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Allelopathic Effects of *Skeletonema* spp. May Influence Interspecific Competition and Bloom Formation of Co–occurring Harmful Flagellates

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We investigated the allelopathic effects of *Skeletonema* spp. on growth and interspecific competition of the co–occurring flagellates *Akashiwo sanguinea, Chattonella* spp., and *Heterosigma akashiwo*, by reviewing field data on fluctuations in their cell densities and through laboratory experiments. From 29 June to 3 September, 2007, three dense blooms were observed in the inner part of the Ariake Sea, Japan. In all the 4 stations, *Skeletonema* spp. were generally detected and contributed to blooms from 20 to 23 July and from 6 to 13 August. *Chattonella* spp. gradually grew from mid–July and formed mixed blooms with *Skeletonema* spp. from 6 to 13 August, and then caused an almost mono–specific bloom in the station 2 and 3 from 20 to 24 August. However, *H. akashiwo* and *A. sanguinea* generally maintained low cell densities. Laboratory experiments showed that when grown in re–enriched culture filtrates of *Skeletonema* sp. (NIES–324) and cultured with *Skeletonema* sp. under non–contact conditions, *A. sanguinea* suffered the highest growth inhibitory effect, followed by *H. akashiwo*, while *C. antiqua* was the most resistant species. In tri–algal culture of these flagellates, the allelopathic effects of *Skeletonema* sp. increased the proportion of *C. antiqua* cells and decreased those of *H. akashiwo* and *A. sanguinea*, perhaps giving *C. antiqua* a competitive advantage over the other 2 flagellates. Our results suggest that allelopathy of *Skeletonema* spp. has potential to influence interspecific competition of these flagellates owing to species–specific growth inhibitory levels, and sometimes may be propitious to bloom formation of *Chattonella* spp. in the field.

Key words: allelopathy, flagellates, interspecific competition, *Skeletonema* spp.

INTRODUCTION

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). In aquatic systems, allelopathy has been emphasized to regulate both community composition and harmful algal bloom (HAB) dynamics (Honjo, 1993; Smayda, 1997; Granéli and Hansen, 2006; Granéli *et al*., 2008). Although allelopathy is becoming increasingly appreciated based upon laboratory results, some recent studies have thrown doubt on its involvement in the process of algal bloom initiation (Flynn, 2008; Jonsson *et al*., 2009). Allelopathic effects in a natural environment, which may be modified by abiotic and biotic factors, may sometimes not be as obvious or even be contrary to that predicted from laboratory studies (e.g. Suikkanen *et al*., 2005; Poulson *et al*., 2010). On the other hand, more and more micro– or mesocosm studies have demonstrated the role of allelopathy in regulating marine microbial communities, and emphasized the importance of considering multiple interactions among species simultaneously in nature (Fistarol *et al*., 2004; Weissbach *et al*., 2011). Further field–based and multi–species studies are therefore needed to investigate the role of allelopathy observed in laboratory–based studies in regulating community composition and bloom formation (Legrand *et al*., 2003; Tillmann *et al*., 2007; Weissbach *et al*. 2011).

Over the last 30 years, HABs have tended to occur more frequently and caused serious damage to aquaculture and fishery production in the Ariake Sea, Japan (Tsutsumi, 2006). The raphidophytes *Heterosigma akashiwo* (Y. Hada) Y. Hada ex Y. Hara and M. Chihara, *Chattonella* spp. [*Chattonella antiqua* (Hada) Ono and *Chattonella marina* (Subrahmanyan) Hara et Chihara] and the dinoflagellate *Akashiwo sanguinea* (K. Hirasaka) G. Hansen & Ø. Moestrup, are representative harmful flagellates in this area, and frequently tend to form blooms from late April to July, from July to late September, and from October to December, respectively (Nakamura and Hirata, 2006; Tsutsumi, 2006; Yamatogi *et al*., 2006). Although these flagellates have been indicated to have a sufficient potential to form blooms in summer in the Ariake Sea from the viewpoint of their physiological characteristics (Matsubara *et al*., 2007; Yamatogi

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et al., 2006), their blooms have rarely been observed to co–occur during this season. Growth interactions through allelopathy were also suggested to have a role in regulating interspecies competition among these flagellates, and *C. antiqua* at higher cell density exhibits some advantages over the 2 other species in forming dominant blooms. However, the combination of initial cell densities of these flagellates are critical in determining the successful species of growth competition in bi–algal cultures, and the species that first reached the early stationary phase tends to strongly inhibit growth of the other alga (Qiu *et al*., 2011a, 2011b). Considering that any member of different phytoplankton species may affect interspecific interactions in a complex plankton community, other co–existing algae may also play roles in determining which particular species will form a dominant bloom.

Diatoms *Skeletonema* spp. are common throughout the year and frequently dominate the phytoplankton community in spring and summer in the Ariake Sea (Tsutsumi, 2006). Previous studies have suggested that *Skeletonema* sp. has species–specific allelopathic effects on phytoplankton (Imada *et al*., 1991; Yamasaki *et al*., 2011) and is strong competitors of harmful flagellates. For example, in spring and summer in Hakozaki Harbor, Japan, bloom of *Skeletonema costatum* (Greville) Cleve played an important role in inhibiting the growth of *A. sanguinea* (Matsubara *et al*., 2008), while *H. akashiwo* and *S. costatum* exhibited mutual inhibitory effects and alternated in forming dense blooms (Honjo *et al*. 1978; Yamasaki *et al*., 2007). During summer in the inner part of the Ariake Sea, *Chattonella* spp. have sometimes formed dense blooms in combination with *Skeletonema* spp., while the cells of other flagellates have generally been at relatively low densities (Matsubara *et al*., 2009; Katano *et al*., 2012). It is therefore reasonable to consider that the allelopathy of *Skeletonema* spp. has the potential to affect bloom formation of these co–occurring flagellates.

