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Algicidal activity of the bacterium, *Pseudomonas fluorescens* SK09, to mitigate *Stephanodiscus hantzschii* (Bacillariophyceae) blooms using field mesocosms

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ABSTRACT

The main goal of this study was to characterize mortality and trophic interactions of *Stephanodiscus hantzschii* (small centric diatoms) in the presence of an algicidal bacterium, *Pseudomonas fluorescens* HYK0210-SK09 (SK09) in outdoor mesocosm experiments with an extremely low temperature and also in direct application in a river. SK09 at an inoculation density of 5×10^6 cells/mL (Experiment 1) and SK09 cells immobilized in activated carbon polyvinyl alcohol sponge carriers (Experiment 2) did not appear to attack *S. hantzschii* in a species-specific manner at temperatures close to freezing. Furthermore, SK09 did not control the diatom blooms when SK09 cells immobilized in the carriers were directly applied in the river. The dynamics of both the heterotrophic flagellate and ciliate abundances showed similar trends to the activity of algicidal bacteria; moreover the heterotrophic protists could not control the total bacterial density. Inorganic nutrient concentrations and conductivity remained unchanged compared with those in the control group. The results obtained in this study and previous results are important for assessing biological control using algicidal bacteria in the wild.

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Introduction

The small centric freshwater diatom *Stephanodiscus hantzschii* Grunow is one of the common species causing blooms in winter worldwide (e.g. Kilham et al. 1986; Kobanova & Genkal 1989). In South Korea, *S. hantzschii* blooms occur annually and can reach a density of over 10^5 cells/mL during winter in the Han, Nakdong, and Yeongsan rivers (Jung et al. 2009b, 2011, 2013; Jeong et al. 2014). These diatom blooms have adverse social, economic, and environmental effects, such as low water transparency, unpleasant water odor and taste, as well as clogging of and/or sedimentation in water treatment equipment (Oksiyuk 1965; Lee et al. 2001; Kolmakov et al. 2002; Hijnen et al. 2007).

One of the most promising techniques for addressing the problems associated with harmful algal blooms (HABs) is biological control. Algicidal bacteria are of particular interest in this context in marine and freshwater environments. For example, we have isolated an algicidal bacterium, *Pseudomonas fluorescens* HYK0210-SK09 (SK09) that can control *S. hantzschii* (Jung et al. 2008). The ecological responses of aquatic organisms to bacterial treatments must be considered if the bacterium to be developed as a biocontrol agent is a potential disturbance to the natural environment. As such,

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in our previous studies, we evaluated SK09 as a biocontrol agent against natural *S. hantzschii* blooms. In indoor and outdoor mesocosm studies (Jung et al. 2008, 2010), SK09 was found to markedly inhibit the proliferation of this diatom. However, its application is associated with several environmental effects, such as changing inorganic nutrient concentrations and the consequent succession of phytoplankton species. To shed further light on this issue, we investigated the immobilization of SK09 cells in activated carbon polyvinyl alcohol sponge (APVAS) carriers (Jung et al. 2009a). Upon field application of SK09 cells immobilized with APVAS carriers, the number of *S. hantzschii* cells and inorganic nutrient concentrations showed a marked decrease (Jung et al. 2013). Against this background, the following question arises: Can SK09 mitigate centric diatom blooms of *S. hantzschii* under any environmental conditions? To answer this question, we implemented two mesocosm experiments and studied direct application of immobilized SK09 cells in extremely low temperatures and analyzed the results. Some of the experimental data referred to in this work were obtained in our previous studies (Jung et al. 2008, 2009a, 2010, 2013).

Methods

Isolation and culture of algicidal bacterium

SK09 was isolated using the algal lawn method by Jung et al. (2008). To obtain a high density of the algicidal bacteria, SK09 cells were incubated with 40 L of nutrient broth (NB) medium (30 rpm at 28 °C) and grown to a density of $5.2 \pm 0.8 \times 10^6$ cells/mL. To immobilize SK09 cells in the carrier, we prepared APVAS carrier purchased from Bio-Carbon Tech. Co. of South Korea. The APVAS carrier was cut into cubes whose sides were 1 cm in length, dried in a dry oven at 60 °C for 48 hours, and sterilized with ultraviolet radiation at a wavelength of approximately 180 to 250 nm for 4 hours. The bacteria were immobilized in the carrier as described previously by Jung et al. (2009a, 2013): the sterilized cubes of the APVAS carrier were placed in 40 L of SK09 cells grown in NB medium in log phase to a cell density of over 10^{10} cells/mL, and then incubated for 4 hours at 25 °C and stirred at 30 rpm in the dark. This approach resulted in a cell density of approximately 10^{10} cells per carrier. Immobilized SK09 cells in the carrier were washed twice in phosphate buffer (pH 7.2) within 24 hours of experimental use.

