academicJournals

Vol. 7(34), pp. 4389-4396, 23 August, 2013
DOI: 10.5897/AJMR2012.2458
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Algicidal activity of *Ulva pertusa* and *Ulva prolifera* on *Prorocentrum donghaiense* under laboratory conditions

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Accepted 6 August, 2013

Allelopathic effects of fresh tissue and dry powder of two bloom-forming green macroalgae *Ulva pertusa* and *Ulva prolifera* on microalga *Prorocentrum donghaiense* were evaluated at different concentrations. The filtrates of macroalga culture medium were investigated for their inhibitory effect on *P. donghaiense* using initial or semi-continuous filtrate addition. Preliminary results using the extracts prepared with four solvents confirmed that allelochemicals were present in the tissue of the two macroalgae. In addition, the effects of the two macroalgae on the microalgae were tested. The results show that the growth of *P. donghaiense* was strongly inhibited by fresh tissue and dry powder of *U. pertusa* and *U. Prolifera*, and that the culture medium filtrates of the two macroalgae exhibited an apparent growth inhibition effect under initial or semi-continuous addition, which suggested that the cells of *P. donghaiense* are sensitive to the allelochemicals. Methanol extracts of the macroalgae were found to strongly inhibit the growth of *P. donghaiense*, while extracts made with three other organic solvents (acetone, ether and chloroform) did not. This suggests that the allelochemicals might be relatively high polar compounds. The methanol extracts were further partitioned using petroleum ether, ethyl acetate, butanol and distilled water by liquid-liquid fractionation. Bioassays of each fraction were carried out on activity against *P. donghaiense*, and the results indicated that the extracts in petroleum ether and ethyl acetate phases had strong algicidal activity.

Key words: Allelopathy, *Ulva pertusa*, *Ulva Prolifera*, macroalgae, red tide microalgae, *Prorocentrum donghaiense*, microcosm experiment, extracts.

INTRODUCTION

Blooms of ulvoid green algae have occurred worldwide, with parts of the Baltic Sea being severely affected by drifting algal mats at a depth of 35 m (Vahteri et al., 2000). Outbreaks of the algae have been reported to impact marine ecosystem in a number of ways including the eradication of sea-grass meadows as well as macroalgal communities (Nelson and Lee, 2001). However, most reports on this phenomenon are descriptive. Moreover, green tides may cause severe economic losses to fishery operations and aquaculture, and have major impacts on the environment and human health, and may result in a decrease in revenue from tourism (Taylor et al., 2001). Severe outbreaks of harmful algal blooms (HABs) have been reported to cause serious problems in effective utilization of water resources such as fisheries and water-supply reservoirs, and affect human health and many socio-economical activities, such as tourism and shellfish production in aquaculture (Oh et al., 2010; Lu et al., 2011). Because of the devastating ecological and economic problems associated with coastal algal blooms, many investigations have been conducted to look into mechanisms that

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enable the bloom-forming algae to outcompete other coastal primary producers and to establish dominant populations, in efforts to develop strategies for the control of HABs (Taylor et al., 2001).

Macroalgal blooms are often associated with eutrophication in the shallow estuaries where light can penetrate to the sea bottom, and during the blooms, macroalgae are able to replace other estuarine producers including microalgae. In the past years, many scientists noticed that green macroalgae could reduce eutrophication in mariculture waters, and improve the productivity, survival rate and feeding efficiency of the culture species, e.g. prawn and shrimp, by means of polyculture (Wang et al., 2001). For example, *Ulva pertusa* has been shown to uptake nutrients from mariculture waters and improve water quality (Nelson et al., 2003). Fong et al. (1993) found a negative correlation between the abundances of macroalgae and microalgae. Various allelopathic effects between macroalgae and microalgae have been demonstrated by a number of researchers (Jeong et al., 2000; Nelson et al., 2003). Allelopathy is a natural phenomenon in aquatic ecosystems, and it has been demonstrated that the structures and succession of aquatic ecosystems can be profoundly affected by allelopathy among primary aquatic producers. The allelopathic effects of certain aquatic plants have been explored for biological control of the growth of undesirable algae and weeds in aquatic ecosystems (Jin et al., 2005; Nelson et al., 2003).

