *P. minimum* in bi-algal culture were almost identical to those in mono-algal culture. In contrast, the presence of *P. minimum* strongly inhibited the growth of *H. akashiwo*, and the cell density of *H. akashiwo* in bi-algal cultures drastically decreased beginning on day 6 (Fig. 2C). In addition, the average cell density of *H. akashiwo* in bi-algal cultures on day 12 was about 0.3% of that in mono-algal cultures.

When initial cell densities of both *P. minimum* and *H. akashiwo* were 10^4 cells ml^{-1} (Fig. 2D), patterns of growth and maximum cell density of *P. minimum* were almost identical in both bi-algal and mono-algal cultures. In contrast, the average maximum cell density of *H. akashiwo* was about 27% of that in mono-algal cultures, and the growth of *H. akashiwo* was strongly suppressed in bi-algal cultures beginning on day 4 (Fig. 2D).

Macronutrient analysis indicated that nitrogen ($NO_2^- + NO_3^-$) and phosphorus (PO_4^{3-}) concentrations decreased to less than 50 μmol l^{-1} (nitrogen) and 2.5 μmol l^{-1} (phosphorus) in all cultures by the end of the 12-d incubations.

Growth simulations of bi-algal cultures

The values of all parameters for Equations (1) and (2) used in the growth simulations are shown in Table 1. Note that the growth patterns of *H. akashiwo* and *P. minimum* predicted using these values are similar to those observed in the bi-algal culture experiments (Fig. 3). We also used the values of the parameters in Table 1 to calculate isoclines (where $dx/dt = 0$ and $dy/dt = 0$) and trajectories of populations of the two species under various initial cell densities (Fig. 4). All simulated trajectories pass through three stages: (1) both species grow, but growth of *H. akashiwo* precedes that of *P. minimum* because the growth rate of *H. akashiwo* is higher than that of *P. minimum* (Fig. 4, area I); (2) *H. akashiwo* numbers gradually decrease and *P. minimum* gradually becomes dominant (Fig. 4, area II); and (3) *P. minimum* finally out-competes *H. akashiwo* (Fig. 4, area III).

Effects of crude extracts on phytoplankton growth

Crude extracts from *P. minimum* culture filtrate inhibited *S. costatum* growth in a concentration-dependent manner. However, these extracts did not affect *H. akashiwo* growth (Fig. 5A). Similarly, crude extracts from *H. akashiwo* culture filtrate inhibited

S. costatum growth in a concentration-dependent manner but did not affect *P. minimum* growth (Fig. 5B).

Relative ROS levels in phytoplankton

To ascertain whether the *H. akashiwo* strain used in bi-algal culture experiments had the ability to produce ROS, as previously reported (Oda et al., 1997; Twiner et al., 2001), we assayed the cell suspensions of *H. akashiwo* kept in modified SWM-3 medium at room temperature for chemiluminescence. After the addition of L-012, a rapid and immediate chemiluminescence response was observed and then the chemiluminescence response gradually decreased (Fig. 6). Similarly, using *C. antiqua* cell suspension as a positive control, a rapid chemiluminescence response was also observed, but its intensity was higher than that of *H. akashiwo* (Fig. 6). Because the chemiluminescence responses of both species were reduced to background levels by the addition of SOD, it appears that the responses were mainly caused by superoxide (O_2^-). No chemiluminescence response was observed for *P. minimum* (Fig. 6).

ROS effects in bi-algal cultures

We grew *C. antiqua*, a known ROS-producing species (Kim et al., 2004, 2005), in bi-algal cultures with *P. minimum* or *S. costatum*, to observe any effects on growth. *Chattonella antiqua* did not affect the growth of *P. minimum* (Fig. 7A) or *S. costatum* (Fig. 7B). Similarly, *C. antiqua* growth was not inhibited by *P. minimum* (Fig. 7A) or *S. costatum* (Fig. 7B).

Discussion

Mono-specific and multi-species blooms have been observed in various coastal waters, and many studies have investigated growth and interactions of two species (Rice, 1984; Cembella, 2003; Legrand et al., 2003; Granéli and Hansen, 2006). However, it is also essential to study the growth and interactions between multiple pairs of species because any number of different phytoplankton species can coexist in the field. Alternating blooms of *P. minimum*, *H. akashiwo* and *S. costatum* have been observed in various coastal waters (Pratt, 1966; Karentz and Smayda, 1984; Kondo et al., 1990; Smayda and Borkman, 2003), and this suggests that growth characteristics and species interactions may contribute to the formation of these alternating blooms.