Outdoor mesocosm and direct application

The location and setting of field mesocosms are important factors to obtain an accurate understanding of the variations of natural biotic and abiotic factors. To prepare outdoor mesocosms, a compatible and/or convenient location should be chosen in the field. For the purposes of this study, the Nakdong River of South Korea is an optimal site for mesocosm study because *S. hantzschii* blooms there repeatedly in winter. Furthermore, this river is relatively shallow and has slow-moving water and a gentle slope into the littoral zone (Jung et al. 2010, 2013). To evaluate the algicidal activities of SK09 (Experiment 1) and immobilized SK09 cells in APVAS carriers (Experiment 2) against *S. hantzschii* blooms and the consequent responses of biotic and abiotic factors, a field mesocosm study was carried out in the Nakdong River (35°22′28.12″ N, 128°38′23.07″ E; Table 1 and Figure 1), when natural *S. hantzschii* cells bloomed at a density of 2.04×10^4 cells/mL. The mesocosms were set up in 3.0 *t* enclosures that were made using non-transparent and cylindrical polyvinyl chloride materials (radius = 1.5 m, height = 1.1 m). Each enclosure contained approximately 2.0 *t* of *S. hantzschii* blooming water. The water in the mesocosms was mixed continuously by wind and exchanged through the sandy bottom. In Experiment 1, SK09 cells were inoculated at a final density of 5×10^6 cells/mL (treatment group) and a control with no addition of SK09 cells was also prepared (control group). In Experiment 2, four thousand cubes of the APVAS carriers in which SK09 cells had been immobilized were added into a mesocosm (treatment group). In addition, a control group that was not subjected to any treatment was prepared (control group). All mesocosm experiments (1 and 2)

Table 1. Overview of purpose and setting of the mesocosm experiments to mitigate natural *S. hantzschii* blooms and comparison with the previous studies.

Experiment	Enclosure volume	Enclosure type	Purpose	Reference
Experiment 1		Outdoor mesocosm	Algicidal activity of SK09 initially inoculated at a density of 5×10^6 cells mL^{-1} in low water temperature when the river surface was covered with ice	This study
Experiment 2	2000-L	Outdoor mesocosm	Algicidal activity of immobilized SK09 with carriers initially inoculated at a density of 5×10^6 cells mL^{-1} in low water temperature when the river surface was covered with ice	This study
Experiment 3	Natural freshwater	Direct application in natural freshwater	Algicidal activity of immobilized SK09 cells in a natural ecosystem	This study
	100-L	Indoor microcosm	Algicidal activity of SK09 initially inoculated at a density of 5×10^6 cells mL^{-1}	Jung et al. (2008)
Previous studies	2000-L	Outdoor mesocosm	Algicidal activity of SK09 initially inoculated at a density of 5×10^6 cells mL^{-1}	Jung et al. (2010)
		Outdoor mesocosm	Algicidal activity of immobilized SK09 with carriers initially inoculated at a density of 5×10^6 cells mL^{-1}	Jung et al. (2013)

were carried out in duplicate for 12 days. The mesocosms were sampled at a depth of 0.5 m twice per day at 10:00 AM and 10:00 PM for the total period of the experiment. In Experiment 3, sub-sampling was performed at a depth of 0.5 m at three locations at intervals of 1 m once per day at 10:00 AM for 11 days.

To investigate the algicidal activity of SK09 cells from direct application in a river (Experiment 3), APVAS carriers with attached SK09 cells were placed in Nakdong River (Figure 1) where natural *S. hantzschii* blooms occurred at a density of approximately 10^4 cells/mL. There were 12,800 immersed APVAS carriers in a net bags with a mesh size of 0.5 cm (SK09 cells were immobilized into the carriers at a density of approximately 10^{10} cells per a carrier) which were suspended in the water column in an iron frame (1 m length × 1 m width and 1.2 m height). We set 16 net bags (approximate inoculation density of SK09 cells = 1.28×10^{14} cells) under the assumption that they would have the capacity to remove the natural *S. hantzschii* blooms within a water volume of 20,000 L in the river. Sub-sampling was performed at a depth of 0.5 m at three sites at intervals of 1 m (Figure 1).

Measurement of biotic and abiotic factors

Water temperature, pH, and conductivity were measured using a portable meter (U-10, Horiba, Japan) with sub-sampling. To analyze inorganic nutrient concentrations (DIN, phosphate, and silicate), a 250 mL sample filtered through a GF/F filter was stored in an acid-cleaned polyethylene bottle in a freezer (−80 °C). Nutrient concentrations were analyzed by a nutrient auto-analyzer (Flow Solution IV, Alpkam, USA) and concentrations were determined according to the modified methods of APHA (1995).

A sub-sample collected in a 50 mL sterilized PE bottle containing 30 mL sample was fixed immediately with glutaraldehyde at a final concentration of 2%. The sample was stored in the dark at 4 °C until analysis. At least 600 bacterial cells were counted for each sample using an epifluorescence microscope (Axioplan, Zeiss, Germany) with 4',6-diaminidino-2-phenylindiole (DAPI) solution at a final concentration of 1 μg/mL (Porter & Feig 1980). To enumerate phytoplankton cells, 100 mL of sample were immediately fixed with glutaraldehyde at a final concentration of 2%. Cell counts and identification of phytoplankton (at least 500 cells per a sample) were made using a Sedgewick-Rafter counting chamber at a 400 × magnification and a light microscope (Axiolab, Zeiss, Germany).