We report the dynamics of some predominant phytoplankton species (diatoms *Skeletonema* spp.; flagellates *A. sanguinea, Chattonella* spp. and *H. akashiwo*) observed in the inner part of the Ariake Sea from 29 June to 3 September of 2007 in detail, from the viewpoint of growth competition among these algae. Through laboratory experiments, we investigated the allelopathy of *Skeletonema* sp. on growth and interspecific interactions of co–occurring harmful flagellates to confirm how the allelopathic effects of this diatom influence interspecific competition and bloom formation among the flagellates.

MATERIALS AND METHODS

Field surveys

We reviewed the phytoplankton fluctuation data at 4 stations (Fig. 1) in the inner part of the Ariake Sea during the summer (29 June to 3 September) of 2007, surveyed by Saga Prefectural Ariake Fisheries Research and Development Center. This field survey was conducted once a week in principle, but, where necessary, additional

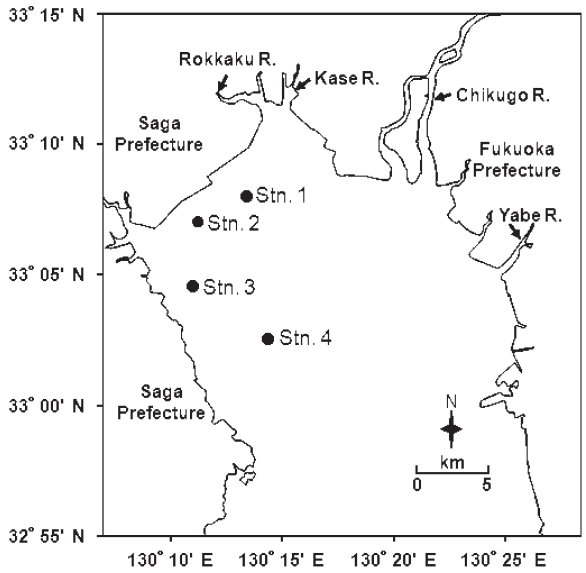

Fig. 1. Location of sampling stations in the inner part of the Ariake Sea, Japan. Arrows indicate main rivers (R.) around this sea area. The filled circles indicate the 4 sampling stations: Stn. 1 (33°08′149″N, 130°13′303″E), Stn. 2 (33°07′184″N, 130°11′121″E), Stn. 3 (33°04′715″N, 130°10′885″E), Stn. 4 (33°02′700″N, 130°14′261″E).

sampling was carried out to follow the development of *Chattonella* spp. blooms (1 to 28 August, 2007). Seawater was sampled from the surface using plastic bottles, and samples were returned to the laboratory within 4 h and treated. Surface water temperatures were measured by a thermometer *in situ*, and water salinity were measured by a digital salinometer (E–202, Tsurumi Seiki, Yokohama, Japan). Dissolved inorganic nitrogen (DIN: NO_2^-, NO_3^- and NH_4^+) and dissolved inorganic phosphorus (DIP: PO_4^{3-}) were measured by an auto analyzer (TRACCS 2000, Bran Luebbe, Hamburg, Germany). The water samples in bottles for counting phytoplankton were gently turned upside down 5 times before removing a subsample for counting. Cells of phytoplankton were counted microscopically in 0.5 ml subsamples, and therefore the detection limit was 2.0 cells ml^{-1}. Thus, our estimates were not suitable for analyzing the diversity of the phytoplankton community, but could be used to analyze the succession of predominant species.

Algal species and culture conditions

Axenic strains of *C. antiqua* (NIES–1), *H. akashiwo* (NIES–10), and *Skeletonema* sp. (NIES–324) were obtained from the National Institute of Environmental Studies (NIES, Japan). *A. sanguinea* was isolated in November 2002 from Hakozaki Harbor in Hakata Bay, Japan, and a demonstrably axenic strain was obtained through repeated washing using capillary pipettes (Matsubara *et al*., 2007). The above strains were verified as axenic using the 4',6–diamidino–2–phenylindole (DAPI) staining method developed for testing for bacterial contamination (Porter and Feig, 1980). Algal cultures were maintained in 70 ml sterile flasks (Nunc, Thermo Fisher Scientific Inc., Suwanee, GA, USA) containing 20 ml of modified SWM–3 medium (Yamasaki *et al*. 2007)

at a salinity of 30. The modified SWM–3 medium contained 0.04% (w/v) of tris(hydroxymethyl)–aminomethane (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to buffer pH during the experiments, and the medium was autoclaved before use (121˚C, 15 min). Stock cultures were conducted in an incubator (FLI–160, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 25˚C under 250±8 µmol photons m^{-2} s^{-1} of cool–white fluorescent illumination at a 12:12 h light:dark cycle. Irradiance in the incubator was measured using a Quantum Scalar Laboratory Irradiance Sensor (QSL–2101, Biospherical Instruments, San Diego, CA, USA).

Effects of re–enriched filtrates from *Skeletonema* sp. cultures on each flagellate

The diatom *Skeletonema* sp. was inoculated at a cell density of 1×10^3 cells ml^{-1} into a 650 ml sterile flask (Nunc) containing 350 ml of modified SWM–3 medium. Culture filtrates were prepared on day 8 once the cell density of *Skeletonema* sp. reached 1×10^6 cells ml^{-1} (100 ml; as a sample of the early stationary phase), on day 20 when cell density reached 6.7×10^5 cells ml^{-1} (100 ml; as a sample of the late stationary phase), and on day 26 when cell density reached 5.2×10^2 cells ml^{-1} (100 ml; as a sample of the death phase), by passing the cultures through a GF/C membrane filter (Whatman International Ltd., Maidstone, UK) on a 47 mm polysulfone holder under gravity filtration. Each filtrate (100% filtrate) was diluted to 50% (v/v) with fresh modified SWM–3 medium. To compensate for nutrients consumed by algal growth, the same quantities of nutrients as in the original modified SWM–3 medium were added (re–enriched) so that the final nutrient concentration of either filtrate (100% or 50% filtrate) was expected to be between 100 and 200% of the original modified SWM–3 medium. The pH of each filtrate was adjusted to 7.8–8.0 using 2 N HCl, and all the media were subsequently passed through 0.22 µm syringe filters (Millipore, Billerica, MA, USA).

