

## ORIGINAL ARTICLE

# Extracellular polysaccharide-protein complexes of a harmful alga mediate the allelopathic control it exerts within the phytoplankton community

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**The goal of this study was to examine the significance of allelopathy by the raphidophyte *Heterosigma akashiwo* in a multispecies phytoplankton community in the field. Towards this aim, we sought allelochemicals of *H. akashiwo*, which had allelopathic effect both in laboratory experiments and in the field. As an initial step, we showed that the allelopathic effects of *H. akashiwo* filtrate were both species-specific and dependent upon the cell density of the target species. Secondly, we found for the first time that extracellular, high-molecular weight allelochemicals [that is, polysaccharide-protein complexes (APPCs)] were produced by a marine phytoplankton species, *H. akashiwo*. Thirdly, we indicated that the purified APPCs selectively inhibited the growth of the diatom *Skeletonema costatum* that is a major competitor of *H. akashiwo*, and thereby tended to promote the formation of monospecific *H. akashiwo* blooms. Furthermore, we demonstrated that the inhibitory effect of APPCs on the growth of the diatoms was determined by binding to the cell surface of the target species. Finally, we succeeded in the detection of APPCs in the field samples at concentrations exceeding their experimentally determined action threshold during the *H. akashiwo* bloom. Strategies for ecosystem control, including mitigation of harmful algal blooms (HABs), should take into account that red-tide organisms like *H. akashiwo* are already part of complex webs involving inter-specific allelopathic inhibition and ecosystem control during their dense blooms.**

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## Introduction

Harmful algal blooms are an increasingly serious environmental problem for aquaculture, fisheries and public health in many coastal waters throughout the world (Anderson, 1997). Although there have been various biological and non-biological studies of the distribution and growth of flagellate

populations (Erga and Heimdal, 1984; Karentz and Smayda, 1984; Haigh *et al.*, 1992; Smayda, 1997; Miralto *et al.*, 1999; Diehl *et al.*, 2002; Ianora *et al.*, 2004; Bruggeman and Kooijman, 2007), it is not always clear how certain phytoplankton species can come to dominate a community. Empirical approaches to studying the allelopathic effects and inter-specific interactions among phytoplankton have developed relatively slowly (Figueredo *et al.*, 2007; Gentien *et al.*, 2007; Strom, 2008).

Allelopathy refers to 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). Most

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allelochemicals, including low-molecular weight (MW) organic acids, phenolic substances, alkaloids and terpenoids, are known as 'plant secondary metabolites'—they exist only in plants and are not directly relevant to the maintenance of life. This is considered to be an important factor for elucidating the mechanism of phytoplankton succession. Both *in situ* and *in vitro* experiments have suggested that allelopathy plays a key role in the growth dynamics of red-tide blooms through inhibitory effects (Cembella, 2003; Legrand *et al.*, 2003; Granéli and Hansen, 2006; Tillmann *et al.*, 2007, 2008; Van Rijssel *et al.*, 2008). However, a recent review of the allelopathy of phytoplankton (Granéli and Hansen, 2006) questioned whether many low-MW allelochemicals exist in the field at concentrations exceeding their action threshold. To confirm that allelopathy plays an ecological role, the existence of allelochemicals should be reported in relation to their threshold concentrations on a case-by-case basis.

The raphidophyte *Heterosigma akashiwo* and the diatom *Skeletonema costatum* form alternating blooms in various coastal waters (Pratt, 1966; Honjo *et al.*, 1978; Honjo and Tabata, 1985). In *in situ* and *in vitro* experiments, high concentrations of *Olisthodiscus luteus* (*H. akashiwo*; Honjo, 1994; Smayda, 1998) inhibit *S. costatum* growth, whereas lower concentrations stimulate *S. costatum* growth (Pratt 1966). Similarly, Honjo *et al.* (1978) reported that *H. akashiwo* and *S. costatum* alternated in forming red-tide blooms in the Hakozi Fishing Port. They found that filtrate from dense cultures of *H. akashiwo*, re-enriched with nutrients, inhibited *S. costatum* growth. Furthermore, Honjo and Tabata (1985) reported an apparent and reciprocal codominance between *O. luteus* and diatoms in a 70 m<sup>3</sup> outdoor tank with flowing coastal water. Recently, we investigated growth interactions between *H. akashiwo* and *S. costatum* using bi-algal cultures under axenic conditions, and our findings indicated that the growth of either species could be suppressed by the unidentified allelochemicals of the other, depending on cell densities (Yamasaki *et al.*, 2007).

In this study, we report that *H. akashiwo* produces allelopathic polysaccharide–protein complexes, and that high-molecular weight allelochemicals exist in the field at concentrations exceeding their action threshold during the *H. akashiwo* bloom. The study clearly demonstrates the significance of allelopathy by *H. akashiwo* in a multispecies phytoplankton community in the field.

## Materials and methods

### *Algal species and culture conditions*

Axenic strains of *S. costatum* (NIES-324) and *H. akashiwo* (NIES-10) were obtained from the National Institute for Environmental Studies (NIES), Japan. An axenic strain of *Thalassiosira*

*rotula* (CCMP 1647) was obtained from the US Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). *Prorocentrum minimum* was isolated from Hakozi Fishing Port in 1996, and the axenic strain was obtained by repeatedly washing with a capillary pipette. All strains were tested for bacterial contamination by the fluorochrome 4',6-diamidino-2-phenylindole staining method (Porter and Feig, 1980), and were verified as axenic. Cultures were maintained in 100-ml flasks containing 50 ml modified SWM-3 medium (Yamasaki *et al.*, 2007) without the addition of calcium pantothenate, nicotinic acid, *p*-aminobenzoic acid, biotin, inositol, folic acid and thymine, at a salinity of 25 at 25 °C under 228 ( $\pm 5$ )  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of cool-white fluorescent illumination on a 12:12 h light/dark cycle. In addition, the modified SWM-3 medium was autoclaved (at 121 °C for 15 min) and contained a Tris (hydroxymethyl) aminomethane buffer (Wako Pure Chemical Industries, Ltd.) to prevent pH change during culture. Irradiance in the incubator was measured with a quantum scalar laboratory irradiance sensor (Biospherical Instruments, San Diego, CA, USA).