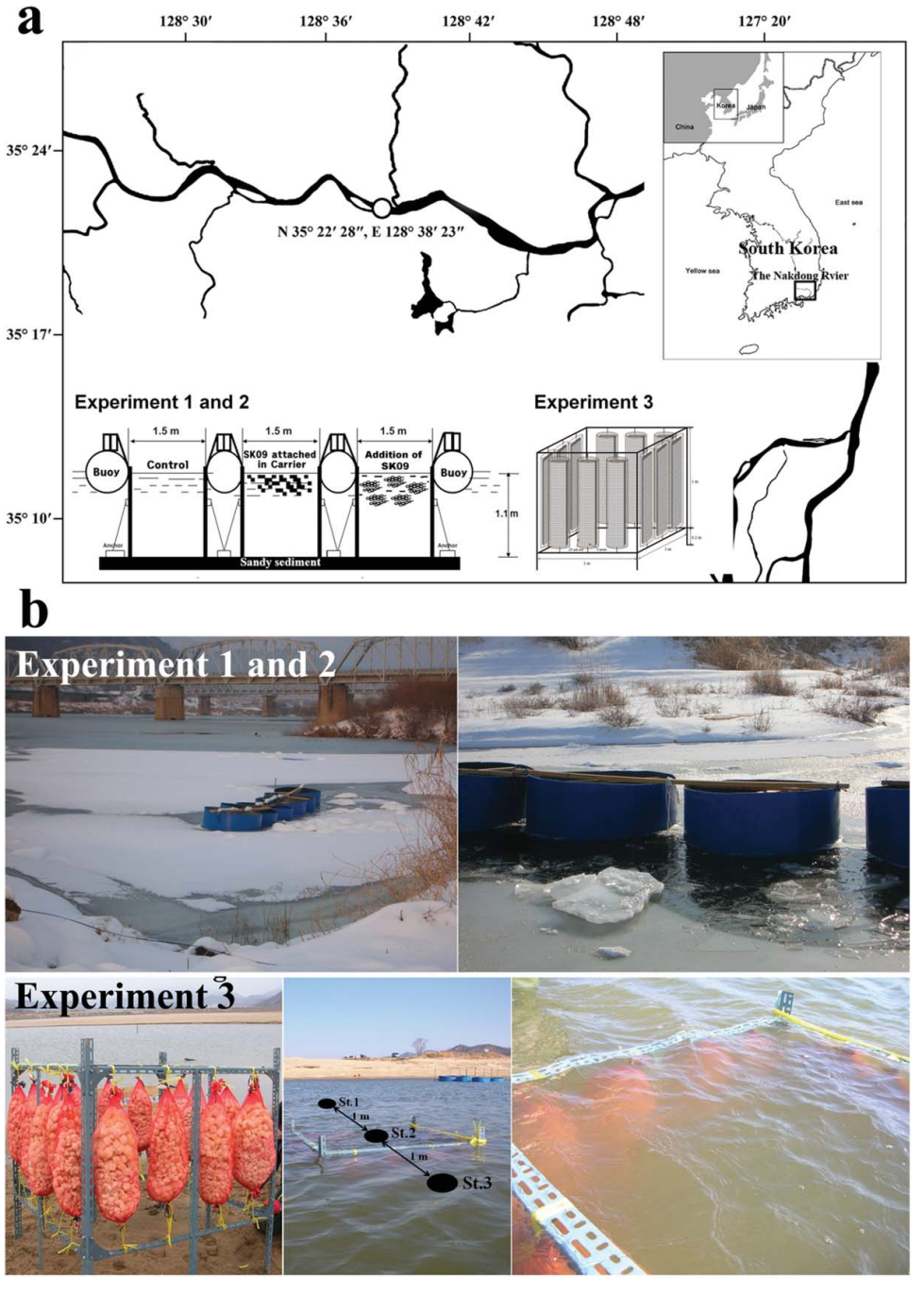

Figure 1. (a) Location, schematic diagrams and (b) photographs of 2000 L outdoor mesocosm and direct application of SK09 cells attached in the activated carbon polyvinyl alcohol sponge (APVAS) carriers in the Nakdong River.

The phytoplankton community present in the sample was categorized into six major taxonomic groups (van den Hoek et al. 1995): bacillariophyceae, chlorophyta, chrysophyta, cryptophyta, cyanophyta, and euglenophyta. To determine chlorophyll *a* concentrations, 300 mL of samples were immediately filtered through a 47 mm Whatman GF/F filter under low vacuum pressure. The filter was soaked in 15 mL cold 90% acetone-distilled water (v/v), then sonicated to break cell walls and

extracted for 24 hours in the dark at 4 °C. Finally, chlorophyll *a* concentrations were measured using a spectrophotometer (Model 8453, Hewlett Packard, USA) according to the equation of Humphrey and Jeffrey (1997). To enumerate heterotrophic flagellates and ciliates, a 100 mL sample was preserved with 25% buffered glutaraldehyde at a final concentration of 1%. The number of heterotrophic flagellates was counted under an epifluorescence microscope following ultra-violet excitation by the primulin staining method (Caron 1983) and the number of ciliates was counted at a 200X magnification under an inverted light microscope (Axioinvert, Zeiss, Germany). For zooplankton enumeration, a 500 mL sample was fixed with neutral formalin at a final concentration of 4% and concentrated by natural sedimentation. Zooplankton abundances were counted using a Bogorov chamber under a stereo-microscope (SZX 12, Olympus, Japan).