For the growth experiment in culture filtrates, *A. sanguinea*, *H. akashiwo*, and *C. antiqua* were cultured separately in 8 ml sterile culture tubes (Evergreen Scientific, Los Angeles, CA, USA) containing 5 ml of a prepared test medium. Each flagellate was cultured alone in modified SWM–3 medium (original medium) as a control, and was also cultured alone in modified SWM–3 medium with the nutrient concentration elevated to 200% of the original medium concentration (re–enriched medium), to evaluate any nutrient inhibitory effect in re–enriched filtrates. The initial cell density of each flagellate was 1×10^2 cells ml^{-1}, with 4 replicate tubes for each treatment. Culture conditions were the same as described in Section 2.2. Relative cell abundances were measured daily using *in vivo* fluorescence (model 10–AU–005–CE fluorometer; Turner Designs, Sunnyvale, CA, USA). The growth rate (d^{-1}) was calculated for each tube from 3 consecutive data points, using the method of Brand *et al*. (1981), and maximum growth rates during the entire incubation period were determined.

Effects of live *Skeletonema* sp. cells on each flagellate under non–contact conditions

For this experiment, the diatom and each flagellate were cultured separately in inner and outer wells separated by a membrane with a pore size of 3.0 µm in 6–well plates (BD35–3091; Becton–Dickinson, Franklin Lakes, NJ, USA), as shown in Fig. 2 (modified from Yamasaki *et al*. 2007). Cells of *A. sanguinea*, *H. akashiwo*, and *C. antiqua* in early stationary phase were diluted with modified SWM–3 medium, and 5 ml of the resulting cell suspension for each flagellate was inoculated into the outer chambers of the well plates (final cell density: 1×10^2 cells ml^{-1}). Subsequently, cells of *Skeletonema* sp. at early stationary phase were diluted with modified SWM–3 medium, and 3 ml of this cell suspension was inoculated into each inner chamber of the well plates (final cell density: 1×10^3 cells ml^{-1}). As controls, 5 ml mono–algal cell suspensions for each flagellate were individually cultured in fresh modified SWM–3 medium (final cell densities: 1×10^2 cells ml^{-1}). Three replicate wells were used for each treatment and culture conditions were the same as described in Section 2.2. At 2 d intervals, 100 µl cultures in the outer chambers (flagellates) and 20 µl cultures in the inner chambers (*Skeletonema* sp.) were taken by a 20–200 µl Pipette (Nichipet EX, Nichiryo CO., Ltd., Koshigaya, Japan), and cell numbers in those subsamples were counted under a light microscope. When cell densities exceeded 2×10^4 cells ml^{-1}, subsamples were diluted 10 to 50 times with fresh modified SWM–3 medium before counting.

Effect of live *Skeletonema* sp. cells on growth competitions among flagellates

This experiment was also conducted using 6–well plates with cell–culture insert plates (Fig. 2). Firstly, cells of *A. sanguinea*, *H. akashiwo*, and *C. antiqua* during the exponential growth phase were individually diluted with modified SWM–3 medium, and then mixed

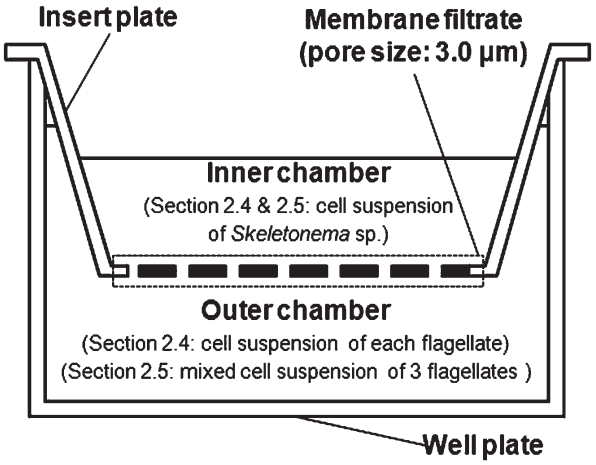

Fig. 2. Cross section of well plate with a BD Falcon cell culture insert used to investigate effects of allelopathic influence of live *Skeletonema* sp. cells on growth of each flagellates (Section 2.4) and on growth competition among flagellates (Section 2.5).

together to make mixed cell suspension of flagellates (final cell density for each flagellate: 1×10^2 cells ml^{-1}). For tri–algal culture of flagellates under the allelopathic influence of live *Skeletonema* sp. cells, 5 ml of resulting mixed cell suspension of flagellates was inoculated into the outer chambers of the well plates. Subsequently, cells of *Skeletonema* sp. at early stationary phase were diluted with modified SWM–3 medium, and 3 ml of this cell suspension was inoculated into the inner chambers of the well plates (final cell density: 1×10^3 cells ml^{-1}). For tri–algal culture of flagellates without the allelopathic influence of live *Skeletonema* sp. cells, 5 ml of mixed cell suspension of flagellates was inoculated into each well plate (without the insert plates). Three replicate wells were used for each treatment and culture conditions were the same as described in Section 2.2. At 2 d intervals, 100 μl subsamples of cultures in the outer chambers were taken by a 20–200 μl Pipette (Nichipet EX, Nichiryo CO., Ltd.), and cell numbers were counted under a light microscope. When cell densities exceeded 2×10^4 cells ml^{-1}, subsamples were diluted 10 to 50 times with fresh modified SWM–3 medium before counting. Growth rates of flagellates during the exponential 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. If the variances were homogeneous, one–way analysis of variance with Dunnett's test was used to test for differences in the maximum growth rate of each flagellate between the control and different treatments in culture filtrate experiments. An independent sample T test was employed to test the differences in growth rate of each flagellate at the exponential phase and the proportion of *C. antiqua* cells between the 2 treatments of tri–algal cultures of flagellates. When there was no proof of data homoscedasticity, the Mann–Whitney *U*–test for nonparametric data was used to compare control and treatments. Statistical analyses were conducted using the Statistical Package for the Social Sciences software (SPSS 13.0; SPSS, Inc., Chicago, IL, USA).

