Allelopathic effects of *Ulva pertusa*, *Corallina pilulifera* and *Sargassum thunbergii* on the growth of the dinoflagellates *Heterosigma akashiwo* and *Alexandrium tamarense*

Renjun Wang · Hui Xiao · Peiyu Zhang ·
Liang Qu · Hengjiang Cai · Xuexi Tang

Received: 21 December 2005 / Revised and Accepted: 26 July 2006 / Published online: 10 November 2006
© Springer Science + Business Media B.V. 2006

Abstract The allelopathic effects of fresh tissue, dry powder and aqueous extracts of three macroalgae, *Ulva pertusa*, *Corallina pilulifera* and *Sargassum thunbergii*, on the growth of the dinoflagellates *Heterosigma akashiwo* and *Alexandrium tamarense* were evaluated using coexistence culture systems in which concentrations of the three macroalga were varied. The results of the coexistence assay showed that the growth of the two microalgae was strongly inhibited by using fresh tissue, dry powder and aqueous extracts of the three macroalga; the allelochemicals were lethal to *H. akashiwo* at relatively higher concentrations of the three macroalga. The macroalgae showing the most allelopathic effect on *H. akashiwo* and *A. tamarense* using fresh tissue were *U. pertusa* and *S. thunbergii*, using dry powder were *S. thunbergii* and *U. pertusa*, and using aqueous extracts were *U. pertusa* and *C. pilulifera*. We also examined the potential allelopathic effect on the two

microalgae of culture filtrate of the three macroalga; culture medium filtrate initially exhibited no inhibitory effects when first added but inhibitory effects became apparent under semi-continuous addition, which suggested that continuous release of small quantities of rapidly degradable allelochemicals from the fresh macroalgal tissue were essential to effectively inhibit the growth of the two microalgae.

Key words allelopathic effects · dinoflagellate · seaweed extract

Introduction

In recent years there has been a growing awareness of the problems associated with red tides, which may induce mass mortalities of cultured fish or shellfish, and cause damage to aquaculture industries such as abalone farming, prawn culture and caged fish culture (Mackenzie 1991; Qi et al. 1993; Honjo 1994; Horner et al. 1997; Kim 1997). Red tides are the result of massive blooms of harmful microalgae (HABs), especially blooms of the dinoflagellates *Heterosigma akashiwo* (Hada) Hada ex Hara et Chihara and *Alexandrium tamarense* (Lebour) Balech, which cause heavy damage almost every year in China and other countries. Moreover, about 2,000 cases of human poisoning resulting from algal toxins are reported every year (Zingone and Enevoldsen 2000).

R. Wang · H. Xiao · P. Zhang ·
L. Qu · H. Cai · X. Tang (✉)
Marine Ecology Laboratory, Ocean University of China,
No. 5, Yu Shan Road, Qingdao, Shandong Province
266003, People's Republic of China
e-mail: tangxx@ouc.edu.cn

P. Zhang
Department of Environmental Science and Engineering,
Qingdao University, Qingdao 266071,
People's Republic of China

Because of the severe economic and public health problems caused by harmful microalgae, many studies about HABs have been conducted and have been well reviewed by Hallegraeff et al. (1995). Rensel (1995) reported several techniques to mitigate the effects of harmful algal blooms on finfish aquaculture. Some promising methods have been developed, including the use of clay to sediment red tide organisms (Na et al. 1996; Choi et al. 1998), chemical agents such as copper sulfate (Steidinger 1983), hydrogen peroxide (Ryu et al. 1998), and some biological control in the form of viruses (Garry et al. 1998) or bacteria (Fukami et al. 1992; Imai et al. 1995). Although these methods seem effective in short-term experiments, they may have potentially dangerous environmental consequences; very few investigations of a direct and specific control of marine harmful algal blooms with few environmental side-effects have been reported (Jeong et al. 2000).

In the search for HAB control agents that are efficient and benign to the environment, scientists are showing interest in allelopathic substances released by other aquatic organisms for growth inhibition of HAB species. Macroalgae and microalgae have long been known to have an antagonistic relationship in both natural and experimental aquatic ecosystems (Hasler and Jones 1949). Hogetsu et al. (1960) surmised that macrophytes released allelochemicals to inhibit algal growth. Several bioactive substances have been extracted and purified successfully for practical HAB control and management (Taft and Martin 1986; Kakisawa et al. 1988; Yu et al. 1992; Suzuki et al. 1998; Nakai et al. 1999; Jeong et al. 2000; Lee et al. 2000; Nakai et al. 2000; Jin and Dong 2003).