The maximum algicidal effect (MAE) of SK09 on *S. hantzschii* was calculated using the following equation: algicidal effect (%) = $(1-T_t/C_t) \times 100$, where T is the abundance of *S. hantzschii* in the presence of SK09 (treatment group), C is the algal abundance in the absence of SK09 (control group), and t is the time at which the number of *S. hantzschii* cells fell to its lowest level. To examine the relationships between the measured parameters, Pearson's correlation analysis was applied. The SPSS (ver. 18) program was used to analyze this data-set.

Results

We performed the experiments in the outdoor mesocosms (Experiment 1 and 2) when the surface of the river was iced over (ice depth of over 10 cm). Water temperature in Experiments 1 and 2 fluctuated between 0.1 °C and 2.7 °C (mean 1.0 °C) and 0.8 °C and 3.0 °C (mean 1.5 °C), respectively (Figure 2). The changes in the abundance of *S. hantzschii* and the MAE of SK09 are presented in Tables 2 and 3. Before the addition of SK09 cells, the phytoplankton communities were dominated by *S. hantzschii* (73.81%–95.24% of the total phytoplankton abundance). The changes in abundance of *S. hantzschii* showed no significant difference between treatment and control groups of Experiments 1 and 2 ($p > 0.05$, Table 2). The MAEs of SK09 in Experiments 1 and 2 were −5.92% and −1.67%, respectively. Upon the inoculation of immobilized SK09 cells in the carriers in the river (Experiment 3), there were no significant differences in the abundance of *S. hantzschii* cells at the three sampling sites (ANOVA, $p > 0.05$). Changes in chlorophyll *a* concentrations were similar to the trends of *S. hantzschii* abundance in all experiments. Specifically, chlorophyll *a* concentration and *S. hantzschii* abundance showed a positive correlation (Figure 3). Figure 4 shows the dynamics of planktonic organisms in the mesocosms. The bacterial cell numbers in all experiments did not show any marked differences among them or compared with the control. The dynamics of both the heterotrophic flagellate, ciliate and zooplankton abundances showed similar trends to the bacterial abundances. Figure 5 summarizes the fluctuations in concentration of dissolved inorganic nutrients (DIN, DIP, and DSi), and conductivity. Thus, concentrations of inorganic nutrients and conductivity remained unchanged compared with those in the control.

Figure 6 shows the conceptual schematic diagram of the ecological responses when SK09 is introduced under different treatment and ecosystem scenarios. Our previous results showed that SK09, when inoculated at optimal concentrations, could effectively mitigate *S. hantzschii* blooms, but these bacteria did not control *S. hantzschii* when the surface of the river was iced (Experiment 1 and 2) or when directly introduced into a river (Experiment 3) because of their short interaction time with the blooms due to the river flow. This indicates that water temperature and interaction time are important factors for inducing the mortality of *S. hantzschii* cells. This also reveals the importance of evaluating the effects of such biological control along the course of a river. Determination of the diffusion of SK09 cells inoculated in water and their effects on algal cell lysis is probably important for clarifying the potential benefits of the algicidal effect in rivers. Our results also revealed that that the bacteria produced a marked increase of the levels of inorganic nutrients released from the lysed cells. However, the APVAS carriers (with the addition of SK09 cells) effectively controlled *S. hantzschii* blooms as well as the levels of inorganic nutrients. This strategy using immobilization could be