RESULTS

Phytoplankton composition and cell density fluctuations

Fluctuations in cell densities of phytoplankton in the 4 stations are shown in Fig. 3. Cell densities of diatoms *Skeletonema* spp., flagellates *Chattonella* spp., *H. akashiwo*, and *A. sanguinea* fluctuated from 0 to 3.1×10^4 (on 6 August in Stn. 4, Fig. 3D), 1.3×10^4 (on 24 August in Stn. 2, Fig. 3B), 1.2×10^4 (on 23 July in Stn. 3, Fig. 3C) and 2.4×10^2 (on 31 August in Stn. 3, Fig. 3C) cells ml^{-1}, respectively. Three mono–specific or mixed blooms were observed from 20 to 23 July (in Stn 1–4, Fig. 3), from 6 to 13 August (in Stn 1–4, Fig. 3), and from 20 to 24 August (in Stn. 2 and 3, Fig. 3B and C). During the current field survey, cells of *Skeletonema* spp. could generally be detected and contributed to the former 2

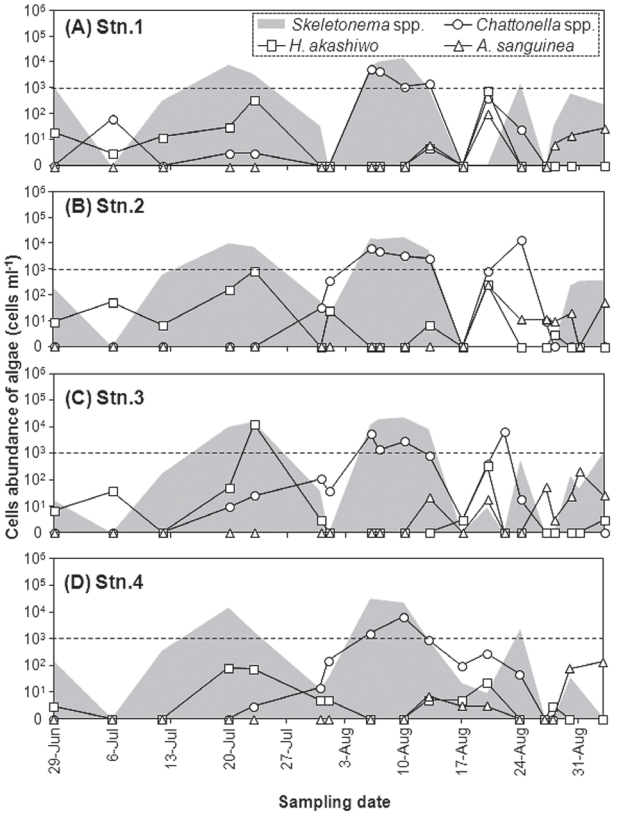

Fig. 3. Fluctuations in cell densities of *Skeletonema* spp. (gray area), *Chattonella* spp. (white circle), *Heterosigma akashiwo* (white box), and *Akashiwo sanguinea* (white triangle) from 29 June to 3 September 2007, in Stn. 1 (A), Stn. 2 (B), Stn. 3 (C) and Stn. 4 (D).

blooms in all the 4 stations (Fig. 3A–D). From 29 June, cells of *H. akashiwo* could be observed in all the 4 stations and contributed to the first bloom only on 23 July in Stn. 3 (Fig. 3C), but most times its cell densities were lower than 1.0×10^3 cells ml^{-1}. From 6 July, cells of *Chattonella* spp. were firstly observed in Stn. 1 and were detected in all the 4 stations after 23 July. On 31 July or 1 August, cells of *Skeletonema* spp., *Chattonella* spp., and *H. akashiwo* were simultaneously observed at low abundance ($<3.4\times10^2$ cells ml^{-1}). Subsequently, *Chattonella* spp. and *Skeletonema* spp. grew rapidly and formed a co–occurring bloom in all the 4 stations from 6 to 13 August (the second bloom, Fig. 3), during when *H. akashiwo* and *A. sanguinea* maintained low cell densities. After the end of the second bloom, *Skeletonema* spp. maintained low abundance ($<2\times10$ cells ml^{-1}) and all these flagellates were simultaneously detected to grow from 17 to 20 August, and then only *Chattonella* spp. formed a dense bloom again from 22 to 24 August in Stn. 2 and 3 (the third bloom, Fig. 3). From 13 August, cells of *A. sanguinea* were firstly observed in Stn. 4 (Fig. 3D), and this flagellate could reach a relatively high cell density (about 2×10^2 cells ml^{-1}) only when cells of other algae maintained low densities (Fig. 3).

During the present field survey, surface water temperature increased from 24.5 to 32.7°C, while the salinity

showed some relatively acute variations after strong precipitations (from 2 to 6 July and from 2 to 4 August) and varied from 6.4 to 29.4. The nutrients concentrations generally decreased after dense blooms of phytoplankton and increased after strong precipitations: the DIN concentrations varied from 0.26 to 77.43 μg at l^{-1}, and the DIP concentration varied from 0.05 to 4.07 μg at l^{-1} (data not shown).

Effects of re–enriched filtrates from *Skeletonema* sp. cultures on each flagellate

Growth inhibitory effects of filtrates prepared from the early stationary phase *Skeletonema* sp. culture (collected on day 8, Fig. 4A) were shown in Fig. 4B. The 100% filtrate significantly reduced the maximum growth rates of *A. sanguinea* (to 84% of that of control, $P<0.05$) and *H. akashiwo* (to 77% of that of control, $P<0.01$); the 50% filtrates significantly decreased the maximum growth rate of *H. akashiwo* (to 90% of that of control, $P<0.01$), while these filtrates of *Skeletonema* sp. had little effect on growth of *C. antiqua*.