In this study, to verify the role of growth and interactions of *P. minimum* and *H. akashiwo* in bloom formation, we conducted bi-algal culture experiments under

axenic conditions using four combinations of initial cell densities of the two species. Our results indicated that either species could suppress the growth of the other, depending on the relative cell densities (Fig. 2). When both species were inoculated at low cell density (10^2 cells ml^{-1}) or high cell density (10^4 cells ml^{-1}), maximum cell densities of *H. akashiwo* were slightly suppressed when *P. minimum* reached the stationary phase (Fig. 2A, D). However, when the initial cell density of *H. akashiwo* (10^4 cells ml^{-1}) was higher than that of *P. minimum* (10^2 cells ml^{-1}), *P. minimum* growth was substantially inhibited. In contrast, when *P. minimum* and *H. akashiwo* were inoculated in bi-algal cultures at 10^4 and 10^2 cells ml^{-1}, respectively (Fig. 2C), *H. akashiwo* growth was substantially inhibited and the inhibitory effect of *P. minimum* on *H. akashiwo* was much stronger than when both species started at the same cell densities (10^2 or 10^4 cells ml^{-1}).

In the field study, we observed alternating blooms of *P. minimum*, *H. akashiwo* and *S. costatum* (Fig. 1) as in previous studies (Pratt, 1966; Karentz and Smayda, 1984; Kondo et al., 1990; Smayda and Borkman, 2003). Interestingly, we found that *P. minimum*, *H. akashiwo* and *S. costatum* could coexist when they all reached high cell density (>10^4 cells ml^{-1}) in late May, but when one species reached high cell density first, the others did not reach high cell density (Fig. 1, from mid-June to late June).

These field observations are in good agreement with the laboratory results from

the bi-algal culture experiments of this study and previous laboratory studies (Yamasaki et al., 2007a; Tameishi et al., 2009); that is, the species first reaching high cell density limited the cell densities of the other species, but when the initial cell densities of *P. minimum*, *H. akashiwo* and *S. costatum* are high, the growth of each species is only weakly suppressed. Thus, in addition to several environmental factors, the growth characteristics and interactions of *P. minimum*, *H. akashiwo* and *S. costatum* could explain why these species alternately bloomed in Hakozaki Fishing Port.

Nitrogen (NO_2^- + NO_3^-) and phosphorus (PO_4^{3-}) concentrations in all bi-algal culture experiments decreased to less than 50 μmol l^{-1} (nitrogen) and 2.5 μmol l^{-1} (phosphorus) by the end of the 12-d incubations, although the growth patterns of *P. minimum* and *H. akashiwo* varied depending on initial densities. For example, when both species were inoculated at high cell densities, the inhibitory effects of each species on growth of the other was weaker than in the other combinations of initial cell densities (Fig. 2). Therefore, it is unlikely that macronutrient limitation was a main factor in the growth interactions between *P. minimum* and *H. akashiwo*.

Previously, Yamasaki et al. (2007a) investigated allelopathic interactions between *S. costatum* and *H. akashiwo* and found that the two species steadily approached a stable equilibrium point of about 3.4×10^5 cells ml^{-1} and 4.8×10^5 cells ml^{-1}, respectively, when they coexisted in culture. In contrast, Tameishi et al.

(2009) examined growth and interactions of *P. minimum* and *S. costatum*, and reported that *P. minimum* out-competed *S. costatum* in all combinations of initial cell densities of the two species. In the present study, we used the parameter values in Table 1 to calculate isoclines and trajectories of populations of *P. minimum* and *H. akashiwo* under various initial cell densities (Fig. 4). This model indicates that *P. minimum* will always out-compete *H. akashiwo* in bi-algal cultures under all experimental conditions because the estimated parameters showed $K_y < aK_x$ and $bK_y < K_x$ (Iwasa, 1998). The growth and interactions of *P. minimum*, *H. akashiwo* and *S. costatum*, as understood from the present and previous studies (Yamasaki et al., 2007a; Tameishi et al., 2009), suggest that *P. minimum* has a survival strategy superior to *H. akashiwo* and *S. costatum*. However, the growth patterns of these species as predicted using values derived from laboratory experiments are not necessarily similar to those observed in the field (e.g. Fig. 1). The parameters used for model predictions are based on results obtained under fixed environmental conditions of temperature, light intensity, photoperiod, pH, salinity and using a culture medium with artificially high nutrient levels. Further study is necessary to determine if the degree of inhibition between these three species is affected by these factors. Furthermore, it is necessary to better integrate parameters for interactions between phytoplankton species into existing models for seasonal phytoplankton blooms (e.g. nutrient–phytoplankton [NP] models) for more accurate prediction of

phytoplankton blooms.

