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Mitigation of Harmful Algal Blooms by Sophorolipid

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Abstract A new method was proposed to control Harmful Algal Blooms (HABs) by a biosurfactant sophorolipid. The effect of sophorolipid on the growth, motility, precipitation, and recovery of algal cells was investigated for four common HAB species, *Scripsiella trochoidea*, *Prorocentrum minimum*, *Cochlodinium polykrikoides*, and *Heterosigma akashiwo*. The motility and growth of algal cells were inhibited significantly at the concentration of 20 and 5 mg/l sophorolipid, respectively, and no recovery was observed under the above concentrations. The concentration of 20 mg/l sophorolipid was considered to be an effective concentration for the mitigation of HABs. A sedimentation test suggested that the maximum precipitation occurred at the end of 1 h, and the algicidal effect of sophorolipid was observed by a microscope. Comparative study showed that sophorolipid had marked algicidal capability. Analysis on biodegradability, toxicity, and cost effectiveness further demonstrated the potential of sophorolipid in future HABs mitigation.

Key words: Harmful algal blooms, mitigation, biosurfactant, sophorolipid

Occurrences of toxic or harmful microalgae represent a significant and seemingly expanding threat to human health, fishery resources, and marine ecosystem throughout the world [1, 20]. In 2001, the loss of the fishery industry was reported to be more than 50 million US dollars in Korea. How to prevent the occurrence and mitigate the harmful effects of HABs has become an imperative problem facing HAB researchers and governments. To date, many methods have been proposed for the control of HABs, such as using ultrasonic waves to destroy algal cells, and killing microalgae directly with chemical agents or predating bloom species by zooplankton. However, none of them has been used in fields due to high costs, secondary pollution, or impracticability [1]. Coagulating HAB organisms with clay minerals is one promising strategy, and has been applied in the field in Korea, but it is still controversial due to unknown risk effects on the environment, especially on the benthos [17, 22]. Furthermore, clay minerals with good coagulating characteristic are not available in some areas. Therefore, it is necessary in the long run to seek new and more efficient ways to mitigate HABs.

Sophorolipid is a glycolipid biosurfactant produced by the yeast *Candida bombicola*, and has the advantages of low toxicity, high biodegradability, and ecological acceptability [6]. It was also reported that some types of glycolipid biosurfactant showed antimicrobial, antifungal, mycoplasmicidal, and antiviral activities [9, 10]. Lang *et al.* [11] tested the effect of a variety of microbially produced glycolipids on fungi as well as Gram-positive and Gram-negative bacteria, and obtained the MIC (Minimum Inhibitory Concentration) values of glycolipids on various microorganisms. Focusing our attention on some of the dominant species of HABs in Korean coastal waters, we investigated for the first time the effect of sophorolipid on the motility inhibition, growth inhibition, recovery, and sedimentation of HAB organisms. When compared with other surfactants, sophorolipid showed better algicidal effects and exhibited excellent potential for future applications. This study shows that HAB mitigation with sophorolipid would provide a new direction for HAB mitigation by biosurfactants.

Materials and Methods

Microorganism and Culture Conditions

The yeast *Candida bombicola* ATCC 22214 from the American Type Culture Collection was maintained on YM

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agar slants and was transferred at regular intervals. For precultivation, a loopful of the culture was used to inoculate 100 ml medium in 500-ml baffled shake flasks. The standard medium for batch cultivation contained (per liter deionized water): glucose 100g, yeast extract 0.5 g, KH_2PO_4 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $CaCl_2 \cdot 2H_2O$ 0.01 g, NaCl 0.01 g, peptone 0.07 g, and corn oil 100 g. Cells were cultivated for 8 days in a 2.5-l jar fermentor at 25°C, 1 vvm aeration rate, and pH 3.5. Sophorolipid was obtained by extracting the culture supernatant with an equal volume of ethyl acetate.

Cultures of HAB Organisms

Four HAB species, *Scripsiella trochoidea*, *Prorocentrum minimum*, *Cochlodinium polykrikoides*, and *Heterosigma akashiwo*, were maintained on *f*/2 medium [5] made with filtered seawater as the base. The seawater was filtered through a 0.2 μm-pore-size cellulose nitrate membrane filter and autoclaved at 121°C for 20 min. Cultures were grown at 20°C under continuous cool white fluorescent light of 5,000-6,000 lux on a 12 h-light-12 h-darkness regimen.