However, we have little knowledge of non-nutrient interactions between macroalgae and microalgae. Thus, in this study, we selected three macroalgae, *Ulva pertusa* Kjellman (Chlorophyta), *Corallina pilulifera* Postels et Ruprecht (Rhodophyta) and *Sargassum thunbergii* (Mertens et Roth) Kuntze (Phaeophyta). The two harmful microalgae used in this study are common HAB species that have been recorded worldwide. *Heterosigma akashiwo* and *Alexandrium tamarense* are well known ichthyologic species through the production of reactive oxygen species or other bioactive metabolites or mucus substances (Kim et al. 1999, 2000; Nagasaki et al. 1999). Macroalgae are common in the coastal waters

of China and collecting abundant macroalgae represents a potentially easy, low cost and relatively environmentally benign means of controlling HABs.

Materials and methods

Algae and experimental conditions

The axenic strains of *H. akashiwo* and *A. tamarense* were provided by the Microalgal Laboratory of Ocean University of China. *U. pertusa*, *C. pilulifera* and *S. thunbergii* were collected from the Taiping region of Qingdao, China. The algae were immediately carefully washed with distilled water to remove attached organisms. They were then treated with a mixture of antibiotics and grown aseptically. All the macroalgae and microalgae were cultured at room temperature with illumination of $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, then prepared in culture for 7 days in f/2 medium (Guillard and Ryther 1962) at 20°C, using a light intensity of about $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 12:12 h light: dark cycle in illuminated incubators. All cultures were shaken twice a day to prevent wall growth. The seawater was obtained from the coast of Qingdao, filtered through 0.22 μm pore size cellulose nitrate membrane filters and autoclaved at 121°C for 20 min.

Microalgae were cultured to exponential phase before subsequent inoculation. The initial cell densities of *H. akashiwo* and *A. tamarense* used in the experiments were approximately 1×10^5 or 8×10^4 cells mL^{-1} , respectively. The culture conditions in the following experiments were the same unless otherwise stated.

Coexistence assays with fresh macroalga tissue

To compare the effects on the two microalgae of different concentrations of fresh tissue from the three macroalgae, coexistence assays were performed using a mixed culture system of one macroalga and one microalga. Different initial inoculation concentrations (0, 0.625, 1.25, 2.5, 5.0, 10 and 15 g-wet wt L^{-1}) of fresh algal tissues were inoculated into 100 mL conical glass flasks containing 40 mL f/2 culture medium. Exponentially growing *H. akashiwo* or *A. tamarense* were added to the fresh tissue seaweed culture in co-culture systems. Controls were prepared by inoculating the microalgae into f/2 culture medium without the addition of a macroalga.

Microalgae samples (1 mL) were taken daily and fixed with acidic Lugol's and counted with a haemocytometer. We conducted the experiment under stable environmental conditions, and then 1 mL nutrition solution, which contained 20 x f ingredients for 40 mL culture medium, was added to each flask to rule out nutrient depletion and avoid nutrient competition between microalgae and macroalgae. The concentration of $\text{NO}_3\text{-N}$ and $\text{PO}_4\text{-P}$ was also routinely measured throughout the experiment. The pH of the culture medium and the wet weight of the macroalga were also measured.

Macroalga culture medium filtrate assays

Initial addition of macroalga culture medium filtrate

The initially added culture solution was prepared by culturing the three macroalgae over 3 days in f/2 medium at a concentration of 80 g-wet wt L^{-1} . This culture solution was added with nutrients equivalent to the medium and filtered through an autoclaved membrane filter (Whatman, 0.22 μm); 40 mL of the filtrate was then immediately added to a 100 mL flask and inoculated with *H. akashiwo* or *A. tamarensis*, while microalgae cultured in fresh f/2 medium were used as controls.

Semi-continuous addition of macroalga culture medium filtrate

The same culture solution as above (40 mL) was added to a 100 mL flask and inoculated with *H. akashiwo* or *A. tamarensis*. Each day 10 mL of the 40 mL culture medium was removed from each flask and 10 mL nutrient re-enriched macroalga culture medium filtrate was added to keep the culture medium volume constant. In the control the filtrate was replaced by fresh f/2 medium.

Boiled macroalga culture medium filtrate assays

The macroalga culture medium was boiled for 20 min at 121°C, filtered through an autoclaved membrane filter (Whatman, 0.22 μm) and re-enriched with f/2 medium. Cells of *H. akashiwo* or *A. tamarensis* were immediately inoculated into the culture medium filtrate of the three macroalgae, while controls were prepared by inoculating microalgae into f/2 medium only.

Coexistence assays

Fresh macroalga tissue (8 g) was ground with some distilled water using a mortar and centrifuged three times using seawater. About 80 mL supernatant was collected and then diluted with seawater to give different concentrations of aqueous extracts (0, 2, 4, 8, 16, 24 g-wet wt L^{-1}). These extracts were inoculated into flasks containing microalgae. Controls were prepared by inoculating only with microalgae without adding the aqueous extraction.