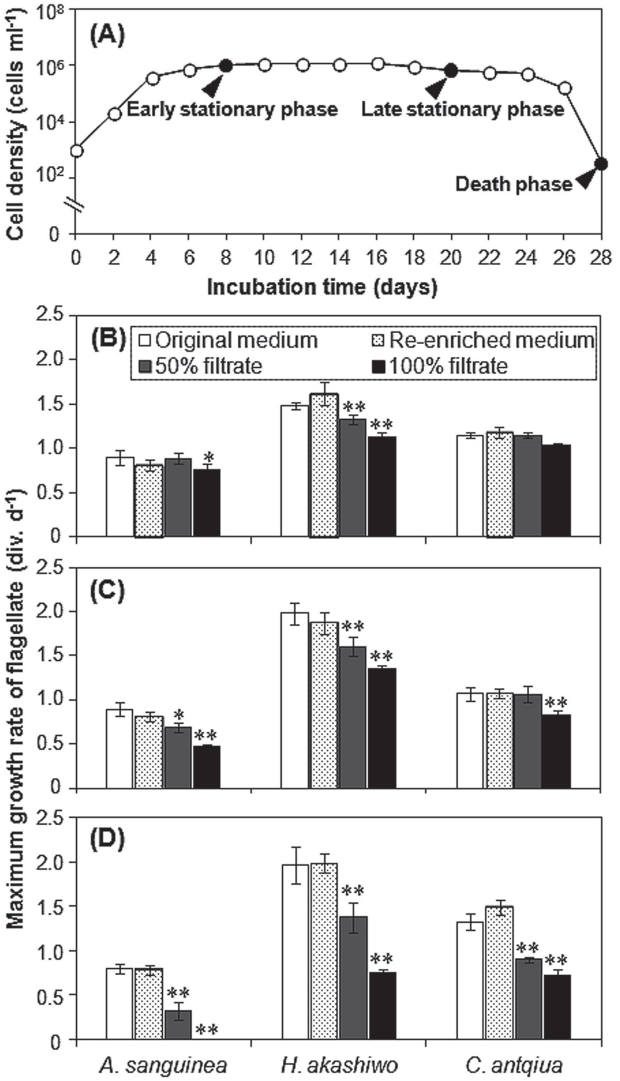

Fig. 4. Effects of filtrates prepared from cultures of *Skeletonema* sp. on the maximum growth rate of flagellates. (A) Growth curve of *Skeletonema* sp. showing collection times of filtrates. (B–D) Inhibitory effects of filtrates prepared from cultures of S. *Skeletonema* sp. at the early stationary phase (B), the late stationary phase (C), and the death phase (D) on the growth of *Akashiwo sanguinea*, *Heterosigma akashiwo*, and *Chattonella antiqua*. Values are means ± SD (n=4) and asterisks indicate values that are significantly different (*: $P<0.05$; **: $P<0.01$) from controls (cultured in fresh modified SWM–3 medium).

Growth inhibitory effects of filtrates prepared from the late stationary phase *Skeletonema* sp. culture (collected on day 20, Fig. 4A) were shown in Fig. 4C. The 100% filtrates significantly reduced the maximum growth rates of *A. sanguine*a (to 53% of that of control, $P<0.01$), *H. akashiwo* (to 68% of that of control, $P<0.01$), and *C. antiqua* (to 78% of that of control, $P<0.01$); the 50% filtrates significantly reduced the maximum growth rates of *A. sanguinea* (to 76% of that of control, $P<0.05$) and *H. akashiwo* (to 81% of that of control, $P<0.01$), but exhibited little inhibitory effect on *C. antiqua* growth (Fig. 4C).

Growth inhibitory effects of filtrates prepared from the death phase *Skeletonema* sp. culture (collected on day 24, Fig. 4A) were shown in Fig. 4D. The 100% filtrates exhibited a lethal effect on the growth of *A. sanguinea*, and significantly reduced the maximum growth rates of *H. akashiwo* (to 38% of that of control, $P<0.01$) and *C. antiqua* (to 54% of that of control, $P<0.01$); the 50% filtrates significantly reduced the maximum growth rates of *A. sanguinea* (to 63% of that of control, $P<0.01$), *H. akashiwo* (to 70% of that of control, $P<0.01$), and *C. antiqua* (to 68% of that of control, $P<0.01$)

The maximum growth rates of the 3 flagellates cultured in re–enriched media were similar to that of cells cultured in the original modified SWM–3 medium (Fig. 4B–D), demonstrating that nutrient concentrations in re–enriched filtrates were not responsible for the observed inhibitory effects. Overall, *A. sanguinea* was the most sensitive species and suffered the highest growth inhibition level, followed by *H. akashiwo*, and *C. antiqua* was the most resistant species.

Effects of live *Skeletonema* sp. cells on each flagellate under non–contact conditions

When cultured with each flagellate under non–contact conditions, the growth of *Skeletonema* sp. was similar to that observed in mono–algal culture (Fig. 5A). In bi–algal culture of *A. sanguinea* and *Skeletonema* sp. under non–contact conditions, the growth of *A. sanguinea* was strongly inhibited soon after the start of the experiment, with the average maximum cell density of *A. sanguinea* (on day 6) reaching about 6% of that in mono–algal culture (on day 12, about 1.4×10^4 cells ml^{-1}, Fig. 5B). In addition, many morphologically abnormal as well as disrupted cells appeared beginning on day 4 when the cell density of the diatom reached 3.4×10^5 cells ml^{-1}. In bi–algal culture of *H. akashiwo* and *Skeletonema* sp. under non–contact conditions, the growth of *H. akashiwo* was strongly inhibited after day 4 when the cell density of the diatom reached 3.3×10^5 cells ml^{-1}, with the average maximum cell density of *H. akashiwo* (on day 6) reaching about 17% of that in mono–algal culture (on day 12, about 7.0×10^5 cells ml^{-1}, Fig. 5C). In bi–algal culture of *C. antiqua* and *Skeletonema* sp. under non–con-