Motility Inhibition

Algal cultures were mixed with various concentrations of sophorolipid in a clean glass test tube. Immotile cells were counted with a Sedgewick-Rafter Counting chamber or Hemocytometer under a light microscope (Olympus BX50). Total cell numbers were determined by fixing the algal cells with Lugol solution. The motility inhibition ratio was calculated by immotile/total cells.

Growth Inhibition

Algae was inoculated into *f*/2 medium containing a certain concentration of sophorolipid and cultured at 20°C under light and dark conditions (flasks were covered with a layer of foil). The cell concentration was determined every day.

Recovery of Motility and Growth

Algal culture was filtered through a 0.2 μm-pore-size cellulose nitrate membrane filter after incubation with sophorolipid for the indicated period of time. The membrane was resuspended in fresh *f*/2 medium and cells were cultured under the above conditions. The motile cell numbers were determined after 1 h of inoculation to calculate the recovery rate. The total cell numbers were determined every day.

Sedimentation of Algal Cells Affected by Sophorolipid

Experiments were conducted in a device shown in Fig. 1. After a certain concentration of sophorolipid was added onto the surface layer, samples from the 4 lateral openings were collected at regular intervals using a syringe, and the cell concentrations were determined.

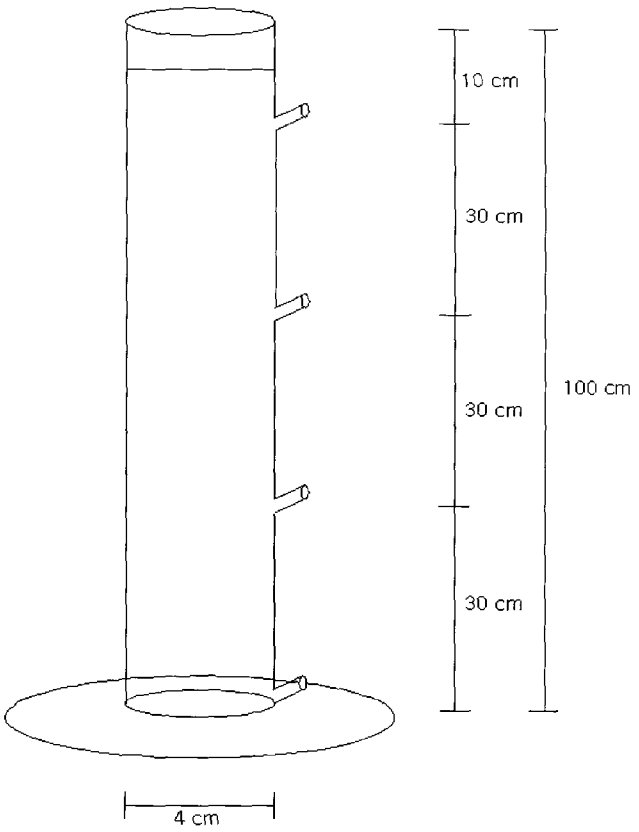

Fig. 1. Schematic diagram of sedimentation test tube.

RESULTS

Inhibition of Sophorolipid on Motility of Algal Cells

Two species of dinoflagellate, *P. minimum* and *C. polykrikoides*, were chosen to investigate the inhibitory effects of sophorolipid on the motility of algal cells. As shown in Fig. 2, motility inhibition by sophorolipid increased with time and concentration. For example, the inhibition efficiency of *P. minimum* was more than 50% after 30 min when sophorolipid concentration was 10 mg/l, and the inhibition was more than 90% after only 10 min at 20 mg/l sophorolipid. Similarly, the inhibition of *C. polykrikoides* was almost 90% at 10 mg/l sophorolipid after 30 min. The above results indicate that sophorolipid could effectively destroy the moving ability of algal cells at relatively low biosurfactant concentrations in a short time.