The method for preparing the aqueous extracts of the macroalgae was the same as described above. About 50 mL solution was boiled for 20 min at 121°C and then diluted with seawater to the desired concentration (8 g-wet wt L^{-1}) and inoculated with exponentially growing microalgae. Controls were prepared by inoculating microalgae in the medium without adding the aqueous macroalga extract.

Coexistence assays with dry macroalga powder

For the convenient use of samples, fresh macroalgal tissue was dried completely for 6 days at room temperature and then ground to a powder using a mortar and pestle. Different amounts of dry powder with an initial concentration of 0, 0.3, 0.6, 1.2 and 2.4 g-dry wt L^{-1} were inoculated into 100 mL flasks, and the dinoflagellates *H. akashiwo* and *A. tamarensis* were also immediately added. The control contained only microalgae.

Statistics

All experiments were repeated at least three times for each independent assay. Mean values were compared to the controls using student's *t*-test. $P < 0.05$ was considered significant.

Results

Sufficient amounts of N and P were available for algae growth. The daily addition of 1 mL 20xf solution resulted in nutrient concentrations of $\text{NO}_3\text{-N} \geq 780 \mu\text{mol L}^{-1}$ and $\text{PO}_4\text{-P} \geq 36 \mu\text{mol L}^{-1}$, which are well above limiting levels for microalgae (Guillard and Ryther 1962; Sharp et al. 1979). An effect of lighting conditions was also ruled out by measuring the light intensity at the bottom of the culture flask; the results

confirmed that inhibition did not occur due to insufficient light (data not shown). The pH measured daily for all culture media was $\text{pH } 8.5 \pm 0.3$, which is well suited for the growth of microalgae (Chen and Durbin 1994).

Effects of fresh macroalgal tissue

Figure 1 shows the growth curve of the three macroalgae coexistence cultures with *H. akashiwo* or *A. tamarensis*. The growth of *H. akashiwo* was

significantly suppressed ($P < 0.05$) by the three macroalgae at the different concentrations tested (Figure 1a–c). Cells of *H. akashiwo* were all dead at concentrations higher than $2.5 \text{ g-wet wt L}^{-1}$ after 3 days with *U. pertusa*, while they were all dead within 8 days with the other two macroalgae when concentrations of $>5 \text{ g-wet wt L}^{-1}$ were used. The growth of *A. tamarensis* was significantly ($P < 0.05$) suppressed by all three macroalgae; however, no lethal effect was observed (Figure 1d–f).

Figure 1 Growth curves of *Heterosigma akashiwo* (a–c) or *Alexandrium tamarensis* (d–f) coexistence assays with different initial inoculation concentrations ($\square$ 0; $\blacksquare$ 0.625; $\blacktriangle$ 1.25; $\times$ 2.5; $\square$ 5; $\bullet$ 10; $\square$ 15 g-wet wt L^{-1}) of fresh tissue of *Ulva pertusa* (a, d), *Corallina pilulifera* (b, e) or *Sargassum thunbergii* (c, f) in coexistence assays. Data are the means $\pm$ SD from at least three independent assays.

Figure 2 Normal maximum growth of *H. akashiwo* (a) or *A. tamarens* (b) in the presence of fresh tissue of *U. pertusa* (▲), *C. pilulifera* (□) or *S. thunbergii* (●). Data are the means $\pm$ SD from at least three independent assays.

Figure 2 shows the respective dose-response relationships between the three macroalgae and the two microalgae. Results were normalized to maximum growth based on previous methods (Nakai et al. 1999). At the concentration used, normal growth of microalgae was inhibited 50% by the macroalga.

The growth of *H. akashiwo* was more strongly suppressed by fresh tissue of *U. pertusa* than fresh tissue of the other two macroalgae (Figure 2a). The EC₅₀ concentration for the fresh tissue of *U. pertusa* was 0.5 g-wet wt L⁻¹. The growth of *A. tamarens* was more strongly suppressed by fresh tissue of *S. thunbergii* than fresh tissue of the other two macroalgae (Figure 2b). The EC₅₀ concentrations for *S. thunbergii*, *U. pertusa* and *C. pilulifera* were 0.8, 1.7 and 4.0 g-wet wt L⁻¹, respectively (Table 1).

Effects of macroalga culture medium filtrate

The growth of *H. akashiwo* was not significantly ($P > 0.05$) inhibited by the culture medium filtrate of *U. pertusa* or *S. thunbergii* under the initial or semi-continuous filtrate addition conditions. The initial addition of *C. pilulifera* culture solution did not inhibit growth of *H. akashiwo*; however, semi-continuous addition significantly inhibited the growth of *H. akashiwo* ($P < 0.05$), and the cells all died (Figure 3a–c).