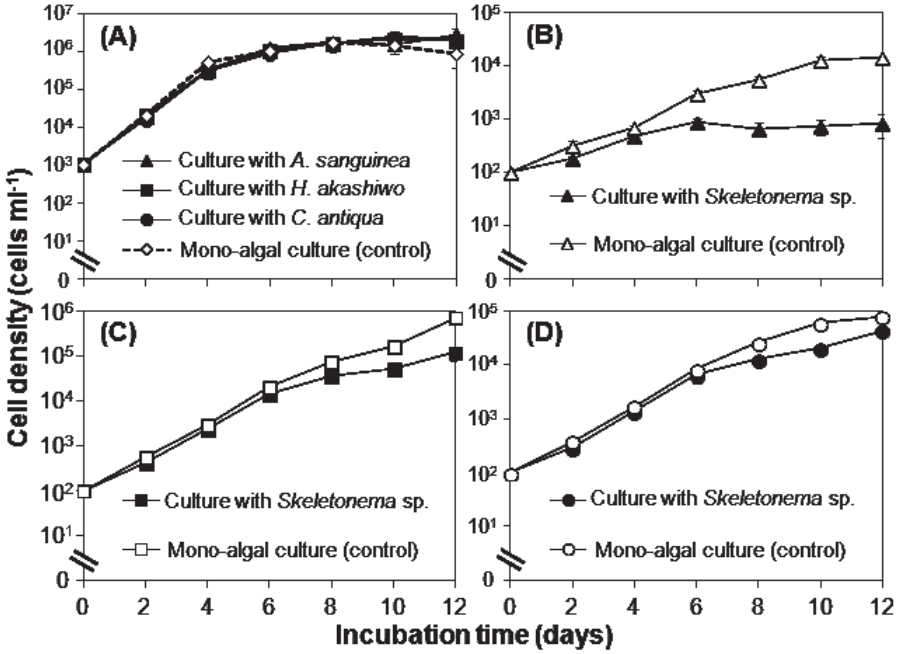

Fig. 5. Growth curves of phytoplankton in mono–algal cultures or bi–algal cultures under non–contact conditions. (A) Growth of *Skeletonema* sp. (initial cell density: 1×10^3 cells ml^{-1}) in mono–algal culture or in bi–algal culture with each flagellate under non–contact conditions. (B–D) Growth of each flagellate (initial cell density: 1×10^2 cells ml^{-1}) in mono–algal culture or in bi–algal culture with *Skeletonema* sp. (initial cell density: 1×10^3 cells ml^{-1}) under non–contact conditions: (B) *Akashiwo sanguinea*, (C) *Heterosigma akashiwo*, (D) *Chattonella antiqua*. Values are means ± SD (n=3).

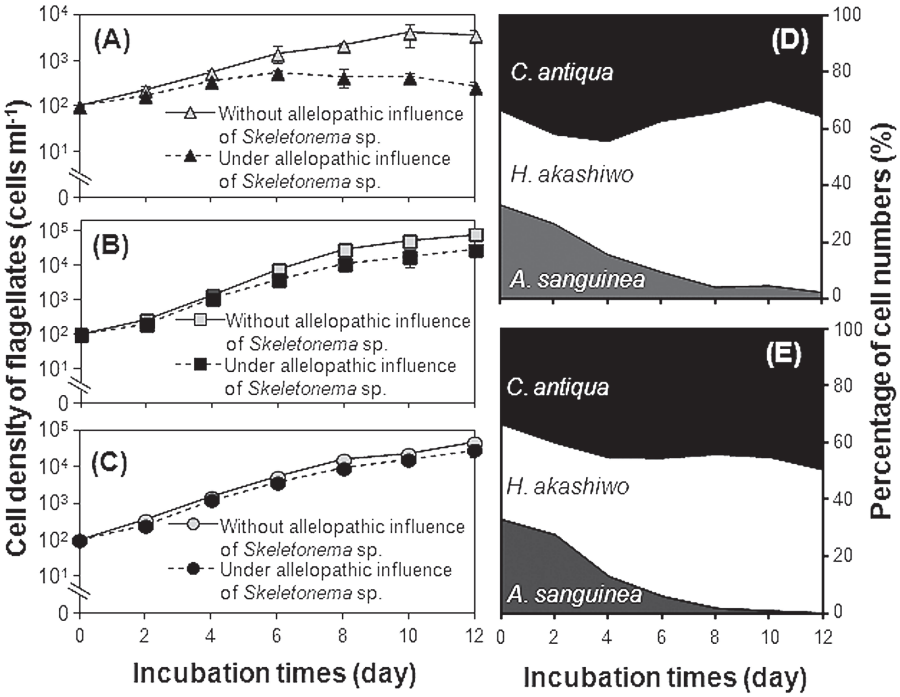

Fig. 6. Effects of allelopathic influence of live *Skeletonema* sp. cells on interspecific interactions among the 3 flagellates. (A–C) Growth of each flagellate (initial cell density: 1×10^2 cells ml^{-1}) in tri–algal cultures without the allelopathic influence of live *Skeletonema* sp. cells (gray symbols) or under the allelopathic influence of live *Skeletonema* sp. cells (black symbols): (A) *Akashiwo sanguinea*, (B) *Heterosigma akashiwo*, (C) *Chattonella antiqua*. Values are means ± SD (n=3). (D–E) Changes in the proportions of cell numbers of *A. sanguinea* (gray areas), *H. akashiwo* (white areas), and *C. antiqua* (black areas) during tri–algal cultures of these flagellates without the allelopathic influence of live *Skeletonema* sp. cells (D), or under the allelopathic influence of live *Skeletonema* sp. cells (E).

tact conditions, the growth of *C. antiqua* was strongly inhibited after day 6 when cell density of the diatom reached 9.4×10^5 cells ml^{-1} (Fig. 5A), with the average maximum cell density of reaching about 55% of that in mono–algal culture (on day 12, about 7.8×10^4 cells ml^{-1}, Fig. 5D). Thus, when cultured together with live *Skeletonema* sp. cells under non–contact conditions, *A. sanguinea* also exhibited the highest sensitivity and suffered the highest growth inhibition level, followed by *H. akashiwo*, while *C. antiqua* was the most resistant species and experienced the lowest level of growth inhibition.