### *Allelopathic effect of H. akashiwo on phytoplankton growth*

*Heterosigma akashiwo* was inoculated at a density of 10<sup>2</sup> cells per ml into 200-ml glass flasks ( $n = 10$ ) containing 100 ml modified SWM-3 medium. After incubation for 17 days (initial cell density, 4–5  $\times 10^5$  cells per ml), each 100-ml sample from the 10 replicate flasks was combined and passed through a 5.0- $\mu\text{m}$  pore-size membrane filter on a 47-mm polysulphone holder under gravity filtration. Then, 1000 ml filtrate was aerated to avoid an increase in pH and to supply dissolved inorganic carbon (DIC). After aeration, nutrients dissolved in Milli-Q water were added to 1000 ml filtrate to give the same final concentration as in the original modified SWM-3 medium. The filtrate was then passed through a 0.22- $\mu\text{m}$  pore-size filter (Millipore, Billerica, MA, USA). The concentration of protein in the filtrate was determined using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). In addition, bovine serum albumin dissolved in modified SWM-3 medium was used as a standard for the determination of protein. This quantitation assay had three replicates.

A sample (0.5 ml) of each cell suspension of *S. costatum*, *T. rotula*, *P. minimum* and *H. akashiwo* at various inoculum cell densities (initial cell densities, 10, 10<sup>2</sup>, 10<sup>3</sup> and 10<sup>4</sup> cells per ml) was added to 4.5 ml of each test solution (described in the above two paragraphs) in polystyrene test tubes (Evergreen Scientific, Los Angeles, CA, USA). As a control, each species was grown in the modified SWM-3 medium without the addition of any sample solution. This growth experiment had 16 test conditions, with four replicates per test condition.

After the start of incubation, the *in vivo* fluorescence of each species was measured daily with an *in vivo* fluorometer (10-AU-005-CE; Turner Designs, Sunnyvale, CA, USA).

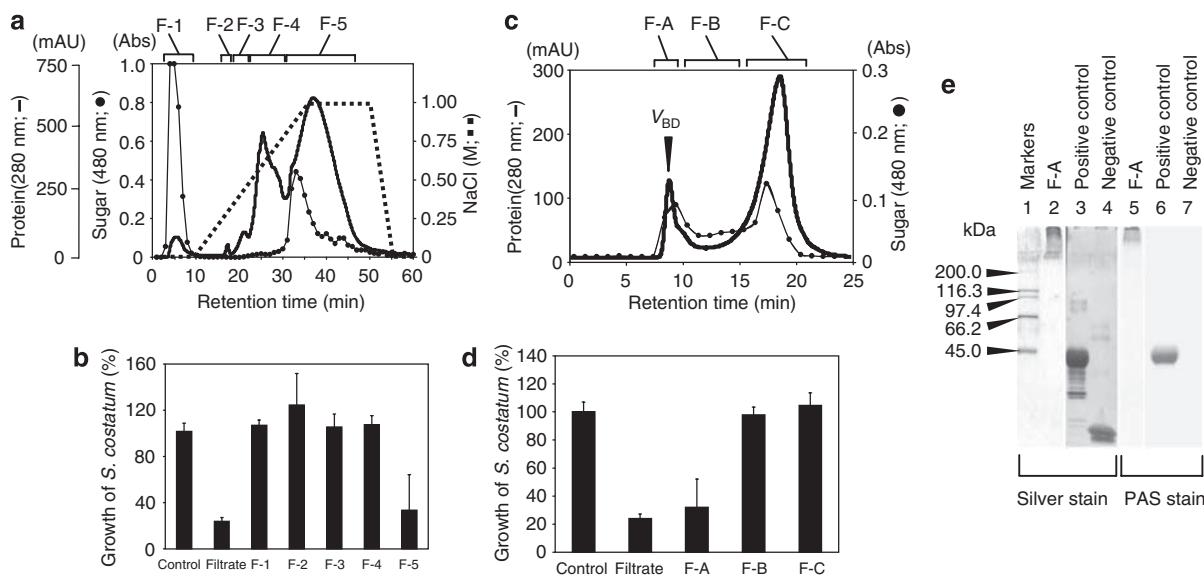
#### Purification of allelochemicals

For the culture, *H. akashiwo* was inoculated at a density of 100 cells per ml into 200-ml glass flasks ( $n=10$ ) containing 100 ml modified SWM-3 medium. After 20 days, 100 ml samples of the culture from each flask were combined to give a total of 1000 ml. This was passed through a 5.0- $\mu\text{m}$  pore-size membrane filter (Millipore) under gravity filtration, and then through a 0.45- $\mu\text{m}$  pore-size membrane filter (Millipore). The filtrate was dialysed against deionized water for 3 days at 4 °C using dialysis membranes with a 3500-Da MW cutoff (MWCO; Spectrum Laboratories, Rancho Dominguez, CA, 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, MO, USA). The crude extract obtained from filtrates of *H. akashiwo* cultures was dissolved in 50 mM Tris-HCl buffer (pH 8.0), and passed through a 0.22- $\mu\text{m}$  syringe filter (Millipore). A sample (2.5 ml) was subjected to a DEAE-Toyopearl Pack column (8 mm I.D.  $\times$  7.5 cm; Tosoh, Tokyo, Japan), which was equilibrated with 50 mM Tris-HCl buffer (pH 8.0), and was eluted with a 0–1 M gradient of NaCl. The flow rate was 1.0 ml min<sup>-1</sup>, and the