A study on the recovery of motility inhibition was further conducted in order to examine the irreversibility of the inhibitory effect of sophorolipid on algal motility. As shown in Table 1, there was no recovery of the motility at 20 mg/l sophorolipid for 3 min contact under light condition and at 10 mg/l sophorolipid under dark condition for 4 species of HAB organisms. When the contact period was extended to 10 min, there was no recovery at 10 mg/l under

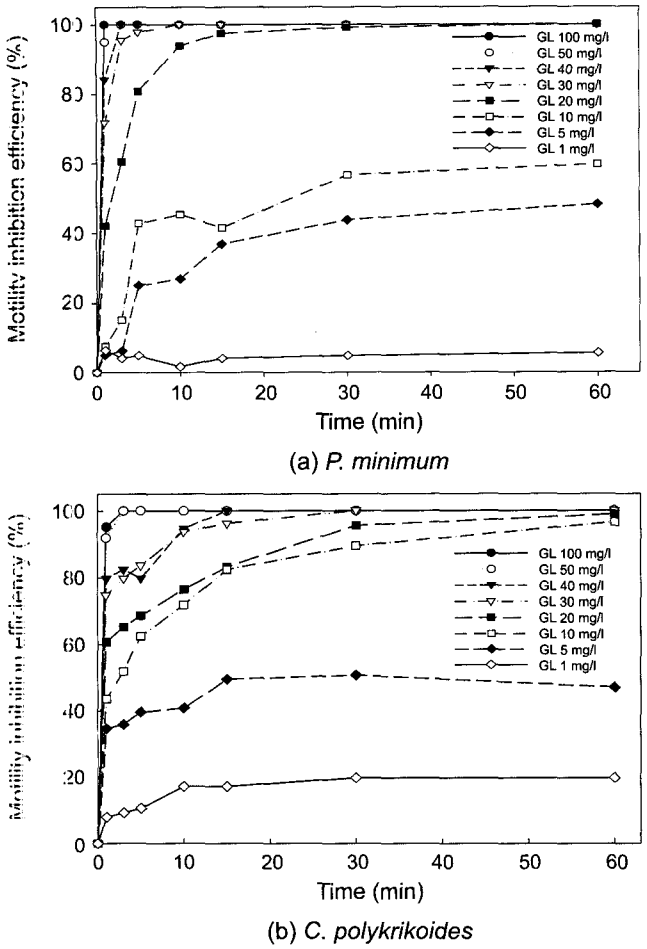

Fig. 2. Effect of sophorolipid concentration and contact time on the motility inhibition of *P. minimum* and *C. polykrikoides* (GL represents sophorolipid).

either light or dark conditions. The above results together suggest that the concentration of 20 mg/l sophorolipid effectively controlled the motility of HAB species and prevented the recovery. Once the cells lost their motility, they generally sank to the bottom in dark condition. Therefore, as shown in Table 1, slower recovery in dark conditions indicates that as low as 10 mg/l of sophorolipid can effectively mitigate HABs.

Inhibition of Growth of HAB Organisms

Figure 3 shows the effect of sophorolipid on the growth of HAB organisms. In light conditions, the control group showed normal lag, exponential, and stationary phases. An addition of 1 mg/l sophorolipid inhibited the growth of microalgae slightly, whereas the inhibition effect was remarkable at the concentration of 5 mg/l sophorolipid. The cell concentration fell to almost zero after culturing for 1 day for *S. trochoidea* and *H. akashiwo*. The growth of *P. minimum* and *C. polykrikoides* stopped after 4 days under the same conditions. The lack of *H. akashiwo* cell wall might have been responsible for the strong inhibition, since the target of surfactants is usually the cell membrane [11]. In dark conditions, the growth of algae in the control group stopped due to the absence of photosynthesis, and the cell concentrations remained almost unchanged during the whole experimental period. Therefore, the algal growth in dark conditions was inhibited at a lower concentration of sophorolipid (1 mg/l). The above results indicate that a low concentration (5 mg/l) of sophorolipid could be used to prevent the occurrence of HABs at the beginning stage.

Figure 4 shows the recovery of algal growth after contact with sophorolipid over an indicated period of time. The results indicate that the possible recovery decreased as the sophorolipid concentration and contact time increased. The growth of algal cells was not observed at 20 mg/l sophorolipid after contacting for 3 min and at 10 mg/l after contacting for 10 min, further suggesting that the concentration of 20 mg/l sophorolipid can mitigate HABs effectively.