The growth of *A. tamarens* was significantly ($P < 0.05$) inhibited by the culture medium filtrate of *U. pertusa* under the initial or semi-continuous filtrate addition conditions, and all cells died at the end. The

effect of the culture medium filtrate of *S. thunbergii* on *A. tamarens* was the same as for *U. pertusa*, but no lethal effect was observed. The growth of *A. tamarens* was not significantly inhibited by the culture medium filtrate of *C. pilulifera* under either initial or semi-continuous addition (Figure 3d–f).

Effects of boiled macroalga culture medium filtrate

The growth of *A. tamarens* was significantly ($P < 0.05$) inhibited by the boiled culture medium filtrate of *S. thunbergii*. However, the inhibitory effect was lower than that of the non-boiled culture medium filtrate and no lethal effect was observed (Figure 4a). The semi-continuous addition of the boiled *C. pilulifera* culture medium filtrate significantly ($P < 0.05$) inhibited the growth of *A. tamarens* but the effect was lower than that observed with non-

Table 1 Summary inhibitory effects (EC₅₀ values) of fresh tissue (g-wet wt L⁻¹) or dry powder (g-dry wt L⁻¹) of *Ulva pertusa*, *Corallina pilulifera* or *Sargassum thunbergii* on *Heterosigma akashiwo* or *Alexandrium tamarens*

Treatment	<i>Heterosigma akashiwo</i>	<i>A. tamarens</i>
Fresh tissue of <i>U. pertusa</i>	0.5	1.7
Fresh tissue of <i>C. pilulifera</i>	1.8	4.0
Fresh tissue of <i>S. thunbergii</i>	2.5	0.8
Dry powder of <i>U. pertusa</i>	0.5	0.6
Dry powder of <i>C. pilulifera</i>	1.0	1.0
Dry powder of <i>S. thunbergii</i>	0.45	1.3

Figure 3 Growth curves of *H. akashiwo* (a–c) or *A. tamarensis* (d–f) under initial (■) or semi-continuous (▲) culture medium filtrate addition of *U. pertusa* (a, d), *C. pilulifera* (b, e) or *S. thunbergii* (c, f) with control (□ initial addition; □ semi-continuous addition). Data are the means $\pm$ SD from at least three independent assays.

boiled filtrate. No lethal effect was observed on *H. akashiwo* (Figure 4b).

Within 5 days, the growth of *A. tamarensis* was significantly lowered by the boiled culture medium filtrate of *U. pertusa* ($P < 0.05$). However, over the period of the experiment (5–8 days), the growth of *A. tamarensis* recovered to normal levels (Figure 4c).

Effects of different concentrations of aqueous extracts

The growth of *H. akashiwo* was significantly reduced ($P < 0.05$) by all tested concentrations of aqueous extracts of *U. pertusa*, *C. pilulifera* and *S. thunbergii* within the first 4, 2 and 2 days, respectively. Inhibition of growth increased with extract concentra-

Figure 4 Growth curves of *A. tamarens* (a, c) or *H. akashiwo* (b) with addition of boiled culture medium filtrate of *S. thunbergii* (a), *C. pilulifera* (b) or *U. pertusa* (c) with control (□ control; ■ treatment). Data are the means $\pm$ SD from at least three independent assays.

tion. Aqueous extracts of all three macroalgae at a concentration of 24 g L^{-1} resulted in the death of *H. akashiwo* within 2 days. The growth of *H. akashiwo* was stimulated at the lower concentration treatments ($2\text{--}8 \text{ g L}^{-1}$). During the 4th and 10th day of the experiment, aqueous extract of *U. pertusa* at a concentration of 2 g L^{-1} and extracts of the two other macroalgae at a concentration of $2\text{--}4 \text{ g L}^{-1}$, resulted in faster and higher growth than in the control culture of *H. akashiwo* (Figure 5a–c).

The growth of *A. tamarens* was significantly ($P < 0.05$) inhibited by all tested concentrations of the aqueous extracts of the three macroalgae within the first 3 days of the experiment. The inhibitory effect of the aqueous extract of *C. pilulifera* was higher than that of the two other macroalgae during the first 2 days of the experiment. Aqueous extracts of *C. pilulifera* and *S. thunbergii* at a concentration of 8 g L^{-1} or 16 g L^{-1} , respectively, resulted in the death

of the *A. tamarens* culture. However, no lethal effects were observed for the aqueous extract of *U. pertusa* (Figure 5d–f).

Effects of boiled aqueous extracts

Within the first 2 days, the growth of *H. akashiwo* was strongly ($P < 0.05$) inhibited by the boiled aqueous extracts of *U. pertusa*. The growth of *H. akashiwo* recovered and was not inhibited significantly ($P > 0.05$) at the end of the experiment (Figure 6a). Growth of *H. akashiwo* and *A. tamarens* was inhibited ($P < 0.05$) by the boiled aqueous extracts of *C. pilulifera*, but the inhibitory effect was weaker than that of the non-boiled aqueous extracts (Figure 6b, c). The growth of *A. tamarens* was inhibited ($P < 0.05$) by boiled aqueous extracts of *S. thunbergii*, and the inhibitory effect was greater than that observed with the non-boiled extracts (Figure 6d).