To identify the factors affecting the growth of and interaction between *P. minimum* and *H. akashiwo*, we examined the effects of crude extracts from cultures of these species on each other and on *S. costatum*. These extracts include the allelochemicals produced by *P. minimum* (polysaccharide[s]; Tameishi et al., 2009) and *H. akashiwo* (polysaccharide–protein complexes; Yamasaki et al., 2009). These crude extracts did not affect the growth of *P. minimum* or *H. akashiwo*, but strongly inhibited the growth of *S. costatum* (Fig. 5), and these results agree with our previous studies (Tameishi et al., 2009; Yamasaki et al., 2009). Thus, these allelochemicals do not appear to be involved in the growth and interactions between *P. minimum* and *H. akashiwo*, although it is also possible that binding of these allelochemicals mitigates allelopathic effects.

On the other hand, *H. akashiwo* is an ROS producer (Oda et al., 1997; Twiner et al., 2001), and ROS produced by raphidophytes are known to affect the growth and survival of the marine bacterium *Vibrio alginolyticus* (Oda et al., 1997; Kim et al., 1999; Marshall et al., 2005). Therefore, we also examined the possible effects of ROS produced by *H. akashiwo* on the growth and interaction between *P. minimum* and *H. akashiwo*, and found that the *H. akashiwo* strain used in this study produced ROS, but the levels produced were lower than from *C. antiqua* (Fig. 6). In addition, *P. minimum* growth was not inhibited by ROS when cultured with *C. antiqua* (Fig. 7A).

Furthermore, the growth of *S. costatum*, which was strongly inhibited by *H. akashiwo*, was not inhibited by ROS when cultured with *C. antiqua* (Fig. 7B). Thus, it is unlikely that ROS are involved to a great degree in the growth and interactions of *P. minimum* and *H. akashiwo* or between *S. costatum* and *H. akashiwo*.

Both *in situ* and *in vitro* experiments have suggested that allelochemicals, mainly LMW substances, play a key role in the growth dynamics of red-tide blooms through inhibitory effects (Rice, 1984; Cembella, 2003; Legrand et al., 2003; Granéli and Hansen, 2006; Van Rijssel et al., 2008). Previous studies also report that *P. minimum* produces LMW substances as extracellular metabolites (Andersen et al., 1980; Trick et al., 1984) and neurotoxin components (Denardou et al., 1995; Grzebyk et al., 1997; Denardou-Queneherve et al., 1999), and that *H. akashiwo* produces LMW substances as neurotoxin components (Khan et al., 1997; Haque and Onoue, 2002) and fatty acids as allelochemicals (Matsuyama et al., 2000). We cannot say whether the strains used in this study produced these metabolites and toxins, because these substances would have been removed by the ultrafiltration and dialysis. Cell contact between phytoplankton species is another possible factor affecting growth and interaction, and is an important consideration for elucidating the mechanisms of phytoplankton succession (Uchida, 2001; Nagasoe et al., 2006; Yamasaki et al., 2007b). Therefore, future studies should examine the effects of metabolites, LMW toxins, and cell contact on the growth of and interaction between *P. minimum* and *H.*

akashiwo.

Conclusions

We demonstrated that interactions between phytoplankton species could mutually affect growth in laboratory experiments, but there are still yet-to-be-defined mechanism(s) behind the growth and interactions of *P. minimum* and *H. akashiwo*. Physical, chemical, and biological factors are all involved in phytoplankton succession and interactions between phytoplankton species in the field. Therefore, it is necessary to clarify the factors that affect the growth of *P. minimum* and *H. akashiwo*, and to verify the role of growth characteristics and species interactions in natural phytoplankton populations.