Effect of live *Skeletonema* sp. cells on growth competition among flagellates

In tri–algal culture of flagellates under the allelopathic influence of live *Skeletonema* sp. cells, the allelopathic influence exhibited higher inhibitory effects on the growth of *A. sanguinea* and *H. akashiwo* than on that of *C. antiqua*, compared with tri–algal culture of flagellates without the allelopathic influence (Fig. 6A–C). The growth rates (from day 2 to day 6) of *A. sanguinea, H. akashiwo*, and *C. antiqua* were 0.83±0.10, 1.64±0.21, and 1.79±0.10 div. d^{-1} in tri–algal culture of flagellates under the allelopathic influence of live *Skeletonema* sp. cells,, which were reduced to 62% ($P<0.01$), 71% ($P<0.05$) and 91% ($P=0.08$) of those in tri–algal culture of flagellates without the allelopathic influence, respectively. The allelopathic influence of live *Skeletonema* sp. cells thus changed the composition of the tri–algal culture of flagellates by increasing the proportion of *C. antiqua* and decreasing that of *A. sanguinea* and *H. akashiwo* (Fig. 6D and E). Changes were particularly marked from day 8 onward ($P<.05$), and on day 12 the proportion of *C. antiqua* in the tri–algal culture of flagellates without the allelopathic influence of live *Skeletonema* sp. cells was 35.6% (total cell density: 1.3×10^5 cells ml^{-1}; Fig. 6D) while that in the tri–algal culture with the allelopathic influence of live *Skeletonema* sp. cells was 49.3% (total cell density: 5.6×10^4 cells ml^{-1}; Fig. 6E).

DISCUSSION

Skeletonema spp. are strong competitor of harmful flagellates in nutrient–limited water (Lomas and Glibert, 2000) and in allelopathic interactions (e.g. Matsubara *et al*., 2008; Yamasaki *et al*., 2011). When grown in re–enriched culture filtrates of *Skeletonema* sp. (Fig. 4) and cultured with *Skeletonema* sp. under non–contact conditions (Fig. 5), growth of the 3 flagellates tested was inhibited at different intensities. Since the pH values in each re–enriched filtrate of *Skeletonema* sp. was adjusted to the level of that in control before testing (see section 2.3), and none of the pH values in bi–algal cultures (see section 2.4) exceeded 8.5 at the end of cultivation (data not shown), these inhibitory effects were not caused by nutrient limitation, elevated pH values (e.g. Lundholm *et al*., 2005), or direct cell contact (Uchida, 2001). Therefore, all these flagellates experienced allelopathic inhibitory effects from the diatom. Although the substances inducing allelopathic effects of *Skeletonema* sp. were not clear, Yamasaki *et al*. (2011) reported that the allelochemicals are likely to be chemically stable, low molecular weight substances. Furthermore, our results also suggested that *A. sanguinea* suffered the highest growth inhibition level from *Skeletonema* sp., followed by *H. akashiwo*, while *C. antiqua* was the most resistant. These results are in good agreement with previous field observations (Honjo *et al*., 1978; Matsubara *et al*., 2008; Yamasaki *et al*., 2010) and strongly support the conclusion that the allelochemicals produced by *Skeletonema* sp. have species–specific effects on phytoplankton (Imada *et al*., 1991; Yamasaki *et al*., 2011).

Allelochemicals can not only benefit the releaser species by decreasing the number of competitors, but also benefit any species that is not so sensitive to the compounds (Fistarol *et al*., 2004). Since *C. antiqua* is more resistant to allelopathic effects of *Skeletonema* sp. than *H. akashiwo* and *A. sanguinea*, the former flagellate may obtain some benefit to from bloom when those algae co–existed. This assumption was confirmed by tri–algal culture of flagellates under the allelopathic influence of live *Skeletonema* sp. cells (Section 2.5): the results showed that allelopathic effects of *Skeletonema* sp. increased the proportion of *C. antiqua* and reduced those of *H. akashiwo* and *A. sanguinea*, compared with that

Table 1. Summary of some physical and chemical conditions affecting the growth of *Akashiwo sanguinea*, *Heterosigma akashiwo*, and *Chattonella antiqua*.

Flagellates	Physical conditions [a]			Chemical conditions (*Ks*, μg at l^{-1}) [b]		
	Temp. (°C)	Salinity	References	Nitrogen	Phosphorus	References
A. sanguinea	10.0–30.0 (25.0)	10.0–40.0 (20.0)	Matsubara *et al*., 2007	0.78–1.22	No data	Kudela *et al*., 2008
C. antiqua	15.0–32.5 (30.0)	16.0–36.0 (28.0)	Yamatogi *et al*., 2006	1.99–2.45	1.9	Nakamura, 1985
H. akashiwo	10.0–32.5 (25.0)	16.0–36.0 (24.0)	Yamatogi *et al*., 2006	1.99–2.45	1.0–1.98	Tomas, 1979

[a] The temperature (Temp.) and salinity values are reported as the ranges for reproduction, and suitable conditions for maximum growth rate are in parentheses.
[b] Values are the half saturation constant (*Ks*, μg at l^{-1}) of the incorporation for nitrogen and phosphorus, respectively.

in tri–algal culture of flagellates without the allelopathic influence of live *Skeletonema* sp. cells (Fig. 6D, E). Our previous studies indicated that the combination of initial cell densities are critical in determining the successful species in growth competition between *C. antiqua* and *H. akashiwo* / *A. sanguinea*: the species that first reached the early stationary phase tended to strongly inhibit growth of the other alga in bi–algal cultures (Qiu *et al*., 2011a, 2011b). Therefore, allelopathy of *Skeletonema* spp. has potential to influence interspecific competition of these flagellates owing to species–specific growth inhibitory effects, and sometimes give *C. antiqua* a competitive advantage against the others.