Many types of marine seaweeds produce unique natural products. Chemical defense by green macroalgae is ecologically related, many of these species are capable of rapid population growth to form large blooms, generating a variety of adverse effects on the structure and diversity of local communities (Jin et al., 2005). The green tide macroalga *Ulva obscura* has been shown to contain certain chemicals (e.g. dopamine) active in anti-herbivore defense (Van Alstyne et al., 2006). Chemicals produced by ulvoid macroalgae have been shown to be responsible for the inhibition of phytoplankton growth. Therefore, it is important to study the potential allelopathic effects of macroalgae aganist microalgae. If these green macroalgae are confirmed to be inhibitory to other undesirable primary producers, such as red tide microalgae, they can be explored to develop new control methods for the undesirable producers.

U. pertusa is one of the most common macroalgae contributing to the phenomenon known as green tides (Taylor et al., 2001). During the bloom of the green alga *U. prolifera* in 2008, huge amounts of floating green algae accumulated along the coast of Qingdao, when the city was preparing for the 2008 Olympic Sailing. In this study, *U. pertusa* and *U. prolifera* were used. *Prorocentrum donghaiense* is one of the most harmful microalgae and it was identified as the causative species of a large-scale bloom that occurred in May 2002 in the East China Sea (Lu et al., 2003). The bloom lasted for about one month and the affected area was over 1000 km^2. In this study, we aimed to evaluate for the allelopathic effects of the two green macroalgae on *P. donghaiense*, to gain insights into the chemical interactions between macroalgae and microalgae isolated from coastal areas, and to verify the allelopathic effects of macroalgal tissue extracts on *P. donghaiense.*

MATERIALS AND METHODS

Algae and experimental conditions

Sterile strains of *P. donghaiense* were obtained from the Microalgal Laboratory, Ocean University of China. They were grown in modified f/2 medium at 20°C under irradiance of 70-μmol m^{-2} s^{-1} with a 12-h light : dark cycle in illuminated incubators. The initial pH and salinity of the culture medium were adjusted to 8.0 and 30‰, respectively. Flasks containing the microalgae were manually shaken twice daily.

The microalgae were cultivated to exponential growth phase before use. The initial cell density of *P. donghaiense* was adjusted to 3×10^4 cells mL^{-1} throughout the experiments, and the culture conditions in the all experiments were the same unless otherwise mentioned.

Macroalgae *U. pertusa* and *U. prolifera* were collected at the Taiping Angle, Qingdao (36°07’N, 120°33’E). They were carefully and immediately washed with distilled water to remove attached organisms. The treated thalli were allowed to adapt to the laboratory environment for 7 days in f/2 medium prior to experiments. Seawater was obtained from coast of Qingdao, filter-sterilized through a nitrate cellulose membrane filter with 0.22 μm pore size. The cultures were maintained at 20°C on a 12 h light : 12 h dark cycle. Illumination was provided by cool-white fluorescent lamps at 60 μmol photons m^{-2} s^{-1}. All cultures were shaken twice daily to prevent the thalli from sinking to the walls of the culture flasks.

Fresh tissue and dry powder

To compare the effects of different initial concentrations of fresh macroalgal tissue on *P. donghaiense*, co-culture assays were performed using a co-culture of one macroalga and one microalga. The fresh thalli of the macroalga were inoculated into 40 ml of microalgal cultures in f/2 medium in 100-ml conical glass flasks to the final contents of 0 (negative control), 0.625, 1.25, 2.5, 5.0 and 10 gfw L^{-1}. The initial cell density of *P. donghaiense* was adjusted to 1.0×10^5 cells mL^{-1}. 1 ml aliquots of microalgae were taken daily from the culture, fixed in acidic lugol's solution and counted under a microscope using a haemocytometer. The experiments were conducted under stable environmental conditions, and 1 mL of nutrition solution, containing f/2 ingredients for the 40 mL culture medium, was added to each flask to compensate then nutrient depletion and avoid nutrient competition of the microalga and macroalga in the co-culture. The concentrations of NO_3-N and PO_4-P were regularly measured throughout the experiments. The pH of the culture medium and wet weights of the macroalga were also measured. This assay protocol was used also for dry powder assays with the two macroalgae, at initial contents of 0, 0.3, 0.6, 1.2 and 2.4 gdw L^{-1}, The assays were conducted for 10 days.