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Figure captions

Fig. 1. Fluctuations in cell densities of *Prorocentrum minimum*, *Heterosigma akashiwo* and *Skeletonema costatum* in May and June 2005 at Hakozaki Fishing Port, Japan.

Fig. 2. Growth interactions between *Prorocentrum minimum* and *Heterosigma akashiwo* showing growth of *P. minimum* when cultured alone (hollow circles) or together with *H. akashiwo* (filled circles), and of *H. akashiwo* when cultured alone (hollow triangles) or together with *P. minimum* (filled triangles). Initial cell density of *P. minimum* 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 means ± SD (cells ml^{-1}) of triplicate measurements.

Fig. 3. Growth simulations of *Prorocentrum minimum* and *Heterosigma akashiwo* in bi-algal cultures for various combinations of initial cell densities. (**A**) *P. minimum*: 10^2 cells ml^{-1}, *H. akashiwo*: 10^2 cells ml^{-1}; (**B**) *P. minimum*: 10^2 cells ml^{-1}, *H. akashiwo*: 10^4 cells ml^{-1}; (**C**) *P. minimum*: 10^4 cells ml^{-1}, *H. akashiwo*: 10^2 cells ml^{-1}; (**D**) *P. minimum*: 10^4 cells ml^{-1}, *H. akashiwo*: 10^4 cells ml^{-1}. Lines show simulated growth curves using Equations 1 and 2 (see text) and the parameters in Table 1, and

symbols show actual data from bi-algal cultures. *P. minimum,* filled circles; *H. akashiwo,* filled triangles.

Fig. 4. Simulations of bi-algal cultures of *Prorocentrum minimum* and *Heterosigma akashiwo* showing isoclines (where dx/dt = 0 or dy/dt = 0) and trajectories of *H. akashiwo* and *P. minimum* populations for various initial cell densities.

Fig. 5. Effects of crude extracts prepared from *Prorocentrum minimum* and *Heterosigma akashiwo* culture filtrates on phytoplankton growth. (**A**) Growth of *H. akashiwo* (hollow bars) and *Skeletonema costatum* (solid bars) exposed to crude *P. minimum* extract. (**B**) Growth of *P. minimum* (hollow bars) and *S. costatum* (solid bars) exposed to crude *H. akashiwo* extract. Data are means ± SD (n = 3).

Fig. 6. Evidence of reactive oxygen species (ROS) production by *Heterosigma akashiwo* and *Chattonella antiqua*. Data shown are the L-012-dependent chemiluminescence responses of *C. antiqua* (filled circles, without added superoxide dismutase [–SOD] and hollow circles, with [+] SOD), *H. akashiwo* (filled triangles, –SOD; hollow triangles, +SOD), *P. minimum* (filled squares) and control (modified SWM-3 medium, hollow diamonds). The chemiluminescence responses at room temperature were recorded for 30 s immediately after the addition of L-012 (final

concentration, 10 μmol l^{-1}). All measurements were performed in triplicate.

Fig. 7. Growth interactions between *Chattonella antiqua* (a known ROS-producer) and (A) *Prorocentrum minimum* or (B) *Skeletonema costatum*. Triangles, *C. antiqua*; circles, *P. minimum* or *S. costatum*. Hollow symbols indicate monocultures, and filled symbols indicate bi-algal cultures. All initial cell densities were 10^2 cells ml^{-1}. Data are means ± SD (cells ml^{-1}) of triplicate measurements.

Table 1 Parameters estimated for bi-algal model simulations using Equations 1 and 2; *a* and *b* are dimensionless. See text for details and definitions.

Species	Carrying capacity (*K*) (cells ml^{-1})	Growth rate (*r*) (divisions h^{-1})	(divisions d^{-1})	Interaction rate *a* or *b*	*A* or *B* (ml $cell^{-1}$ s^{-1})
P. minimum	273,800	0.033	0.784	0.358	1.2×10^{-11}
H. akashiwo	632,604	0.051	1.220	2.810	6.3×10^{-12}