Sedimentation of Algal Cells Affected by Sophorolipid

In order to avoid the heterogeneous distribution of algal cells in seawater, samples were taken from 4 layers (5, 35, 65, 95 cm) after adding various concentrations of sophorolipid (device shown in Fig. 1). As shown in Fig. 5, at low sophorolipid concentration (5 mg/l), algal numbers varied slightly compared to the control group, except that the cell concentration in the bottom layer increased due to sedimentation of immotile cells. With the increase of sophorolipid to 10-20 mg/l, the cell concentration in the surface layer decreased immediately. The concentrations of the other three layers, however, showed the trend of first

Table 1. Recovery efficiency of algal motility after contacting with sophorolipid.

HAB species	Recovery after 3 min contact (%)							Recovery after 10 min contact (%)						
	Light condition				Dark condition			Light condition				Dark condition		
	Sophorolipid (mg/l)													
	1	5	10	20	1	5	10	1	5	10	20	1	5	10
Scripsiella trochoidea	83	63	33	0	63	31	0	67	45	0	0	46	18	0
Prorocentrum minimum	86	72	45	0	66	33	0	61	41	0	0	41	15	0
Heterosigma akashiwo	80	66	20	0	60	24	0	68	39	0	0	45	19	0
Cochlodinium polykrikoides	89	68	48	0	59	36	0	63	48	0	0	49	13	0