Figure 5 Growth curves of *H. akashiwo* (a–c) or *A. tamarense* (d–f) coexistence assays with different initial concentrations ($\square 0$; $\blacksquare 2$; $\blacktriangle 4$; $\times 8$; $\square 16$; $\bullet 24$ g-wet L^{-1}) of aqueous extracts of *U. pertusa* (a, d), *C. pilulifera* (b, e) or *S. thunbergii* (c, f). Data are the means $\pm$ SD from at least three independent assays.

Effects of dry macroalga powder

In the first 6 days, the growth of *H. akashiwo* was significantly ($P < 0.05$) inhibited by all tested concentrations of dry powder of *U. pertusa* and *S. thunbergii* and at the two highest concentrations of dry powder (1.2 and 2.4 g-dry wt L^{-1}) all the cells of *H. akashiwo*

died within 1 day. The inhibition effect of the dry powder of *Corallina* was lower than for the two other macroalgae; at high concentrations (1.2 g-dry wt L^{-1}), all cells of *H. akashiwo* died within 5 days, whereas at lower concentrations (0.3–1.2 g-dry wt L^{-1}), the growth of *H. akashiwo* was not significantly inhibited. In contrast, the growth of *H. akashiwo*

Figure 6 Growth curves of *H. akashiwo* (a, b) or *A. tamarensis* (c, d) coexistence assays with boiled aqueous extracts of *U. pertusa* (a), *C. pilulifera* (b, c) and *S. thunbergii* (d). Data are the means $\pm$ SD from at least three independent assays.

was significantly increased at a dry powder concentration of 0.3 g-dry wt L $^{-1}$ (Figure 7a–c).

In the 10-day coexistence assays, the growth of *A. tamarensis* was significantly inhibited at all concentrations of dry powder of *U. pertusa* and *S. thunbergii*. At the highest concentrations of the dry powder of *U. pertusa*, the biomass of *A. tamarensis* was lower and no lethal effect was observed. However, all cells of *A. tamarensis* died when 2.4 g-dry wt L $^{-1}$ of the dry powder of *S. thunbergii* was added (Figure 7d, f). The growth of *A. tamarensis* was not significantly inhibited by the dry powder of *C. pilulifera* at concentrations lower than 0.6 g-dry wt L $^{-1}$. On the contrary, at the end of the experiment, the growth of *A. tamarensis* was significantly heightened in comparison with the control. At the highest concentrations (1.2 and 2.4 g-dry wt L $^{-1}$) *A. tamarensis* died within 1 day (Figure 7e).

The growth of *H. akashiwo* was more strongly inhibited by the dry powder of *S. thunbergii* than by the two other macroalgae (Figure 8a). The EC $_{50}$

concentrations of the dry powder of *S. thunbergii*, *U. pertusa* and *C. pilulifera* on *H. akashiwo* were 0.45, 0.5 and 1.0 g-dry wt L $^{-1}$, respectively. The growth of *A. tamarensis* was more strongly inhibited by the dry powder of *U. pertusa* than by the two other macroalgae (Figure 8b), and the EC $_{50}$ concentrations of *U. pertusa*, *C. pilulifera* and *S. thunbergii*, on *A. tamarensis* were 0.6, 1.0 and 1.3 g-dry wt L $^{-1}$, respectively (Table 1).

Discussion

Allelopathy is a common natural phenomenon in aquatic ecosystems, being seen in many biochemical interactions among higher plants and between higher plants and microorganisms, and including both stimulatory and inhibitory interactions (Molisch 1937). However, it is difficult for researchers to study allelopathic effects among aquatic organisms under natural conditions because factors such as nutrient

Figure 7 Growth curves of *H. akashiwo* (a–c) or *A. tamarensis* (d–f) coexistence assays with different initial concentrations ($\square$ 0; $\blacksquare$ 0.3; $\square$ 0.6; $\times$ 1.2; $\bullet$ 2.4 g-dry wt L^{-1}) of dry powder of *U. pertusa* (a, d), *C. pilulifera* (b, e) or *S. thunbergii* (c, f). Data are the means $\pm$ SD from at least three independent assays.

and light competition, temperature and pH changes can totally mask allelopathic effects (Keating 1977).

We conducted laboratory experiments under stable environmental conditions, precluding nutrient and light competition, in order to investigate the allelopathic effects of three macroalgae on two microalgae. The growth of *Heterosigma akashiwo* and *Alexandrium tamarensis* was strongly inhibited by the fresh

tissues of three macroalgae, with cells of *H. akashiwo* being completely killed at higher concentrations of macroalgae. *Ulva pertusa* and *Sargassum thunbergii* culture medium filtrate did not exhibit an apparent inhibitory effect on the growth of *H. akashiwo* under the initial or semi-continuous addition conditions; however, they did exhibit significant growth inhibition on *A. tamarensis*.