Fig. 1

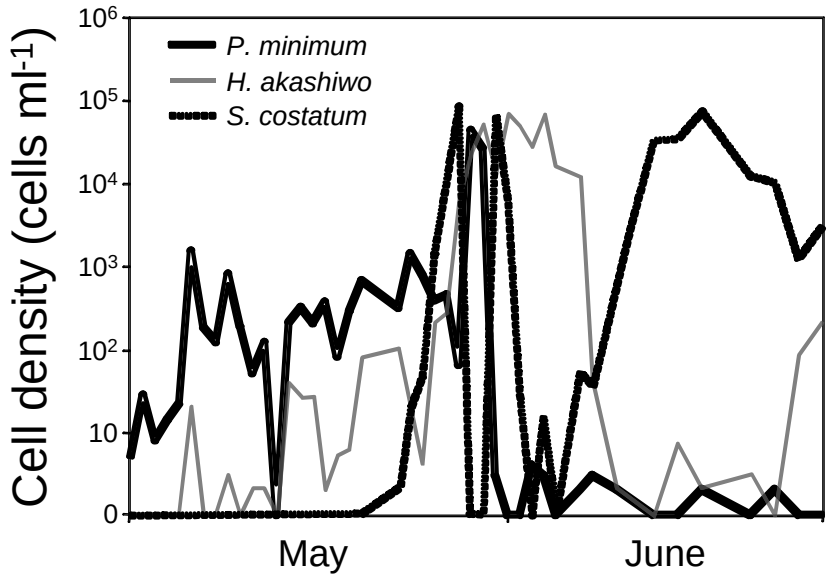

Cell density (cells ml-1)
10^6
10^5
10^4
10^3
10^2
10
0
P. minimum
H. akashiwo
S. costatum
May
June

Fig. 2

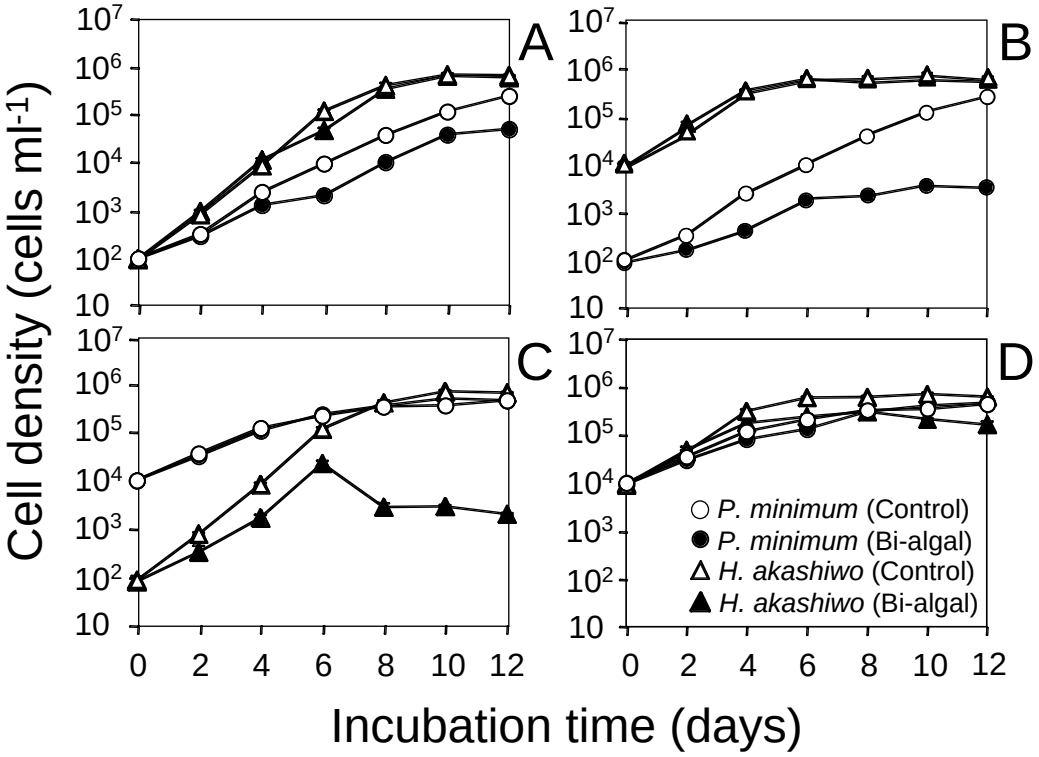

A
B
C
D
Cell density (cells ml-1)
Incubation time (days)
P. minimum (Control)
P. minimum (Bi-algal)
H. akashiwo (Control)
H. akashiwo (Bi-algal)