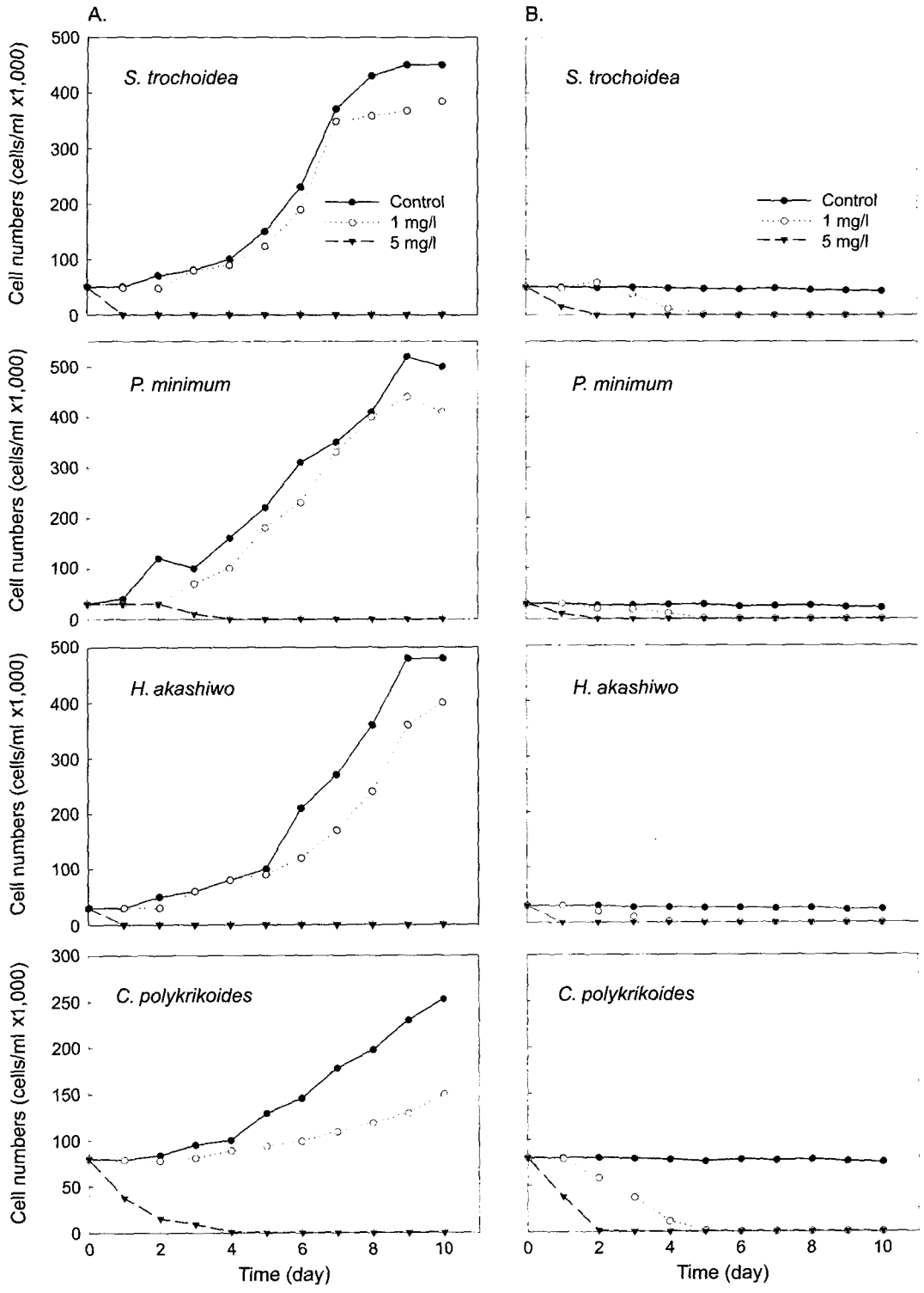

Fig. 3. Effect of sophorolipid on the growth of HAB organisms.
(A) Light condition. (B) Dark condition.

increasing followed by decreasing. Probably, the algal cells lost motility and gradually precipitated to the bottom, resulting in decreased cell numbers in the surface layer and proportionate increase in the other three layers. As the time increased, cell lysis increased, resulting in decreased cell concentrations in all layers. Peak cell numbers in the