eluate was monitored at 280 nm. The total sugar in 1.0 ml of each fraction was determined by the modified phenol-sulphuric acid method (Mckelvy and Lee, 1969). Fraction 5 (Figure 1a) obtained by the anion-exchange chromatography was pooled and dialysed against deionized water for 3 days at 4 °C using dialysis membranes with a 3500-Da MWCO (Spectrum Laboratories). After dialysis, the inner solution was frozen at  $-30$  °C and lyophilized with a Free Zone 2.5 freeze-drying apparatus (Labconco). The freeze-dried sample was dissolved in 50 mM Tris-HCl buffer (pH 7.0) including 200 mM NaCl, and was passed through a 0.22- $\mu\text{m}$  syringe filter (Millipore). The 300- $\mu\text{l}$  sample was then subjected to a TSKgel G-4000SW<sub>XL</sub> column (7.8 mm I.D.  $\times$  30 cm; Tosoh), which was equilibrated with 50 mM Tris-HCl buffer (pH 7.0). The flow rate was 0.8 ml min<sup>-1</sup>, and the eluate was monitored at 280 nm. The total sugar in 0.8 ml of each fraction was determined by the modified phenol-sulphuric acid method (Mckelvy and Lee, 1969). In addition, the inhibitory effect of each fraction obtained from the anion-exchange chromatography and the gel-filtration chromatography on *S. costatum* growth was determined by the bioassay.

#### Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The fraction-A (Figure 1c; F-A) obtained from gel filtration was analysed by SDS-PAGE on 5% gels



**Figure 1** Purification of *H. akashiwo* allelochemicals. (a) Anion-exchange chromatography of a preparation from the culture filtrate of *H. akashiwo* on a high-performance liquid chromatography (HPLC) system equipped with a DEAE-Toyopearl Pack column. (b) Effect of each fraction obtained from anion-exchange chromatography on the growth of *S. costatum* examined using a 48-well plate. This bioassay had three replicates. (c) Gel-filtration profile of the fraction (F-5) obtained from anion-exchange chromatography on a HPLC system equipped with a TSK gel G-4000SW<sub>XL</sub> column. The arrowhead ( $V_{BD}$ ) indicates the elution time of blue dextran (MW  $2 \times 10^6$ ). (d) Effect of each fraction obtained from gel-filtration chromatography on the growth of *S. costatum* examined using a 48-well plate. This bioassay had three replicates. (e) SDS-PAGE of F-A conducted using 5% polyacrylamide gel. After electrophoresis, the gels were stained with either silver stain (lane 1–4) or PAS stain (lane 5–7). Lane 1, the molecular mass standard markers; lane 2, 5, F-A obtained from gel-filtration chromatography; lane 3, 6, positive control (horseradish peroxidase); and lane 4, 7, negative control (soybean trypsin inhibitor).

according to the Laemmli method (Laemmli, 1970). After electrophoresis, the gels were silver-stained and periodic acid-Schiff (PAS)-stained for visualization of protein and glycoprotein bands, respectively.

For deglycosylation, the fraction-A (Figure 1c; F-A) was treated with glycopeptidase F (TaKaRa) under denaturing conditions at 37 °C for 24 h (Tarentino *et al.*, 1985), and was treated with alkaline (Morelle *et al.*, 1998), respectively. After deglycosylation with glycopeptidase F and alkaline treatments, each sample was analysed by SDS-PAGE as described above.

#### *Amino-acid and sugar-composition analyses*

The amino-acid and sugar compositions of fraction-A (Figure 1c; F-A) were analysed by the ninhydrin method (Moore and Stein, 1948) and post-column fluorometric detection (Mikami and Ishida, 1983), respectively.

#### *Bioassays using 48-well plates*

*Skeletonema costatum* cells in the stationary phase (10 to 12 × 10<sup>5</sup> cells per ml) were diluted to 10<sup>4</sup> cells per ml with modified SWM-3 medium. A sample (10 µl) of the *S. costatum* cell suspension was added to 990 µl of each sample solution to be tested in 48-well plates (initial cell density, 10<sup>2</sup> cells per ml). Each bioassay had three replicates. As a control, *S. costatum* was grown in modified SWM-3 medium without the addition of any sample solution. After incubation for 5 days, the cells in each of five 10-µl subsamples from each well were counted microscopically. If necessary, the samples were diluted by a factor of 10–20 with modified SWM-3 medium.

*Antibody production against allelopathic polysaccharide–protein complexes (APPCs) in a rabbit* APPCs (F-A, Figure 1c) was purified as described above. The F-A fractions were pooled, dialysed and lyophilized. An adult New Zealand white rabbit was used to produce polyclonal antibodies against APPCs of *H. akashiwo*. Immunological cross-reaction of polyclonal antibody against 11 species of phytoplankters was examined by the dot-blot analysis. Details of methods are given in the Supplementary Figure 1.

#### *Indirect fluorescent-antibody test (IFAT)*

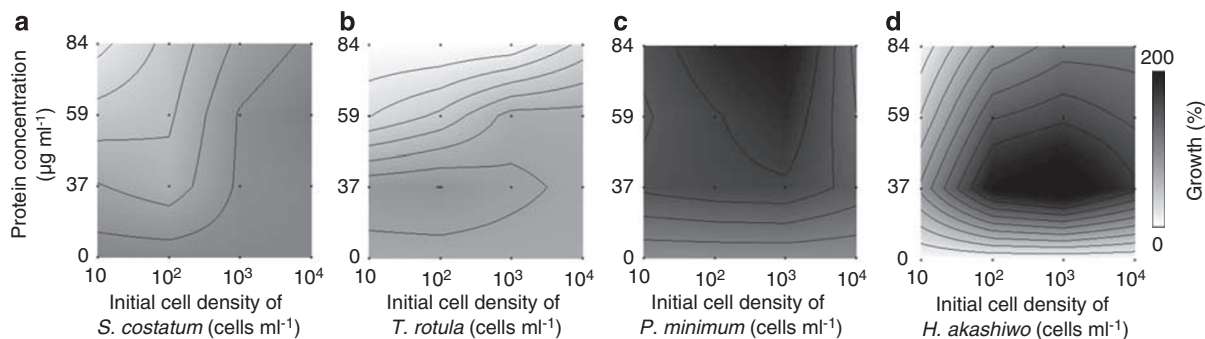
*Skeletonema costatum* (7 × 10<sup>5</sup> cells per ml), *T. rotula* (13 × 10<sup>4</sup> cells per ml) and *P. minimum* (4 × 10<sup>5</sup> cells per ml) were collected from each 10 ml culture by centrifugation, and were treated with 1 ml of APPCs (F-A, Figure 1c) solution dissolved in modified SWM-3 medium (100 µg per ml) as the antigen. After incubation for 90 min at 25 °C, the APPCs solution was removed from each tube and the cells were washed three times in modified SWM-3 medium. The modified SWM-3 medium was then removed, and the first antibody solution, which was produced by dissolving 100 µl antiserum