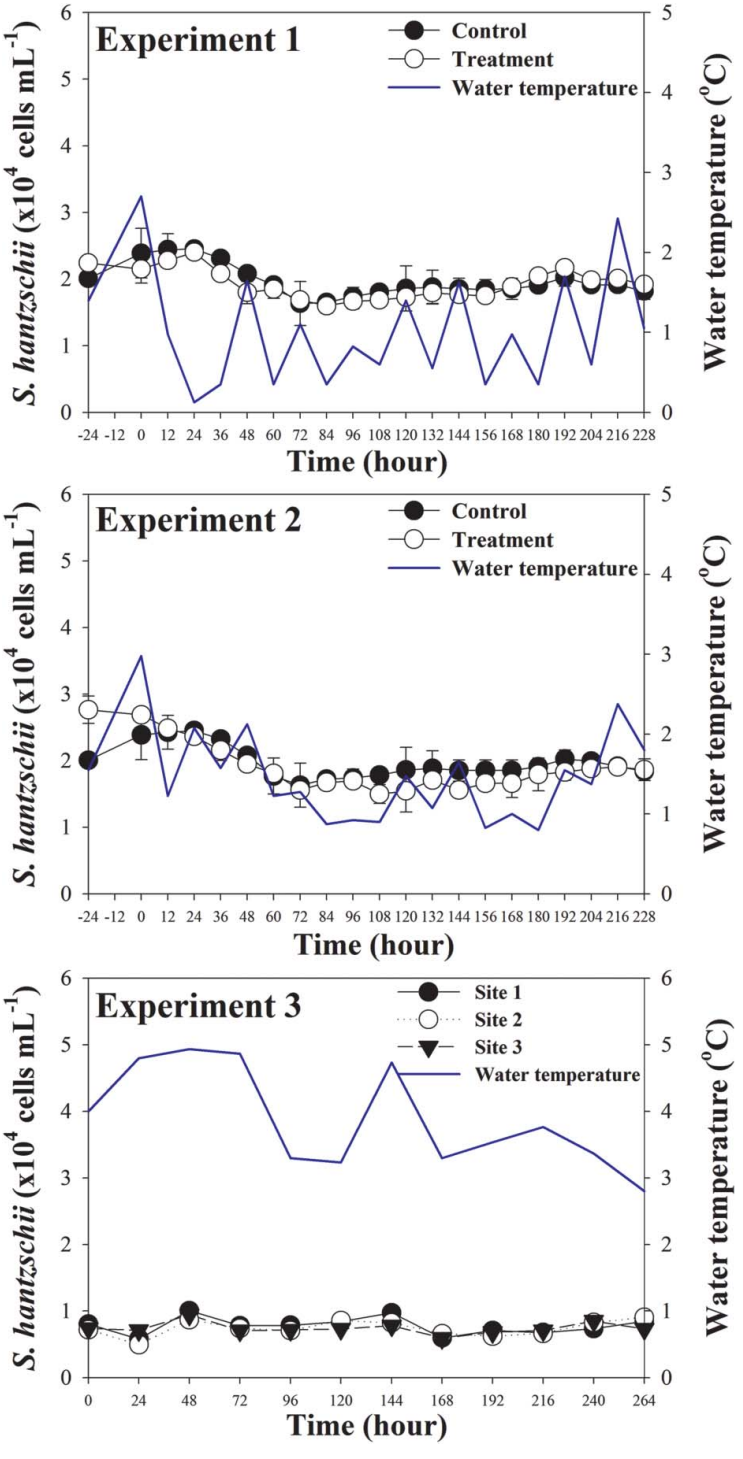

Figure 2. Changes in abundances of *Stephanodiscus hantzschii* and water temperature in mesocosm experiments and direct application in the wild.

Table 2. Effect of algicidal bacteria *Pseudomonas fluorescens* HYK0210-SK09 against *Stephanodiscus hantzschii* the mesocosm experiments and comparison with the previous studies.

	Abundance of *S. hantzschii* ($\times 10^4$ cells/mL)				Chlorophyll*a* concentration (μg/L)				
Experiment	Treatment	Control	*t*-statistic	Maximum algicidal effect (%)	Treatment	Control	*t*-statistic	Maximum algicidal effect (%)	Reference
Experiment 1	1.92±0.08	1.82±0.13	NS	−5.92±12.07	36.8±6.5	36.4±0.0	NS	−0.4±17.6	This study
Experiment 2	1.87±0.16	1.84±0.08	3.14*	−1.67±13.53	42.5±1.3	36.8±6.5	−3.47*	−17.4±24.1	This study
Previous studies	0.72±0.01	3.08±0.09	−9.09**	76.60±0.46	23.1±0.0	137.4±2.2	−11.27**	83.2±0.3	Jung et al. (2008)
	0.76±0.00	2.69±0.00	10.64**	71.81±0.16	37.6±0.1	120.5±0.2	9.24**	68.8±0.1	Jung et al. (2010)
	0.92±0.04	2.69±0.00	10.96**	65.81±1.43	61.2±14.4	120.5±0.2	9.46*	49.2±12.0	Jung et al. (2013)

Note: Treatment (group) is the presence of SK09, but control (group) is the absence of SK09.
t-statistic in Experiments 1 and 2 was performed between treatment and control, $^*p < 0.01$, $^{**}p < 0.001$, NS: not significant.

Table 3. Effect of immobilized algicidal bacteria *Pseudomonas fluorescens* HYK0210-SK09 against *Stephanodiscus hantzschii* as direct application in natural freshwater.

	Abundance of *S. hantzschii* ($\times 10^4$ cells/mL)				Chlorophyll *a* concentration (μg/L)			
Experiment	Site 1	Site 2	Site 3	ANOVA test	Site 1	Site 2	Site 3	ANOVA test
Experiment 3	0.84[a]	0.90[a]	0.73[a]	NS	45.0[a]	44.3[a]	40.5[a]	NS

Note: Results in Experiment 3 were analyzed by one-way ANOVA and Scheffe's *post hoc* test.
[a]Indicates no significant difference among Sites 1–3.