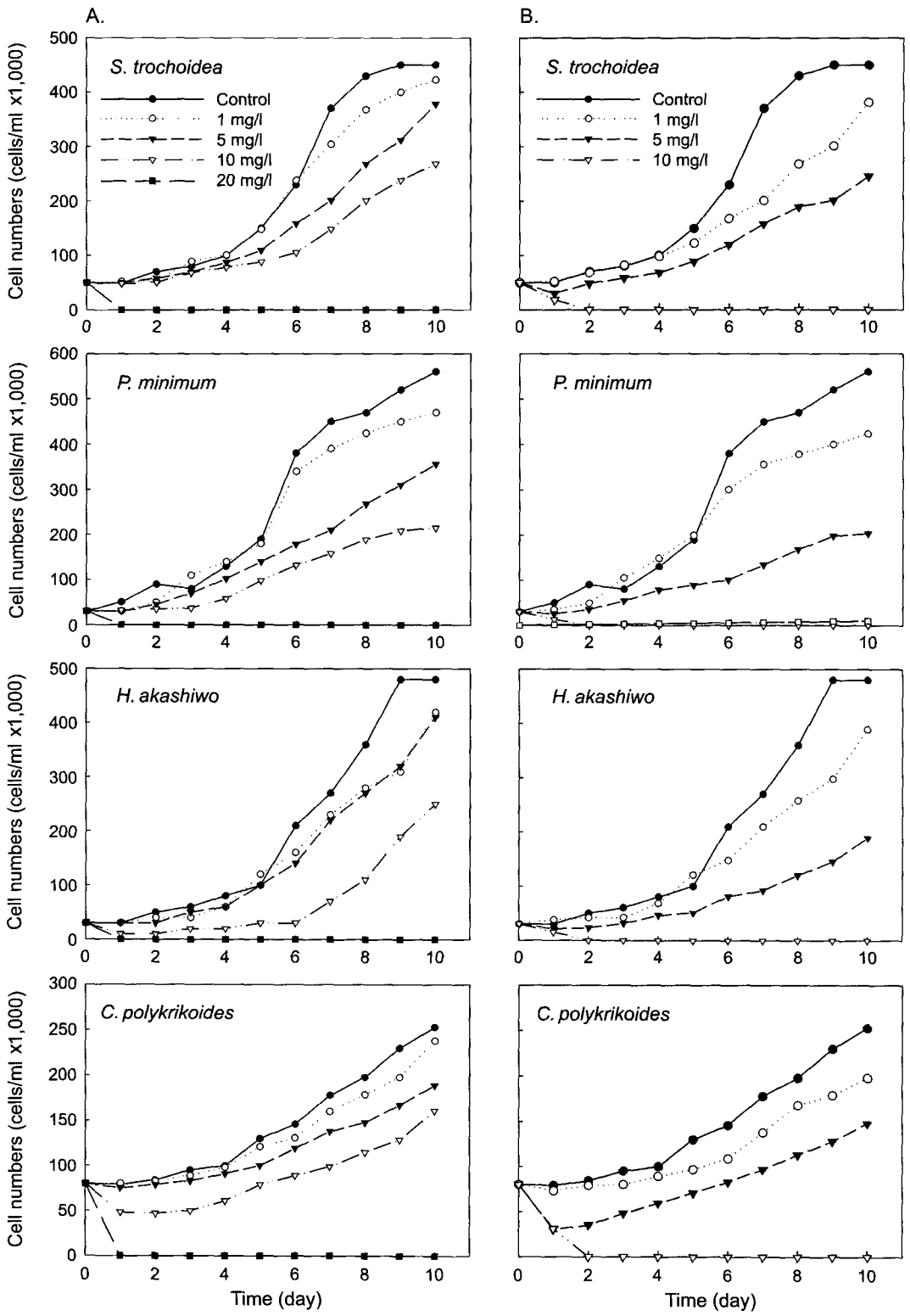

Fig. 4. Effect of contact time with sophorolipid on the growth of HAB organisms.
(A) Contact time was 3 min. (B) Contact time was 10 min.

bottom layer appeared after 1 h, which corresponded to the maximum sedimentation of algal cells at this point. At the end of 10 h, the cell numbers in the whole waterbody decreased significantly at 10 and 20 mg/l sophorolipid,