in 900 µl-modified SWM-3 medium, was added. After incubation for 50 min at 25 °C, the first antibody solution was removed from each tube, and the cells were washed three times in modified SWM-3 medium. The modified SWM-3 medium was then removed, and the second antibody solution (fluorescein isothiocyanate-conjugated anti-rabbit γ-globulin IgG of goat; ZYMED Laboratories, South San Francisco, CA, USA), which was produced by dissolving 20 µl antiserum in 980 µl modified SWM-3 medium, was added. After incubation for 50 min at 30 °C under dark conditions, the second antibody solution was removed from each tube and the cells were washed three times in modified SWM-3 medium. They were then observed using a fluorescent microscope (BZ-9000; Keyence, Osaka, Japan) under the following conditions: excitation wavelength, 488 nm; absorption wavelength, 530 nm; exposure time, 0.17 s for *S. costatum*, *T. rotula* and *P. minimum*, and 0.33 s for *H. akashiwo*. In addition, *H. akashiwo* was tested using the method described above in the absence of APPCs solution.

#### *Field study*

Surface seawater was sampled at Hakozaki Fishing Port from May to June 2007. The sampling was conducted every 3 or 4 days, or daily during the *H. akashiwo* bloom. After sampling, the numbers of phytoplankters in each of the two 500-µl subsamples were counted microscopically without fixation. After sampling, a 10-ml portion of each 1000 ml sample was passed through a 0.22-µm pore-size filter, and frozen at –80 °C until use. APPCs (F-A, Figure 1c) of *H. akashiwo* in seawater samples were detected by dot-blot analysis.

A 300-ml seawater sample from each of the *H. akashiwo* and *S. costatum* blooms was passed through a 5.0-µm pore-size membrane filter (Millipore) under gravity filtration to remove particulates, including phytoplankton, and then through a 0.45-µm pore-size membrane filter (Millipore). These filtrates were dialysed and lyophilized as described above. A bioassay was conducted on each sampling day, to estimate the effect of the collected seawater samples on *S. costatum* growth. A 50-ml portion of each 1000 ml sample was passed through a 0.45-µm pore-size filter (Steriflip; Millipore) to remove microparticles. Nutrients dissolved in Milli-Q water were added to each 50 ml sample of filtrate to give the same final concentration as in the original modified SWM-3 medium. The filtrate was then passed through a 0.22-µm pore-size filter (Millipore). The bioassay was conducted using 48-well plates (Corning, Corning, NY, USA), and had three replicates. Crude extracts from the laboratory culture of *H. akashiwo* and red-tide seawater from *H. akashiwo* were dissolved in modified SWM-3 medium. The protein concentration in each sample was determined by a BCA



**Figure 2** Species-specific allelopathic effects of *H. akashiwo* filtrate. Allelopathic effect of *H. akashiwo* on phytoplankton growth [(a), *S. costatum*; (b), *T. rotula*; (c), *P. minimum*; (d), *H. akashiwo*]. Allelopathic effects were examined as described in the text, and the experiment had four replicates.

protein assay kit (Thermo Fisher Scientific Inc.). Crude extracts from red-tide seawater samples from *S. costatum* (80 µg dry weight) were dissolved in 1 ml modified SWM-3. The effects of each sample on *S. costatum* growth were examined using 48-well plates.

#### Dot-blot analysis

A dry nitrocellulose sheet (Bio-Rad, Hercules, CA, USA) was pre-wetted in Tris-buffered saline (TBS; 20 mM Tris and 500 mM NaCl, pH 7.5), and the pre-wetted membrane was placed in the Bio-Dot SF apparatus (Bio-Rad). To ensure uniform binding of the antigen, the membrane was rehydrated with 100 µl TBS per well. After rehydration, each sample was applied at 5 µl per well, and the entire samples were filtered through the membrane under a gentle vacuum. Each sample well was then washed with 200 µl TBS under a gentle vacuum. After the wells had completely drained, the membrane was removed from the apparatus and placed in the blocking solution (3% gelatin-TBS). The membrane in the blocking solution was gently agitated using an orbital shaker platform and incubated for 40 min at room temperature (RT). After incubation, the blocking solution was removed, and TBS containing 0.05% Tween-20 (TTBS) was added to the membrane. The membrane was washed in TTBS for 10 min with gentle agitation at RT. After washing, the TTBS was removed and first antibody solution, which was a 1 per 100 dilution of the antiserum in antibody buffer (1% gelatin-TTBS), was added to the membrane. After incubation for 2 h under gentle agitation at RT, the unbound first antibody was removed by washing the membrane with TTBS for 5 min at RT, and then with fresh TTBS for 5 min at RT. The TTBS was removed and the second antibody solution, which was a 0.033 per 100 dilution of the antibody conjugate (goat anti-rabbit IgG (H+L) alkaline phosphatase (AP) conjugate; Bio-Rad) in 100 ml antibody buffer, was added. After incubation for 2 h under gentle agitation at RT, the conjugate solution was removed and the membrane was

washed in TTBS for 5 min with gentle agitation at RT. The TTBS was then removed, and the membrane was washed with fresh TTBS for 5 min at RT. The membrane was then washed with TBS for 5 min with gentle agitation to remove residual Tween-20 from the surface. The TBS was removed and the membrane was immersed in colour development solution (Immun-Blot Assay Kit; Bio-Rad). The colour development was stopped by immersing the membrane in distilled water for 10 min with gentle agitation at RT.