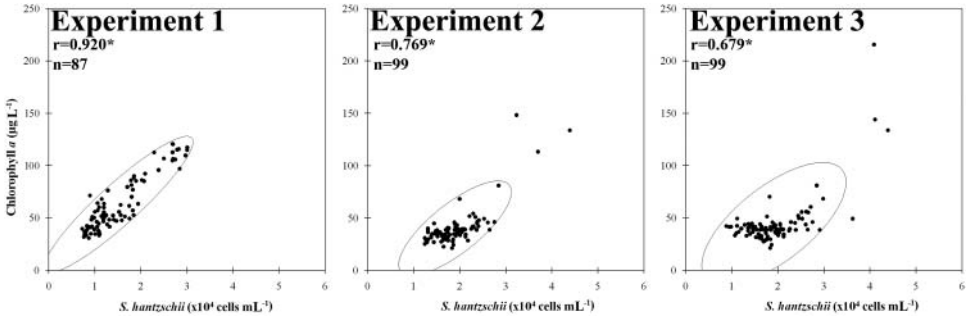

Figure 3. Pearson correlation analyses changes in chlorophyll *a* concentration and abundance of *Stephanodiscus hantzschii* in mesocosm experiments. An ellipse solid line in each figure showed confidence level of 95%. $^*p < 0.001$.

important as one of the practical methods for controlling harmful diatom blooms without disturbing the ecology of the local area.

Discussion

S. hantzschii blooms in water at low temperatures, below 15 °C (Jung et al. 2009b). Therefore, the ability to function at a low temperature is an essential requirement for SK09 to express its algicidal activity. The previous study confirmed that SK09 grows well between 10 °C and 30 °C (Jung et al. 2008) and showed the powerful algicidal activity between 5 °C and 15 °C in the wild (Jung et al. 2010, 2013). Thus, it is possible to apply this bacterium to naturally occurring *Stephanodiscus* blooms. However, there was no activity of SK09 in water with an extremely low temperature. Therefore, when considering whether to apply SK09, the environmental conditions should be taken into account, in particular the water temperature, even though SK09 has strong algicidal activity at a fairly low temperature.

The bacterial cell numbers in all experiments did not show any marked differences among them or compared with the control. However, the total numbers of bacterial cells after SK09 inoculation

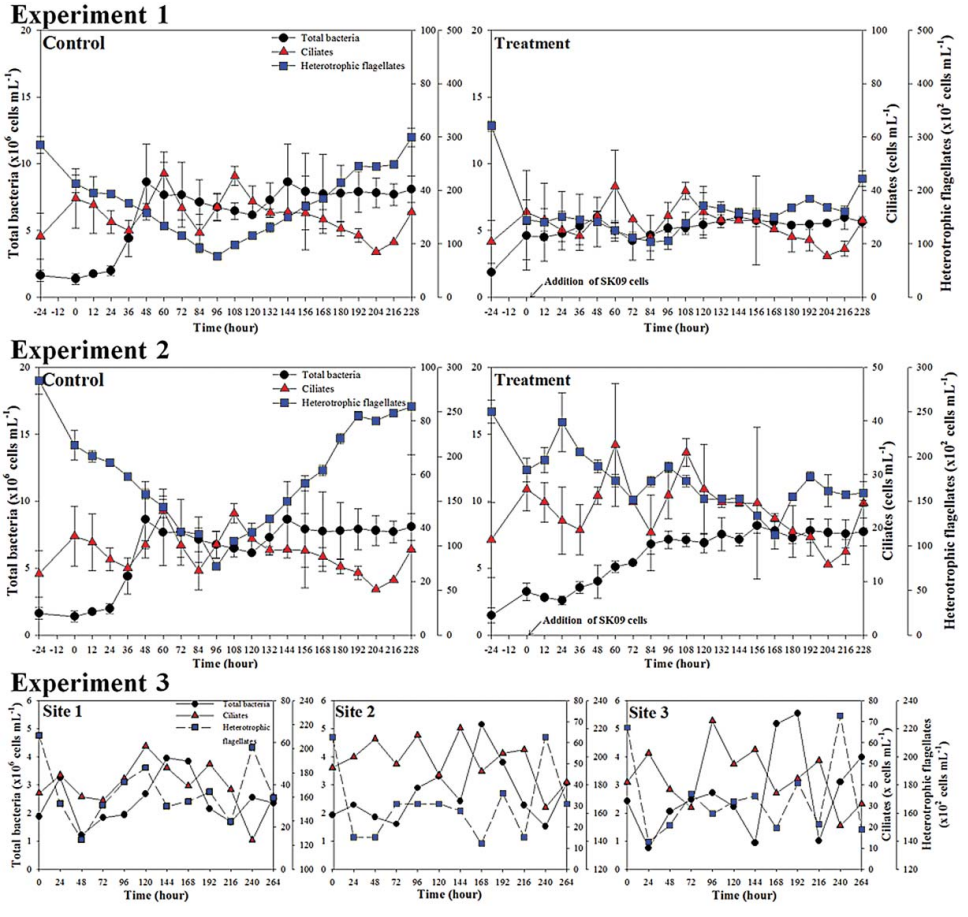

Figure 4. Changes in abundances of total bacteria, heterotrophic flagellates, and ciliates in mesocosm experiments and direct application in the wild.