In the Ariake Sea, Japan, physicochemical factors certainly play large roles in bloom formations of phytoplankton (e.g. Nakamura and Hirata, 2006; Tsutsumi, 2006). A summary of some physical and chemical factors affecting the growth of these 3 flagellates is shown in Table 1. The fluctuations in physicochemical factors during the current field survey have been described in detail by Matsubara *et al*., 2009. The average surface water temperature ranged from 24.8 to 29.8°C, and most times the average salinity ranged from 15 to 30. Under these conditions, the growth rate of *C. antiqua* and *H. akashiwo* isolated from the Ariake Sea was reported at 1.0–1.43 and 1.2–1.64 div. day^{-1}, respectively (Yamatogi *et al*., 2006), the growth rate of *A. sanguinea* isolated from Hakata Bay (near the Ariake Sea) ranged from 0.4 to 1.13 div. day^{-1} but drastically decreased if the salinity was <20 (Matsubara *et al*., 2007). Therefore, most times the water temperature and salinity were suitable for the growth of these flagellates except the periods of relatively low salinity (from 6 to 12 July and around 6 August) after strong precipitations. The concentrations of dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorous (DIP) in the surface water, which were more than 50 μg at l^{-1} and 2.5 μg at l^{-1} just before the first blooms (12 July), decreased to less than 10 μg at l^{-1} and 1 μg at l^{-1}, respectively, during these blooms (from 23 July to 20 August, Matsubara *et al*., 2009). However, when the cell density of *Skeletonema* spp. was $\geq 1.0\times10^3$ cells ml^{-1}, that of *H. akashiwo* was usually $<1.0\times10^3$ cells ml^{-1} (except in Stn. 3, Fig. 3C) and that of *A. sanguinea* was generally below detection level, while *Chattonella* spp. could reach high density and form mixed blooms with the diatoms (Fig. 3). Therefore, Blooms of *Skeletonema* spp. may also have a role in regulating algal succession and bloom formation of co–occurring flagellates in the Ariake Sea, associated with changes in physicochemical factors such as relatively low salinity or nutrient concentrations.

The present laboratory experiments have suggested that *Skeletonema* spp. may influence bloom formation of co–occurring flagellates in 2 ways by its species–specific allelopathic effects: (1) directly suppress their growth at different levels; (2) influence interspecific interactions among flagellates and give *C. antiqua* a competitive advantage over *H. akashiwo* and *A. sanguinea*. These results are in accordance with fluctuations in cell densities of these flagellates observed during the field surveys. From 12 June to 23 July, 2007, the allelopathic effects of bloomed *Skeletonema* spp. may play a role in inhibiting the growth of *H. akashiwo* and *A. sanguinea* and keeping their cell densities at low levels or below detection level (Fig. 3). After appearance (germination) of *Chattonella* spp., the allelopathic effects of *Skeletonema* spp. may give *Chattonella* spp. an advantage and enable them to form dense blooms, while growth of other 2 flagellates was strongly suppressed by the inhibitory effects from mixed blooms of *Chattonella* spp. and *Skeletonema* spp. (from 6 to 13 August, Fig. 3).

In this field survey, the maximum density of *Skeletonema* spp. was $<5\times10^4$ cells ml^{-1} (Fig. 3); much lower than the 'threshold value' for strong inhibition of flagellate growth observed in the laboratory experiments ($>10^5$ cells ml^{-1}, Fig. 5). Mikhail (2007) reported that a bloom of *C. antiqua* was preceded by increased numbers of *Skeletonema*, and that during the bloom of *C. antiqua* in combination with *Skeletonema* (about 10^3cells ml^{-1}) the cell density of *H. akashiwo* did not exceed 150 cells l^{-1} during summer of 2006 in waters of Alexandria, Egypt. Katano *et al*. (2012) reported that the total abundance of dinoflagellates (including *A. sanguinea*) was always at low level during an alternating bloom of *Chattonella* and *Skeletonema*, while cell density of *Chattonella* also declined when that of *Skeletonema* reached at $>10^5$ cells ml^{-1} during summer of 2010 in the Ariake Sea. It is likely that only a suitable abundance of *Skeletonema* spp. ($<10^5$ cells ml^{-1}) will give *Chattonella* spp. a competitive advantage to form a bloom against other flagellates, and a higher abundance of the diatoms may also strongly inhibit growth of *Chattonella* by allelopathic effects and nutrient competition (Lomas and Glibert, 2000). It should be remembered, however, that the laboratory experiments were conducted under axenic conditions in a culture medium with artificially high nutrient levels, and culture conditions such as temperature, light intensity, photoperiod, and salinity were artificially fixed. Further studies of how variations in such environmental factors and direct cell contact between algae (Uchida, 2001) affect allelopathic effects of *Skeletonema* spp. are necessary.

Overall, our study has demonstrated that the allelopathy of *Skeletonema* spp. at a suitable abundance has potential to mediate interspecific interactions and change community composition of co–occurring flagellates owing to species–specific growth inhibition levels, which may have a role in regulating bloom formation during summer in the Ariake Sea, Japan. To clarify the proportion of allelopathy of *Skeletonema* spp. in mediating bloom formations of these co–occurring species, it is necessary to determine the effects of various environmental factors on the production and transmission of allelochemicals, and to verify the role of allelopathy in a further study of a natural phytoplankton population.

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