## Results and discussion

#### Allelopathic effect of *H. akashiwo* on phytoplankton growth

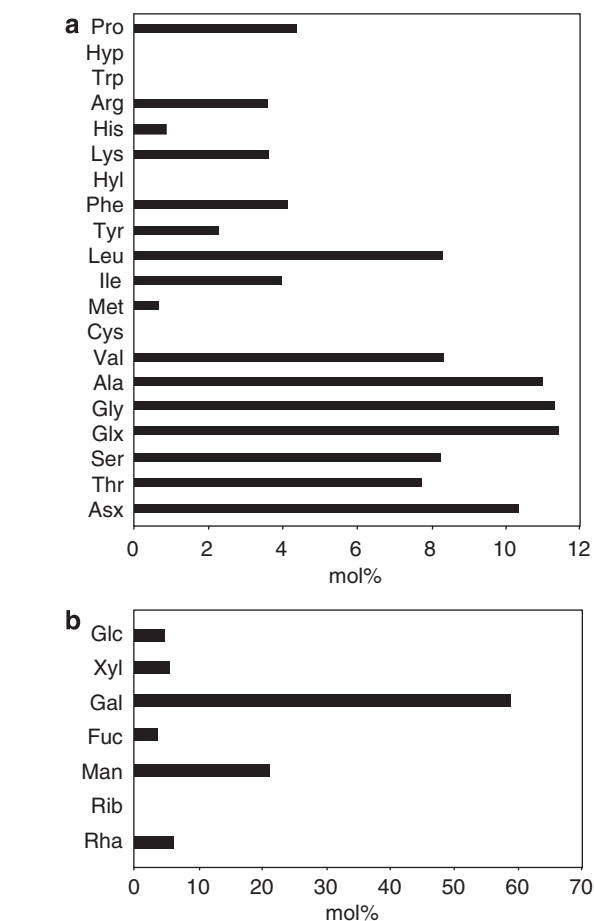
When *S. costatum* was inoculated at low-cell densities and exposed to the filtrate from dense culture of *H. akashiwo*, its growth was markedly inhibited. By contrast, *S. costatum* growth was not inhibited by the filtrate of *H. akashiwo* at all of the concentrations (37–84 µg protein per ml) when it was inoculated at high cell densities (Figure 2a, Supplementary Figure 2). Thus, it is likely that the allelopathic effects of *H. akashiwo* were both species-specific and dependent upon the cell density of the target species. This supported the finding of a previous study (Yamasaki *et al.*, 2007), and suggested that allelopathy drives the alternation of the two species in the field. Similarly, the diatom *T. rotula* was also strongly inhibited depending upon the concentration of protein dissolved in the filtrate of *H. akashiwo* (Figure 2, Supplementary Figure 2). However, *Prorocentrum minimum*, which does occur naturally with *H. akashiwo*, was stimulated by the filtrate of *H. akashiwo* at all of the concentrations (37–84 µg protein per ml) and the target cell densities (10–10<sup>4</sup> cells per ml) tested (Figure 2, Supplementary Figure 2). This result was similar to previous studies, which showed that *P. minimum* was stimulated by organic substances such as humic acids (Kondo *et al.*, 1990; Heil, 2005). Therefore, the stimulation effect of allelochemicals

on *P. minimum* growth might be caused by the utilization of allelochemicals as nutrient sources. On the other hand, the filtrate of *H. akashiwo* at all of the concentrations (37–84 µg protein per ml) did not inhibit *H. akashiwo* at inoculum densities > 10<sup>2</sup> cells per ml (Figure 2d, Supplementary Figure 2).

#### Purification and chemical composition analysis of APPCs

The high-molecular weight fraction obtained from the filtrate of *H. akashiwo* culture was subjected to a DEAE-Toyopearl anion-exchange column chromatography. Based on the UV (280 nm)-monitored protein elution profile, five fractions (F-1 to F-5) were collected (Figure 1a). The elution pattern plotted by the phenol-sulphuric acid method indicated that F-1 and F-5 contained abundant sugar, whereas other fractions (F-2 to F-4) were almost sugarless (Figure 1a). When the allelopathic effect of each fraction was examined by the bioassay using *S. costatum*, its effect was exclusively detected in F-5, whereas no such effect was detected in other fractions (Figure 1b). For further purification and characterization, the fraction F-5 was dialysed against deionized water, lyophilized, dissolved in 50 mM Tris-HCl buffer (pH 7.0) and subjected to gel-filtration chromatography on a TSKgel G-4000SW<sub>XL</sub> column. The three fractions (F-A to F-C), all of which contain sugar, were collected (Figure 1c) and the allelopathic effect against *S. costatum* was examined. Among the three fractions, most of the allelopathic activity was detected in F-A (Figure 1d). As the active fraction (F-A) appeared in the void volume of TSKgel G-4000SW<sub>XL</sub> column, which has an exclusion limit of 10<sup>6</sup>–10<sup>7</sup> Da, the molecular sizes of allelochemical substances produced by *H. akashiwo* were estimated to be > 10<sup>6</sup> Da (Figure 1c). The extremely large molecular sizes of the allelochemicals (F-A) were also suggested by a silver-stained SDS-PAGE gel, which showed a broad band in the stacking gel, not migrating into the separation gel (Figure 1e, lane 2). The broad band was also visualized by PAS staining (Figure 1e, lane 5), suggesting that the allelochemicals are polydisperse glycoproteins, as is the case with mucin, which has molecular masses ranging from 2 × 10<sup>6</sup> Da to 5 × 10<sup>7</sup> Da (Bansil and Turner, 2006). We therefore proceeded to analyse chemical compositions of the allelochemicals (F-A) obtained at the step of gel filtration, without further purification.