in the previous microcosm and mesocosm experiments increased for the first 2 days and then decreased for the rest of the experimental period and the time lag in the changes in abundance of heterotrophic flagellates and ciliates might be associated with fluctuation in the total density of all bacteria (Jung et al. 2008, 2010, 2013). In microbial loops, bacterivores play important roles in controlling bacterial communities (Jung et al. 2012, 2015). In the previous results (Jung et al. 2010, 2013), heterotrophic protist communities responded quickly to the increase of bacterial cells after the inoculation of SK09. However, bacterivores in this study could not change their abundances due to no increase of bacterial cells at an extremely low temperature. This is an important interaction between protists and bacteria, similar to Mayali and Azam (2004) who reported that heterotrophic protists prevented the lysis of harmful algal species by grazing on algicidal bacteria. In addition, Lee et al. (2008) demonstrated that heterotrophic nanoflagellates inhibited the activity of algicidal bacteria against a harmful alga. In this regard, the time lag of the changes in heterotrophic protists could help to control the total bacterial density. If the inoculated bacterial cells were immediately utilized by heterotrophic protists, biological control would fail. In contrast, if the grazing did not occur upon the application of the bacterial cells, the ecosystem would be disturbed by a foreign organism. Thus, grazing pressure on algicidal bacteria plays a crucial role in the success of biological control and is important for the successful application of algicidal bacteria in the wild.

In this study, concentrations of inorganic nutrient and conductivity remained unchanged compared with those in the control. However, in our previous results (Jung et al. 2008, 2009a, 2010,

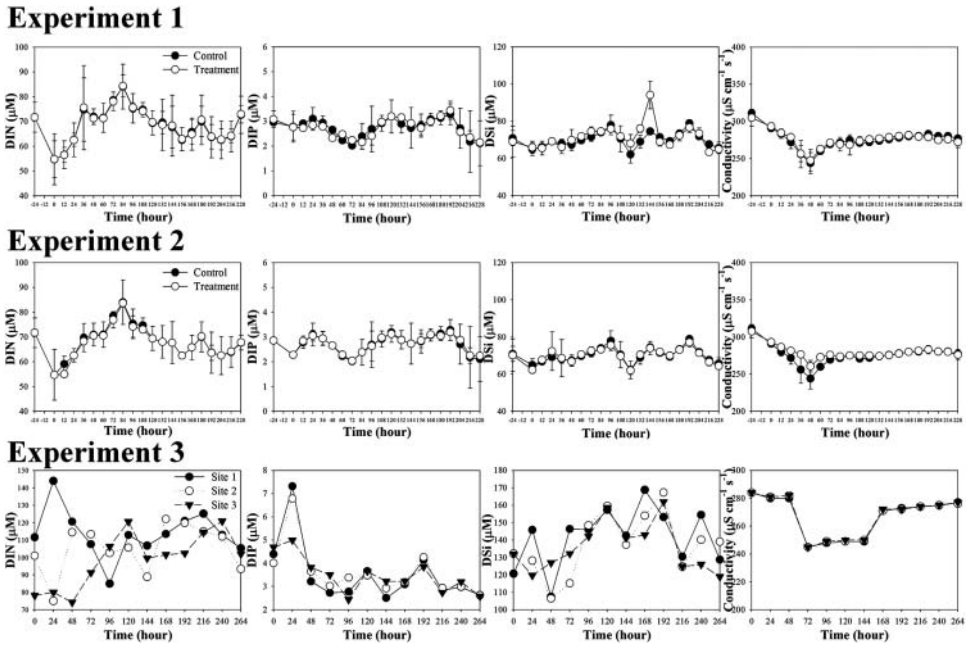

Figure 5. Changes in concentrations of dissolved inorganic nutrients [dissolved inorganic nitrogen (DIN), dissolved inorganic phosphorus (DIP), and dissolved silica (DSi)] and conductivity in mesocosm experiments and direct application in the wild.

2013), inorganic nutrient concentrations were significantly increased after the inoculation of SK09 cells in microcosm and mesocosm experiments. In line with this, as comparison between our previous and this study, the order of nutrient concentrations after treatment with algicidal bacteria was as follows: closed microcosm (Jung et al. 2008) > semi-open mesocosm (Jung et al. 2010) > carrier-treated semi-open mesocosm (Jung et al. 2013), but SK09 in this study could not control *S. hantzschii* blooms through inhibition of algicidal activity. Possible explanations for this include the release of inorganic substances resulting from the lysis of *S. hantzschii* cells by the algicidal activity of SK09 and lower nutrient uptake of phytoplankton. In addition, all the nutrient concentrations in the semi-open mesocosms were lower than those in the closed microcosms. Moreover, DIP concentration did not increase in the semi-open mesocosms, while it did markedly increase in the closed ones. The presence of sediment may have contributed to this difference; sediment is in general associated with a number of important mechanisms via the sequestering particulate matter to the bottom, the control of the supply of nutrients to benthos, and via the influence of epilimnetic nutrient regeneration (Hutchinson 1975; Lerman & Lietzke 1975). In particular, bacteria and meiofauna in sediment play significant roles in the decomposition of detritus and the uptake of nutrients. However, organic particles and inorganic nutrients produced by the destruction of *S. hantzschii* cells in closed microcosms may not have been eliminated by the activities of benthos, as these closed microcosms did not have a sediment layer. Upon addition of the APVAS carriers, DSi concentrations significantly decreased in the mesocosms supplemented with the algicidal bacteria. One of the key features for successful nutrient removal is the characteristics of the carrier, which can promote or inhibit nutrient removal (Aguilar et al. 2002). For example, Sublette et al. (1982) reported that powdered activated carbon did improve the nutrient removal effects.