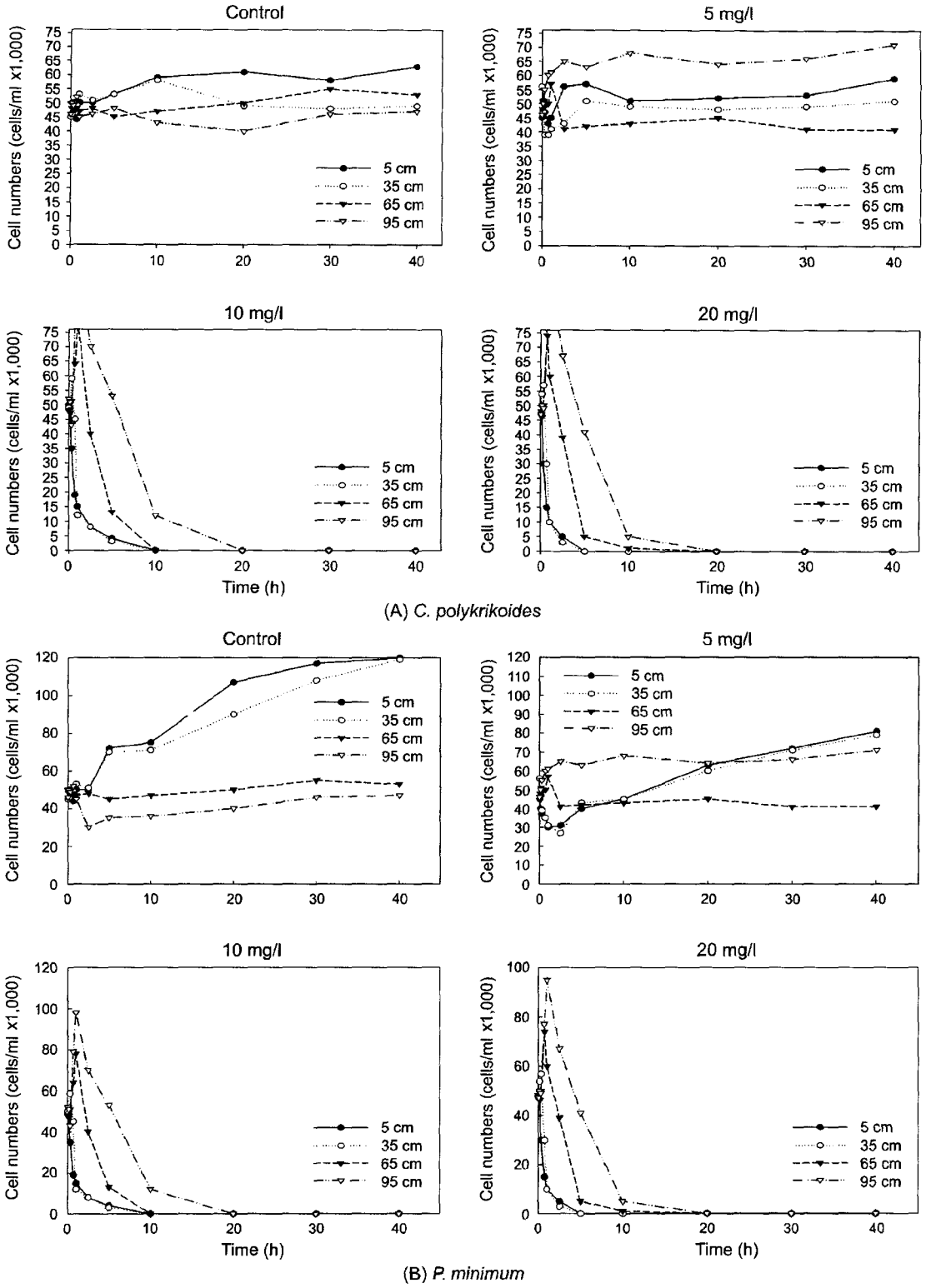

Fig. 5. Sedimentation of algal cells affected by sophorolipid.
(A) *C. polykrikoides*. (B) *P. minimum*.

indicating the lysis of most of the algal cells. The microscope features demonstrate the above phenomena in Fig. 6. The whole process of sophorolipid on algal cells can be described as follows: normal cells→cells losing motility→

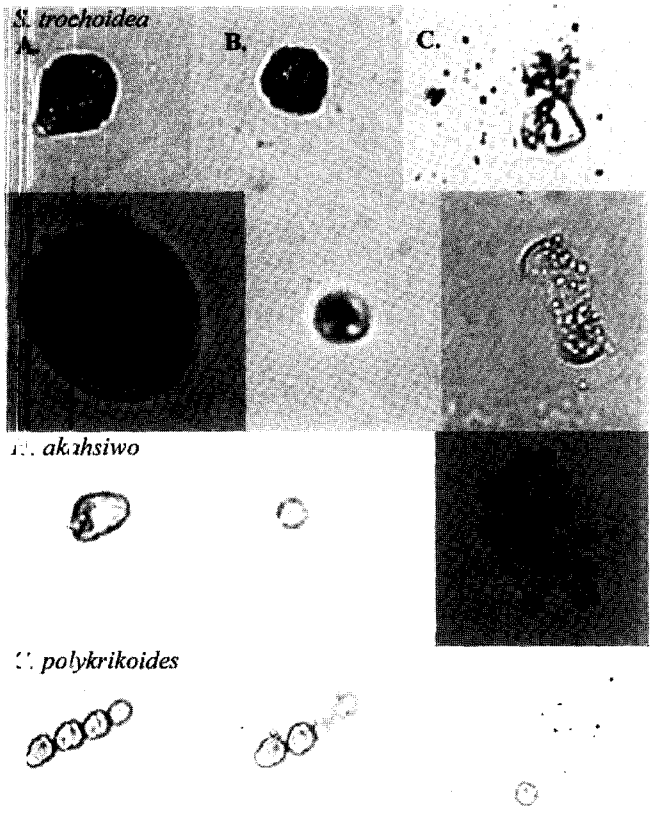

Fig. 6. Microscope pictures on cell lysis induced by 20 mg/l sophorolipid.
(A) The control. (B) After 5 min. (C) After 10 min.

swelling cells→cell lysis, thus proving that sophorolipid prevents HABs by directly killing algal cells.

Comparison Between Sophorolipid and Other Surfactants

As depicted in Table 2, sophorolipid showed excellent algicidal capability, compared with other surfactants. Also, the removal ratios of HAB organisms were even more than 10 times higher than those of other surfactants at the concentration of 20 mg/l sophorolipid, indicating great potential application in HAB mitigation.

DISCUSSION

Harmful algal blooms, especially toxic dinoflagellate red tide, is one of the most serious marine environmental problems to have aroused the world's attention. In this study, biosurfactant sophorolipid was used in HABs mitigation for the first time, which not only provided a new way to bring about HABs mitigation with bioactive materials, but also broadened the application area of sophorolipid.