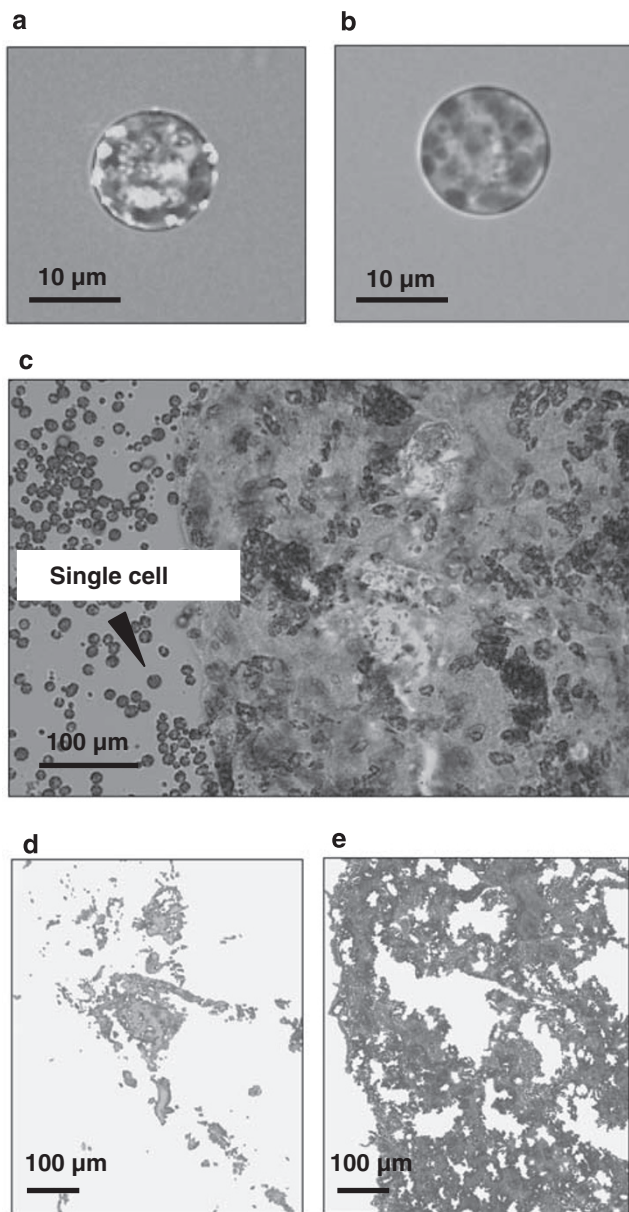
Amino acid and sugar compositions of the allelochemicals (F-A) showed enrichment in Asx, Thr, Ser, Glx, Gly, Ala, Val, Leu (around 10 mol% per each amino acid) as protein constituents (Figure 3a), Gal (60 mol%) and Man (20 mol%) for sugar constituents (Figure 3b). Because Asx and Ser/Thr are relatively dominant amino acids (10 and 16 mol%, respectively), the presence of a great number of *N*- and *O*-linked glycans were expected as seen in macromolecular glycoproteins, such as



**Figure 3** Chemical composition analysis of F-A. (a and b) Amino-acid (a) and sugar (b) compositions of F-A produced by *H. akashiwo*.

mucin, collagen and proteoglycans. Contrary to expectation, the allelochemicals (in fraction F-A) were resistant to glycopeptidase F and alkaline treatments (data not shown). These observations suggest that the *H. akashiwo* allelochemicals are most likely macromolecular glycoproteins, but are distinct from any known glycoproteins, having characteristic chemical compositions (Figure 3). Although further biochemical analyses are needed to clarify the structural aspects of *H. akashiwo* allelochemicals, we tentatively designate these allelochemicals as APPCs (allelopathic polysaccharide–protein complexes).

All the allelochemicals reported to date in land and aquatic plants are small compounds (< 10<sup>4</sup> Da) and most of them have been identified as secondary metabolites (Maestrini and Bonin, 1981; Rice, 1984; Cembella, 2003; Legrand *et al.*, 2003; Granéli and Hansen, 2006). To our knowledge, this is the first report demonstrating the presence of extremely large allelochemicals (> 10<sup>6</sup> Da). Thus, APPCs produced by *H. akashiwo* are novel members of allelochemicals, meaning that APPCs form a new category of allelopathic agents. Although chemical structures of APPCs and mechanisms by which APPCs express



**Figure 4** Localization of APPCs on the surface of *H. akashiwo* cells using the fluorescent-antibody method. IFAT was conducted as described in the text. (a) *H. akashiwo* was tested by IFAT without addition of APPCs. (b) As a control, *H. akashiwo* was tested by the addition of normal serum. Each cell and glycocalyx of *H. akashiwo* was tested by the IFAT (c–e); (c) Single cell and glycocalyx of *H. akashiwo*; (d) Antigen-positive part of glycocalyx; (e) Antigen-negative part of glycocalyx.

allelopathic functions are unclear, viscoelastic, colloidal and/or selectively adhesive properties of APPCs could be important features of certain exopolymeric APPCs, which would not be exerted by small allelochemicals.

#### Localization of APPCs on the *H. akashiwo* cells

To investigate localization of APPCs on the *H. akashiwo* cells, we developed an antibody against

purified APPCs (F-A, Figure 1c). An indirect fluorescent-antibody test (IFAT) indicated that APPCs were present on the cell surface of *H. akashiwo* (Figure 4a). In addition, immunological cross-reaction of the second antibody against *H. akashiwo* was not detected under experimental conditions (Figure 4b). Furthermore, IFAT using the cell pellet of *H. akashiwo* indicated that APPCs were present in the glycocalyx (Yokote *et al.*, 1985) of *H. akashiwo* (Figures 4c–e). On the other hand, APPCs were present not only in the glycocalyx and/or on the cell surface of *H. akashiwo*, but also in the filtrate when this species was cultured alone (Supplementary Figure 3). It is therefore likely that *H. akashiwo* continually releases APPCs from its cell surface into the environment.