An essential goal of studies on biological control using algicidal bacteria is to apply such bacteria in the wild, but ecosystems are highly complex and are affected by various biotic and abiotic factors. Many environmental, chemical, and biological scientists are only now beginning to implement such methods of biological control and therefore require more detailed knowledge about the ecosystems

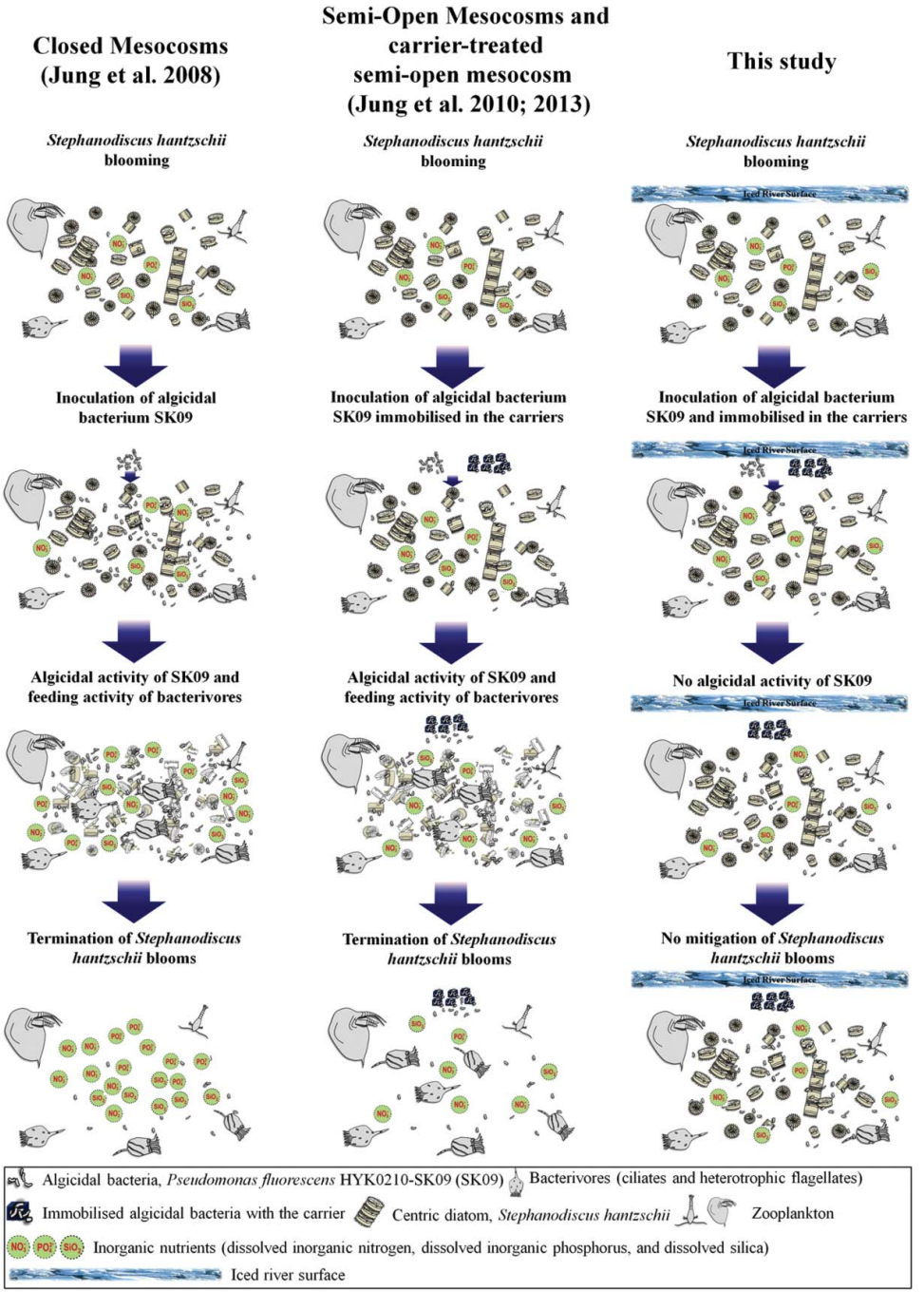

Figure 6. Conceptual schematic diagram of the ecological responses when SK09 is introduced under different treatment and ecosystem scenarios in various mesocosm experiments.

they are targeting. Most studies on algicidal bacteria in algal and bacterial culture-dependent experiments in the laboratory have been successful, but the actual assessment of HABs mitigation using algicidal bacteria must be carried out in the wild. As such, the results of this study assessing biological control using algicidal bacteria in the wild are significant. Future work should focus on

developing effective carriers as well as algicidal bacteria that rapidly induce HAB mortality for practical approaches to the control of such blooms and improvement of environmental stewardship.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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