Figure 8 Normal maximum growth of *H. akashiwo* (a) or *A. tamarense* (b) in the presence of dry powder of *U. pertusa* (▲), *C. pilulifera* (□) or *S. thunbergii* (●). Data are the means $\pm$ SD from at least three independent assays.

Schmidt and Hansen (2001) investigated the effects of pH on the immobilization of *Heterocapsa triquetra* cells by *Chrysochromulina polylepis* and noted that pH had a dramatic effect on *H. triquetra*. Macroalgae may change the pH of the culture medium during growth, making it unsuitable for microalgal growth. In the coexistence assays, we measured the pH of the culture medium at the beginning and the end of the experiment and there was no evidence that a change in pH of the culture medium played an important role in the growth inhibition of the two microalgae. Hence, the secretion of allelopathic substances by the three macroalgae is the most likely explanation for the observed growth inhibition.

The results from both coexistence and macroalga culture medium filtrate assays indicate that the three macroalgae release rapidly degradable allelopathic substances, and that a continuous allelochemical secretion from fresh tissue is essential to effectively inhibit the growth of the microalgae. Nakai et al. (1999) demonstrated that the growth inhibition of cyanobacteria by the macrophyte *Miriophyllum spicatum* required continuous secretion of some unstable, growth-inhibitory allelopathic compounds. They also found that growth of *Microcystis aeruginosa* was not apparently inhibited by the initial addition of culture solution of *Miriophyllum spicatum*, whereas a quasi-continuous addition did lead to inhibitory effects on growth. Jin and Dong (2003) reported the same phenomenon, indicating that a very small amount of the hypothetical rapidly degradable allelochemicals was continuously released into the culture medium by *Ulva pertusa*. Van Donk and van

de Bund (2002) found that growth reduction of *Scenedesmus acutus* by *Chara aspera* occurred only when *C. aspera* was actually present in the medium during the experiment; when the algae were inoculated into medium in which *C. aspera* had been grown but removed before the experiment, no effect could be demonstrated. The phenomenon observed in the latter experiment is similar to our observations. Although the investigations mentioned above indicated the continuous secretion of unstable growth inhibitory allelochemicals in freshwater ecosystems, we speculate that this phenomenon may also exist in marine ecosystems. Sanna et al. (2004) reported that the sensitivity of an organism might also depend on the nature of the allelochemicals to which it is exposed, since the same organism may respond differently to filtrates from different algae. In our experiment, we believe that the inhibitory effect of the macroalga culture medium filtrate on microalgae is species-specific.

The inhibitory effects of fresh tissue and dry powder of *Ulva pertusa* on the growth of *Heterosigma akashiwo* and *Alexandrium tamarense* were considerably stronger than those of the two other macroalgae, with the exception of the inhibitory effect of dry powder of *Sargassum thunbergii* on *A. tamarense*, which can be inferred from the EC₅₀ (Table 1). Therefore, we think that our experiments indicate that *U. pertusa* is a broad-spectrum macroalga that exhibits an inhibitory effect on these two HABs, but that *S. thunbergii* is a differential macroalga. The dry powder of the three macroalga inhibited the growth of the two microalgae considerably more than the fresh tissue of the macroalgae, with the

exception of the effect of the dry powder of *S. thunbergii* on *A. tamarense*. This was probably because the allelochemicals in the dry powder were added to the culture medium of the microalgae as a large pulse, so that the initial allelochemical concentrations were much higher than those in the culture media of the two microalgae coexisting with the fresh tissue of the macroalga, even though the allelochemical supply pattern was not continuous.

We observed that high concentrations of the dry powder and aqueous extracts of the three macroalga strongly inhibited the growth of the microalgae and killed all the cells of *H. akashiwo* and *A. tamarense*. At lower concentrations neither macroalga affected growth, or they in fact stimulated microalgae growth. This was probably because, at higher concentrations, the microalgae cells were all killed by the allelopathic substances in a short time interval, while at lower concentrations some microalgal cells were still alive after degradation of a part of the allelochemicals, enabling the growth of cells that survived the allelochemicals to be supplied by the large quantity of nutrients released from dead microalgae cells by physical leaching. Jin and Dong (2003) reported the effect of dry powder of the nonsexual strain of *U. pertusa* on the growth of *H. akashiwo*. Their observations were similar to ours.