Sophorolipid prevented HABs by killing algal cells directly, thus avoiding recurrence of HABs. According to Lang and Wagner [12], the cell membranes are primarily affected by the biosurfactant. This same notion was proposed by Rosenberg and Ron [19]. The different cell-wall structures of the organisms and the different amphiphile character of the biosurfactant (e.g., ionic charge, hydrophilic/hydrophobic moieties) are responsible for the different effects. Upon treatment of *Bacillus subtilus* with sophorolipid, an intracellular TCA cycle enzyme, i.e., malate dehydrogenase, was released outside the cell [9]. This indicates that the sophorolipid increased the membrane permeability of Gram-positive bacteria. It is suggested that the lipopeptide, a biosurfactant molecule, might penetrate into the cellular membrane, resulting in a leakage of cytoplasm material and/or cell lysis [15, 16]. This same mechanism probably causes the swelling and lysis of HAB organisms, however, further work needs to be conducted in order to elucidate the interaction of sophorolipid with the cellular membrane.

According to the review of Yu *et al.* [21], HAB organisms have the character of phototaxis, and usually move to the surface during the daytime. Therefore, the treatment with HAB should be conducted at the surface layer. Accordingly, the surfactant can become one kind of anti-HAB agent. This method can be efficient in the case of high density of algal cells, and has the advantage of a high rate of killing and removing HAB organisms, with little damage to other environmental organisms. It is well known that migratory behaviour is a kind of adaptive strategy for HAB organisms. The motility of algae is a key

Table 2. Comparison of algicidal effect between sophorolipid and other surfactants.

HAB species	Inhibition by Surfactants[a] (%)								
		Sucrose mono							
	Sophorolipid	aleate	olate	Rhamnolipid	Span80	Tween80	OIMP[b]	AOS[c]	MPS[d]
Scripsiella trochoidea	100	41	43	28	79	45	7	10	35
Prorocentrum minimum	100	78	39	12	69	8	6	5	38
Heterosigma akashiwo	100	83	79	35	85	43	18	43	41
Cochlodinium polykrikoides	100	100	81	37	89	46	16	12	46

[a] The concentrations of sophorolipid and other surfactants were 20 mg/l.
[b] OIMP: 1-methyl-1-oleylamidoethyl-2-oleylmidazolinium methosulfate.
[c] AOS: oleffinsulfonate.
[d] MPS: esterquart.

factor affecting the nutrient uptake and photosynthesis of HAB species, which result in their dominance during bloom events in some instances. Some species can adapt to exploit fluctuating irradiance associated with rapid vertical mixing [8, 13]. Therefore, the inhibition on algal motility would directly influence the physiological activities of the microalgae. In our study, sophorolipid showed excellent features with regard to the inhibition of algal motility, growth, and algicidal effects at the concentration of 20 mg/l. Moreover, there was no recovery from the inhibition, which avoided the reoccurrence of HABs.

The main factors that should be considered in selecting anti-HAB agents are effectiveness, cost, biodegradability, degradation products, and their toxicity to humans, environment, and environmental organisms. As mentioned above, sophorolipid has the advantages of low toxicity, a biodegradable nature and ecological acceptability. Up to now, many studies have described the biodegradability of sophorolipid [4, 6, 14], and our study showed that sophorolipid was degraded faster than other chemical surfactants [10]. Also, our study on the acute toxicity of sophorolipid on marine organisms indicated that there was almost no mortality of *Mytilus edulis*, *Scapharca subcrenata*, *Mugil cephalus*, and *Sebastes schlegeli* at maximum concentrations of 700, 70, and 40 mg/l sophorolipid after 24 h (detailed toxicity data will be published in a separate paper), which were far higher than the effective concentrations observed in the above laboratory experiments. Using crude microbial surfactants for toxicity on *Daphnia magna*, Zajic and Gerson [23] found that some microbial surfactants appeared to serve as a nutrient to the *Daphnia*, and death rates in the absence of the surfactant exceeded those with biosurfactants. Toxicity tests with brine crayfish *Corophium volutator* (field test) or with *Artenia larva* (laboratory test) also indicated the superiority of the biosurfactants [2, 3]. The above findings strongly demonstrate the low toxicity of sophorolipid on the marine environment.

As for the cost of sophorolipid, Rau *et al.* [18] gave an estimated price of \$1-\$3/ kg, based on their production facilities yielding more than 300 g/l of sophorolipid at maximum productivity of 76 g/l/d. With the progress of efficient cultivation and the development of new production techniques, the cost of sophorolipid would further decrease, thus making it even more promising in HAB mitigation [5].

Acknowledgments

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