Fig. 3

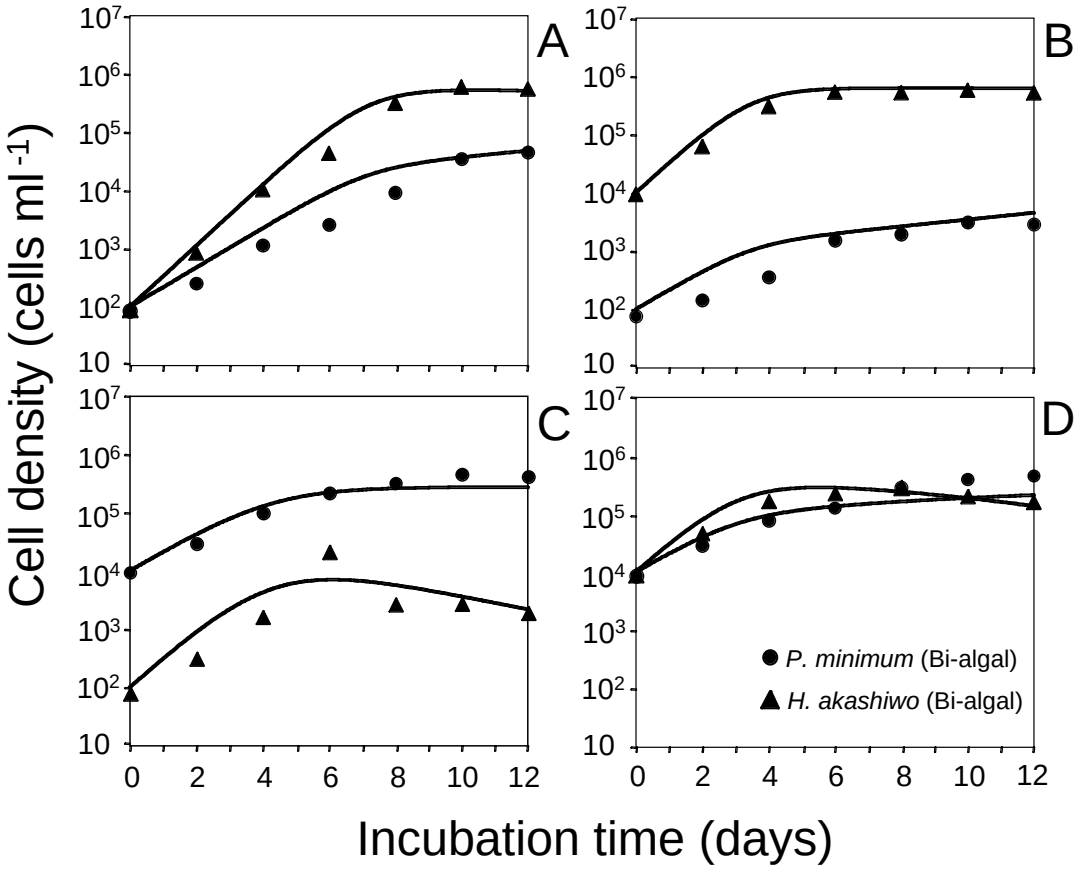

A
B
C
D
Cell density (cells ml-1)
Incubation time (days)
P. minimum (Bi-algal)
H. akashiwo (Bi-algal)