Heterosigma akashiwo and *A. tamarense* showed different responses when coexisting with the fresh tissue of the macroalgae. Relatively low concentrations had lethal effects on *H. akashiwo*, whereas the cells of *A. tamarense* were not killed completely even at the highest concentration of macroalgae. Guo (1994) states that there is no cell wall around *H. akashiwo* cells and that the outermost layer of the cell is a naked cell membrane, whereas the *A. tamarense* cell is covered by a hard wall (Qi and Qian 1994). The difference in the cell surface structures between *H. akashiwo* and *A. tamarense* cells may account for the different responses to allelochemicals. Kakisawa et al. (1988) reported that the brown alga *Cladosiphon okamuranus* produced some allelopathic substances against several HAB algae including *H. akashiwo*. They discovered that these allelochemicals were active against phytoplankton without cell coverings and inactive to those with rigid cell walls.

Macroalgae such as *U. pertusa*, *C. pilulifera* and *S. thunbergii* are widely dispersed. Collection and cultivation of abundant macroalgae species represents an

easy, economical and environmentally benign means of potential HAB control in confined areas (Jeong et al. 2000). In this study, our results have demonstrated that three macroalgae from the Chlorophyta, Phaeophyta and Rhodophyta can release some allelochemicals that effectively inhibit the growth of the dinoflagellates *H. akashiwo* and *A. tamarense*. Application of allelopathy in the control of microalgae blooms may require identification of the allelochemicals; however, such identification of natural products is often difficult. It is paramount in this case because it would instigate the development of ecologically desirable, highly specific, biological algacides. These three macroalgae possess several allelopathic substances, and purification of the active substances is now in progress.

Further research is also needed to elucidate the mechanism of selective allelopathic effects against harmful red tide microalgae. Although it is possible to control algal growth in an actual ecosystem by the addition of macroalgae, their overgrowth would have a negative impact (Nakai et al. 1999), thus the algicidal effect of macroalgae extracts, powders or culture medium filtrates should be encouraged for the control of red tides in confined coastal areas.

Acknowledgements We thank Prof. Guangxi Xing for his useful comments on the manuscript and for improving an earlier vision of this paper. This work was supported by The National Natural Science Foundation of China (No. 30270258 and No. 40506028) and the Encouraging Foundation for Outstanding Youth Scientists of Shangdong Province (No. 03BS 120).

References

- Chen CY, Durbin EG (1994) Effects of pH on the growth and carbon uptake of marine phytoplankton. *Mar Ecol Prog Ser* 100:83–94
- Choi H, Kim P, Lee W, Yun S (1998) Removal efficiency of *Cochlodinium polykrikoides* by yellow loess. *J Korea Fish Soc* 31:109–113
- Fukami K, Yuzawa A, Nishijima J, Hata Y (1992) Isolation and properties of a bacterium inhibiting the growth of *Gymnodinium nagasakiense*. *Nippon Suisan Gakkaishi* 58:1073–1077
- Garry RT, Hearing P, Cosper EM (1998) Characterization of a lytic virus infective to the bloom-forming microalga *Aureococcus anophagefferens* (Pelagophyceae). *J Phycol* 34:616–621
- Guillard RL, Ryther JH (1962) Studies on marine planktonic diatoms: I. *Cyclotella nana* (Hustedt) and *Detonula confervaceae* (Cleve). *Can J Microbiol* 8:229–239