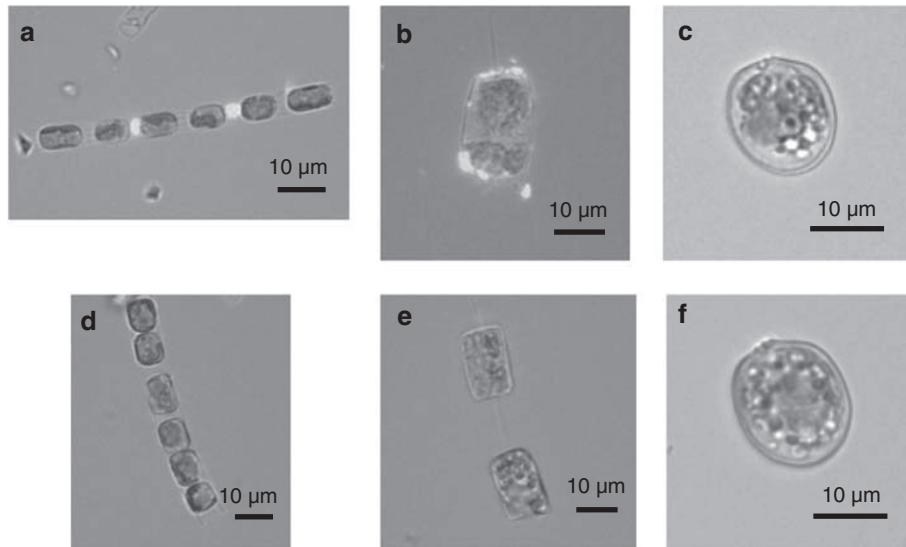
#### Possible mechanism of growth inhibition by APPCs

An indirect fluorescent-antibody test (IFAT) indicated that the growth inhibition of diatoms could be induced by specific binding of APPCs to the cell surface of the target species (Figure 5). In addition, immunological cross-reaction of polyclonal antibody against *S. costatum*, *T. rotula* and *P. minimum* was not detected under experimental conditions (Figures 5d–f). The APPCs might therefore have bound to the cell surfaces of *S. costatum* and *T. rotula* (Figures 5a and b), which were inhibited by *H. akashiwo* (Figures 2a and b), but not *P. minimum* (Figure 5c), which was not inhibited (Figure 2c). These factors—that is, the amounts of APPCs produced for each cell and whether they bound to the cell surface—might therefore have acted as triggers, and induced the particular effects of allelopathy as evidenced by two physiological processes: photosynthesis and enzyme activity (Gross, 2003). Alternatively, it remains possible that the cells bound specifically with APPCs get clogged by the APPCs, and suffer from a reduced gas exchange.

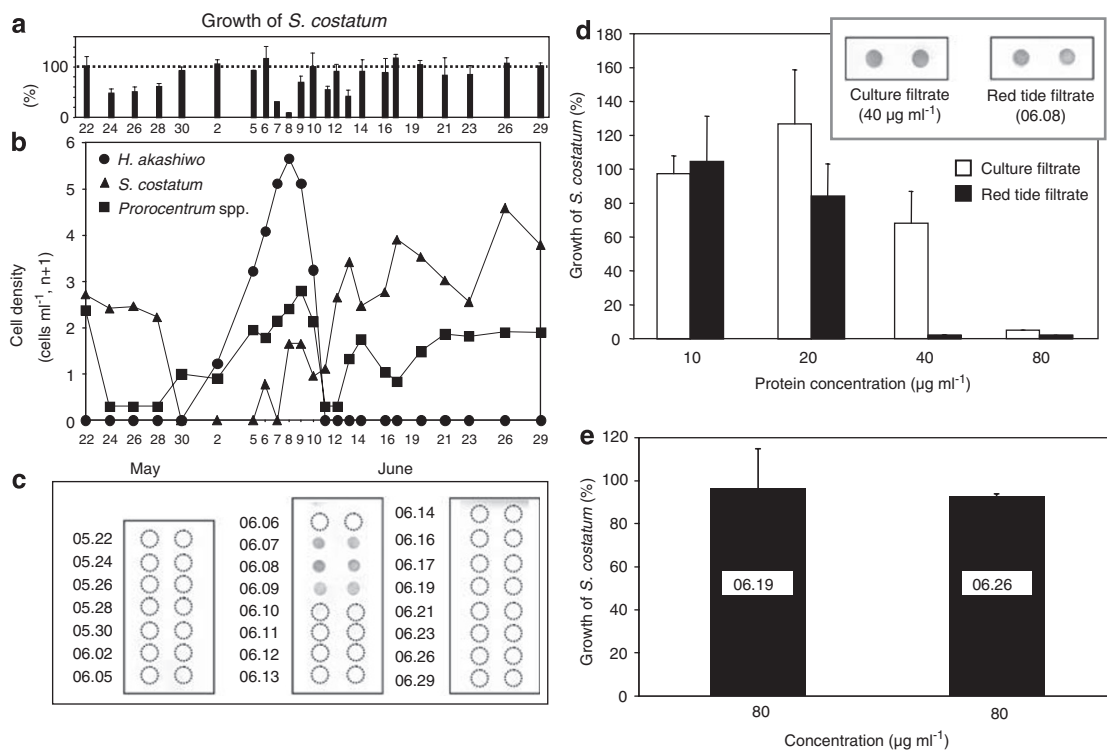
However, it may be difficult to explain species-specific allelopathic effects of APPCs produced by *H. akashiwo* by such mechanisms. It is much more likely that the mode of action of APPCs is similar to growth factors (Mendelsohn and Baselga, 2003) in having specific receptors comparable with those on the surface of target mammalian cells. Further study will be required to clarify the mode of action of APPCs on the growth of phytoplankters.