Fig. 4

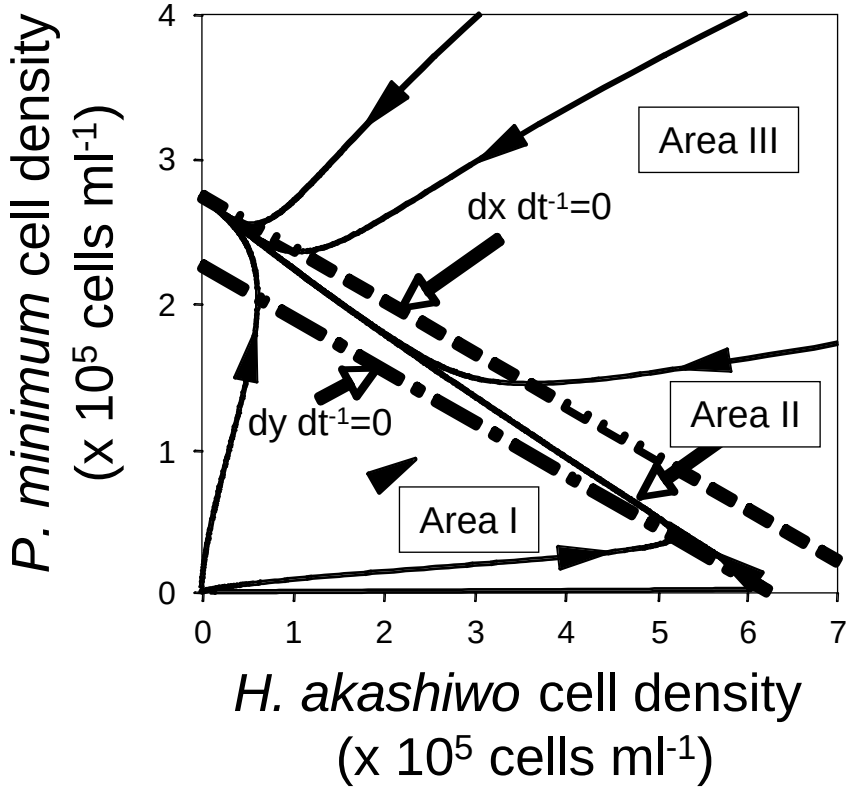

P. minimum cell density
(x 10^5 cells ml^-1)
H. akashiwo cell density
(x 10^5 cells ml^-1)
Area III
dx dt^-1=0
dy dt^-1=0
Area II
Area I
0
1
2
3
4
5
6
7

Fig. 5

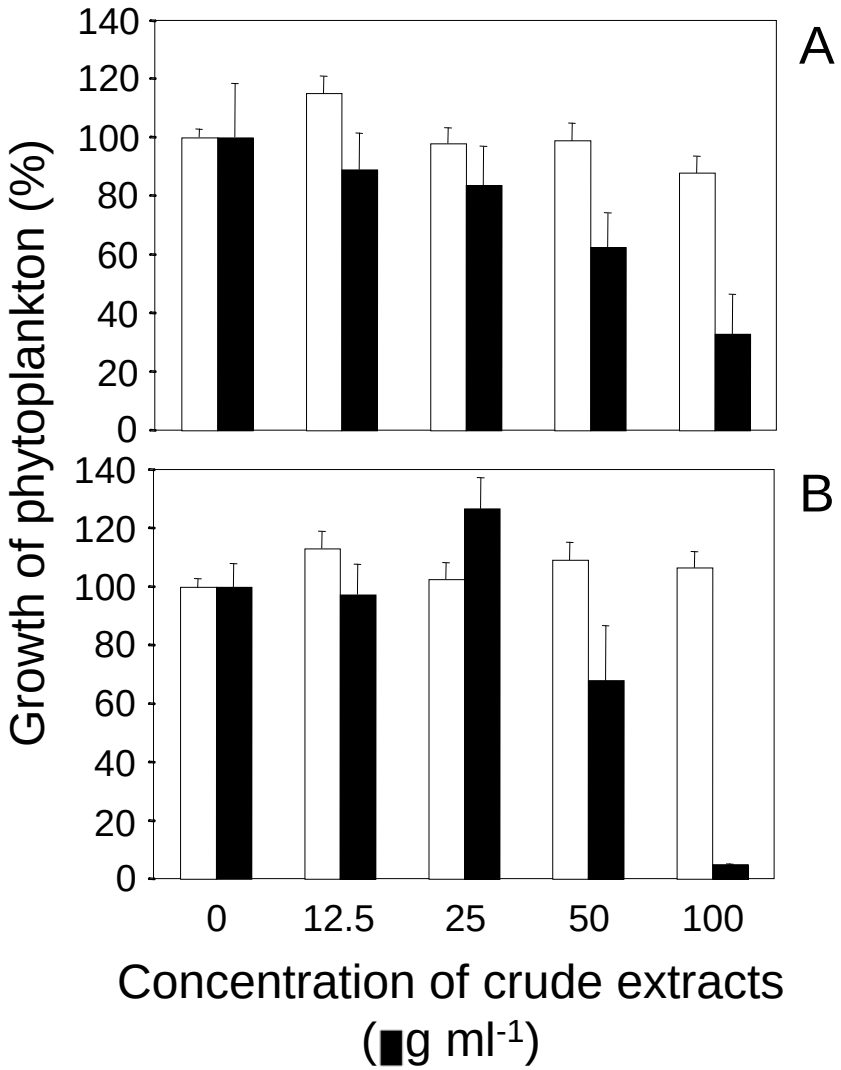

A
B
Growth of phytoplankton (%)
140
120
100
80
60
40
20
0
0
12.5
25
50
100
Concentration of crude extracts
(μg ml-1)