- Guo Y (1994) Studies on *Heterosigma akashiwo* (Hada) Hada in the Dalian Bight, Liaoning, China. *Oceanol Limnol Sin* 25:211–215
- Hallegraeff GM, Anderson DM, Cembella AD, Enevoldsen HO (eds) (1995) Manual on harmful marine microalgae, Intergovernmental Oceanographic Commission. UNESCO, Paris
- Hasler AD, Jones E (1949) Demonstration of the antagonistic action of large aquatic plants on algae and rotifers. *Ecology* 30:359–354
- Hogetsu K, Okanishi R, Sugawara H (1960) Studies on the antagonistic between phytoplankton and rooted aquatic plants. *Jpn J Limnol* 21:124–130
- Honjo T (1994) The biology and prediction of representative red tides associated with fish kills in Japan. *Rev Fish Sci* 2:225–253
- Horner RA, Garrison DL, Plumley FG (1997) Harmful algal blooms and red tide problems on the U.S. west coast. *Limnol Oceanogr* 42:1076–1088
- Imai I, Ishida Y, Sakaguchi K, Hata Y (1995) Algicidal marine bacteria isolated from northern Hiroshima bay, Japan. *Fish Sci* 61:628–636
- Jeong JH, Jin HJ, Sohn CH, Suh KH, Hong YK (2000) Algicidal activity of the seaweed *Corallina pilulifera* against red tide microalgae. *J Appl Phycol* 12:37–43
- Jin Q, Dong ShL (2003) Comparative studies on the allelopathic effects of two different strains of *Ulva pertusa* on *Heterosigma akashiwo* and *Alexandrium tamarense*. *J Exp Mar Biol Ecol* 293:41–45
- Kakisawa H, Asari F, Kusumi T, Toma T, Sakuria T, Hara Y, Chihara M (1988) An allelopathic fatty acid from the brown alga *Aladosiphon okamuranus*. *Phytochemistry* 27:731–735
- Keating KI (1977) Allelopathic influence on blue-green bloom sequence in a eutrophic lake. *Science* 196:885–887
- Kim HG (1997) Recent harmful algal blooms and mitigation strategies in Korea. *Ocean Res* 19:185–192
- Kim CS, Lee SG, Lee CK, Kim HG, Jung J (1999) Reactive oxygen species as causative agents in the ichthyotoxicity of the red tide dinoflagellate *Cochlodinium polykrikoides*. *J Plankton Res* 21:2105–2115
- Kim CS, Lee SG, Kim HG, Jung J (2000) Biochemical responses of fish exposed to a harmful dinoflagellate *Cochlodinium polykrikoides*. *J Exp Mar Biol Ecol* 254:3–4
- Lee SJ, Kim Y, Kim HG, Seo GM, Jeong JH, Hong YK (2000) Algalytic activity of amannosidase on harmful marine microalgae. *J Appl Phycol* 12:191–193
- MacKenzie L (1991) Toxic and noxious phytoplankton in Big Glory Bay, Stewart Island, New Zealand. *J Appl Phycol* 3:19–34
- Molisch H (1937) Der Einfluss einer Pflanze auf die andere-Allelopathie. Fischer Verlag, Jena, Germany
- Na G, Choi W, Chun Y (1996) A study on red tide control with loess suspension. *Kor J Aquacult* 9:239–245
- Nagasaki K, Tarutano K, Yamagiecio M (1999) Growth characteristics of *Heterosigma akashiwo* virus and its possible use as a microbiological agent for red tide control. *Appl Environ Microbiol* 65:898–902
- Nakai S, Inoue Y, Hosomi M, Murakami A (1999) Growth inhibition of blue-green algae by allelopathic effects of macrophytes. *Water Sci Technol* 39:47–53
- Nakai S, Inoue Y, Hosomi M, Murakami A (2000) *Myriophyllum spicatum*-released allelopathic polyphenols inhibiting growth of blue-green algae *Microcystis aeruginosa*. *Water Res* 34:3026–3032
- Qi Y, Qian F (1994) Taxonomic studies on red tide causative dinoflagellates in Dapeng Bay, South China Sea. *Oceanol Limnol Sin* 25:206–211
- Qi Y, Zhang Z, Hong Y, Lu S, Zhu C, Li Y (1993) Occurrence of red tides on the coasts of China Sea. *Toxic Phytoplankton Blooms Sea* 3:43–46
- Rensel JE (1995) Management of finfish aquaculture resources. In: Hallegraeff GM, Anderson DM, Cembella AD, Enevoldsen HO (eds) Manual on harmful marine microalgae, Intergovernmental Oceanographic Commission, UNESCO, pp 463–464
- Ryu H, Shim J, Bang J, Lee C (1998) Experimental chemical treatments for the control of dinoflagellate, *Cochlodinium polykrikoides* in the land-based culture of olive flounder *Paralichthys olivaceus*. *Kor J Aquacult* 11:285–294
- Sanna S, Giovana O, Edna G (2004) Allelopathic effects of the Baltic cyanobacteria *Nodularia spumigena*, *Aphanizomenon flos-aquae* and *Anabaena lemmermannii* on algal monocultures. *J Exp Mar Biol Ecol* 308:85–101
- Schmidt LE, Hansen PJ (2001) Allelopathy in the prymnesiophyte *Chrysochromulina polylepis*: effect of cell concentration, growth phase and pH. *Mar Ecol Prog Ser* 216:67–81
- Sharp JH, Underhill PA, Hughers D (1979) Interaction (allelopathy) between marine diatoms: *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*. *J Phycol* 15:353–362
- Steidinger KA (1983) A re-evaluation of toxic dinoflagellates biology and ecology. *Prog Phycol Res* 2:147–188
- Suzuki Y, Takabayashi T, Kawaguchi T, Matsunaga K (1998) Isolation of an allelopathic substance from the crustose coralline algae, *Lithophyllum* spp., and its effect on the brown alga, *Laminaria religiosa* Miyabe (Phaeophyta). *J Exp Mar Biol Ecol* 225:9–77
- Taft WH, Martin DF (1986) The potential for managing a Florida red tide. *J Environ Sci Health* 21:107–127
- Van Donk E, van de Bund WJ (2002) Impact of submerged macrophytes including charophytes on phyto- and zooplankton communities: allelopathy versus other mechanisms. *Aquat Bot* 72:261–274
- Yu Zh, Sun W, Guo K, Yu S (1992) Allelopathic effects of several aquatic plants on algae. *Acta Hydrobiol Sin* 16:1–7
- Zingone A, Enevoldsen HO (2000) The diversity of harmful algal blooms: a challenge for science and management. *Ocean Coast Manage* 43:725–748