#### Significance of APPCs in the field

A field study was carried out in Hakozaiki Fishing Port, Fukuoka, Japan (latitude 34°24'58"N, longitude 130°12'20"W), in May and June 2007. To elucidate the role of allelopathy in the field, we evaluated whether several factors affected the growth dynamics of phytoplankton in Hakozaiki Fishing Port. During the field study, most of the environmental factors were suitable for *S. costatum* growth



**Figure 5** Binding specificity of *H. akashiwo* APPCs. Detection of APPCs from the cell surfaces of *S. costatum*, *T. rotula* and *P. minimum* using IFAT. IFAT was conducted as described in the text. *S. costatum* (a), *T. rotula* (b) and *P. minimum* (c) were exposed by APPCs and were tested by IFAT. As a control, *S. costatum* (d), *T. rotula* (e) and *P. minimum* (f) were tested without the addition of APPCs.



**Figure 6** Detection of APPCs in the field during *H. akashiwo* bloom. (a and b) A bioassay was conducted on each sampling day using seawater samples (a), and phytoplankters were counted microscopically (b). (c) Dot-blot analysis conducted with two replicates using seawater samples. All samples were spotted at 5 µl per spot. (d) Effects of crude extracts from the laboratory culture of *H. akashiwo* and red-tide seawater from *H. akashiwo* on *S. costatum* growth examined using 48-well plates. (e) Effects of crude extracts from red-tide seawater samples from *S. costatum* on its growth examined using 48-well plates. This bioassay had three replicates (100% growth as that in control medium).

(Supplementary Figures 4, 5); however, *S. costatum* declined from 28 to 30 May due to unknown factors apparently unrelated to allelopathy of *H. akashiwo* (Figure 6b).

Interestingly, APPCs were detected by a dot-blot analysis of three seawater samples taken from 7 to 9 June (Figure 6c), when *S. costatum* growth was inhibited according to the results of the bioassay



(Figure 6a), but were below the detection limit of dot-blot analysis (Figure 6c, Supplementary Figure 3a). Similarly, APPCs were detected by a dot-blot analysis of axenic cultures of *H. akashiwo* at densities  $1 \times 10^5$  cells per ml (Supplementary Figure 3b), at which *H. akashiwo* can inhibit *S. costatum* growth in the laboratory experiments (Yamasaki *et al.*, 2007); these findings supported the conclusion drawn from the field study (Figures 6b and c) that APPCs can affect the growth of other phytoplankton in the field. A comparison of dot-blot analysis from  $40 \mu\text{g ml}^{-1}$  culture filtrate and from the dense bloom sampled on 8 June (Figure 6d) showed a similar stain density in both, indicating a similar amount of allelopathic activity, which was sufficient to suppress *S. costatum* growth. In fact, the inhibitory effect of the filtrate from the dense bloom was greater than that of the crude extract of axenic cultures of *H. akashiwo* (Figure 6d). However, two crude extracts collected from seawater samples during the *S. costatum* bloom did not affect its growth (Figure 6e). By contrast, *S. costatum* growth in the field and in the laboratory experiment recovered concurrently (compare Figures 6a and b) when *H. akashiwo* declined and APPCs were no longer detected by the dot-blot analysis (Figure 6c). Furthermore, we showed that the APPCs became deactivated over time (time-scale of days) (Supplementary Figure 6), which was comparable to the reported reduction of activity by the allelopathic material secreted by the red-tide dinoflagellate *Karenia mikimotoi* (Gentien *et al.*, 2007). These results suggest that APPCs of *H. akashiwo* affect the growth of phytoplankton during dense *H. akashiwo* blooms.

#### Final conclusions

This is the first report that phytoplankton-secreted allelochemicals, which have previously been found in the laboratory, exist in nature at concentrations exceeding their action threshold. Allelopathic polysaccharide–protein complexes (APPCs, MW  $> 10^6$ ) produced by *H. akashiwo* (Figures 1 and 3), inhibited the growth of primary producers such as *S. costatum* and *T. rotula* (Figure 2, Supplementary Figure 2), but stimulated or were neutral to the harmful dinoflagellates, *Prorocentrum* spp. and *Heterocapsa circularisquama* (Figure 2, Supplementary Figure 7), during the dense *H. akashiwo* bloom (Figure 5b, Supplementary Figure 8). In addition to the traditional concept of allelopathy between phytoplankton species, APPCs produced by *H. akashiwo* may have another role. Shikata *et al.* (2008) reported that the density of *H. akashiwo* cysts in the bottom sediment of the Hakozaki Fishing Port sharply increased from the time of the peak to the decline phase of a bloom in the water. Thus, APPCs may not only contribute to growth competition with other species during the late exponential phase of the bloom as reported by Pratt (1966), Honjo *et al.*

(1978), and Honjo and Tabata (1985), but also contribute to maintenance of monospecific *H. akashiwo* blooms through inhibitory effects on competitors. By reducing competition from organisms such as diatoms and attacks from predators, *H. akashiwo* succeeds in establishing dense cyst beds, which give the next generation an advantage by allowing extensive re-inoculation of the water column. Increases in the frequency and severity of harmful algal blooms have become a global-scale problem, associated with climate warming, (Peperzak, 2003) and eutrophication (Wu, 1999). Furthermore, the escalation of global environmental problems in the coming years will likely exacerbate the problem of harmful algal blooms including *H. akashiwo* to socio-economic activity, especially in developing countries (Wu, 1999). Our results suggest that, as harmful algal blooms increase in severity and frequency, the target-specific effects of APPCs produced by *H. akashiwo* will increasingly control the structure of plankton ecosystems.

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