Fig. 6

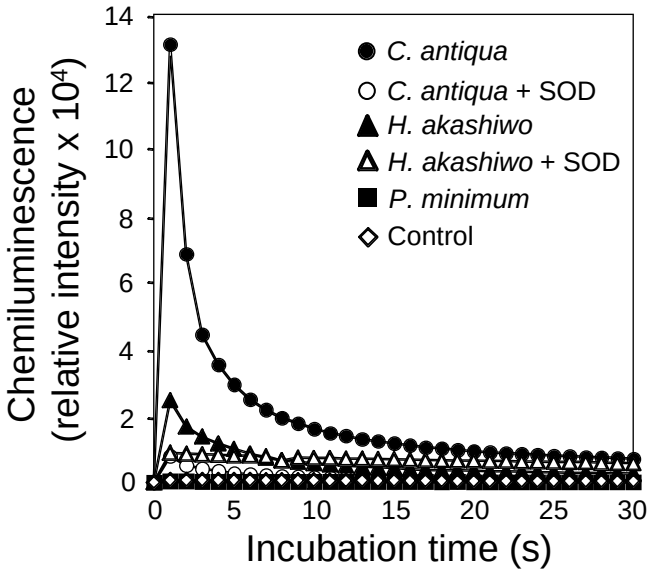
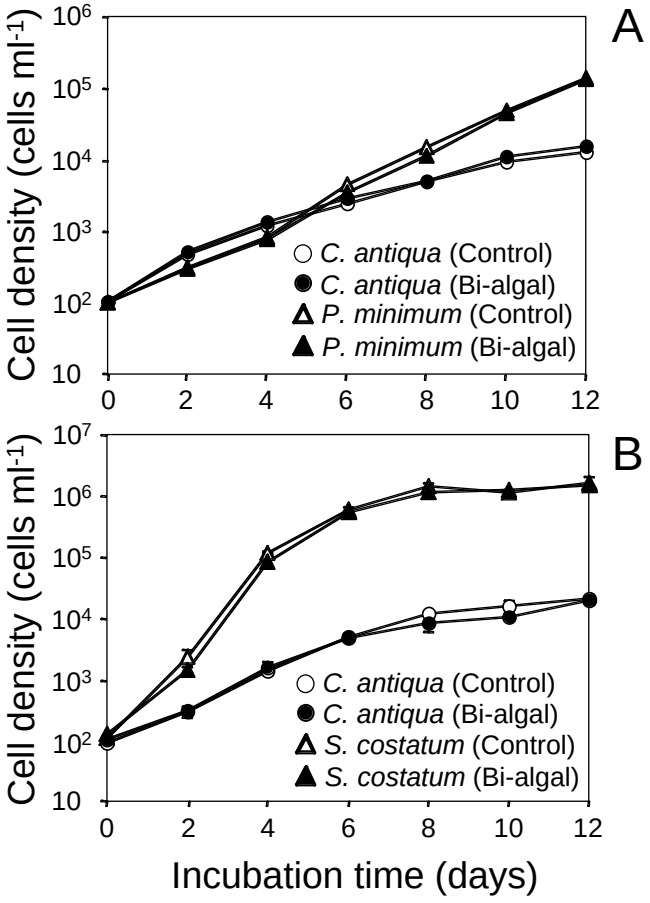

Chemiluminescence
(relative intensity x 10⁴)
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
Incubation time (s)
C. antiqua
C. antiqua + SOD
H. akashiwo
H. akashiwo + SOD
P. minimum
Control

Fig. 7


A
B
Cell density (cells ml-1)
Incubation time (days)
C. antiqua (Control)
C. antiqua (Bi-algal)
P. minimum (Control)
P. minimum (Bi-algal)
S. costatum (Control)
S. costatum (Bi-algal)