Culture medium filtrate

To determine the effect of macroalgal secretions on microalgal

growth, filtrates from macroalgal culture medium were added to the microalgal cultures in two manners, one was a one time addition at the beginning of the culture (initial addition), and the other was interval addition during the culture (semi-continuous addition) as described below:

For the initial addition, macroalgae at an initial density of 80 gfw L^{-1} were cultivated for 3 days and culture medium was collected and filtered through a 0.22-μm pore-size membrane filter (Whatman) and supplemented with stock f/2 medium. 40 ml of the filtrate was added to culture of *P. donghaiense* at a density of 3×10^4 cells mL^{-1} in a 100 mL flask. Addition of f/2 medium was used as negative control.

For semi-continuous addition, experiments were conducted under the same conditions as described for initial addition experiments, except that 10 ml of f/2 medium-enriched filtrate was added daily after 10 ml of culture medium was removed from the culture.

Preparation of macroalgal extracts

Fresh macroalga was air dried for 6 days at room temperature and then ground into fine powder with a mortar and pastel. For methanol extraction, 50 g of the dry powder was mixed with 1000 mL methanol and shaken in darkness for 24 h at 20°C. The mixture was separated by centrifugation (6000 ×*g* for 15 min at 20°C) and the pellet was extracted one more time. The microalga was inoculated into 100 mL flasks containing 40 ml of fresh f/2-enriched seawater. The macroalgal methanol extract was diluted to a macroalgal contents equivalent to 25, 50, 100, 200 and 400 gL^{-1}. 40 μL of the diluted extract was added into 40 mL of the microalgal cultures at a final concentration between 0.025 and 0.4 gL^{-1}. The same procedure was used for three other organic solvents (acetone, ether and chloroform). The same volumes of the solvents were used as controls. 1 mL aliquots of the cultures were taken daily for 15 days, fixed acidic Lugol's solution and counted under a microscope using a haemocytometer. Results were normalized to maximum growth following a method reported by Nakai et al. (1999) to obtain the concentration of macroalgal extracts which reduced normal growth of the microalgae by 50% (EC_{50} value).

Isolation of allelochemicals

1000 g dry powder of *U. pertusa* or *U. prolifera* was mixed with 5 L 100% methanol and shaken in darkness for 24 h at 20°C. The mixture was separated by high speed centrifugation and the power was extracted twice. The extracts were combined and evaporated to dryness under reduced pressure in a rotary evaporator, and 80 g dried methanol extract was obtained. The extract was dissolved in 90% methanol and petroleum ether was added to perform liquid-liquid fractionation. The resultant petroleum ether extracts were combined and evaporated to dryness under reduced pressure at 40°C in a rotary evaporator, generating 40 g of extract. The combined methanol phase was evaporated and the extract was dissolved in water, which was then partitioned with ethyl acetate. The combined ethyl acetate extract was evaporated to dryness and 10 g of extract was obtained. For the water phase, butanol was added to partition and the combined butanol or distilled water extracts were evaporated to dryness and 5 or 1 g of extracts were obtained, respectively. All liquid fractionations were done three times. The four types of extracts were investigated for their biological activity.

Bioassays

Two microalgae were used to investigate the allelopathic activity of the extracted compounds. The four extracts obtained as described above were dissolved in methanol and diluted to concentrations of 0.1, 1, 10 and 100 g L^{-1}, respectively, 40 μL aliquots of the diluted extract were added to 100 mL of the microalga cultures in flasks to final concentrations of 0.1, 1, 10 and 100 mg L^{-1}, while controls received the same volume of corresponding solvents. The assays lasted for 6 h. After 1 h, 1 ml aliquots of cells were taken, fixed and counted immediately under a light microscope (Olympus BX50) at 100× magnification. The motility inhibition was calculated as the ratio of numbers of the immotile to total cells.

Statistical analysis

The experiments were repeated at least three times for each independent assay, and the data was analyzed by one-way ANOVA and significance of the differences between the treated groups and the control was tested by Tukey's post-hoc test. Difference was considered to be significant if the probability is less than 0.05.

RESULTS

The availability of N and P sufficient for algae growth was confirmed by chemical analysis. The daily addition of 1 mL f/2 solution resulted in concentrations of NO_3-N≥780 μmol L^{-1} and PO_4-P≥36 μmol L^{-1}, which are well above limiting levels for microalgae as previously shown (Guillard and Ryther, 1975; Sharp et al., 1979). The effect of lighting conditions was ruled out by measuring the light intensity at the bottom of the culture flasks and results confirmed that the inhibition was not due to insufficient light (data not shown). The pH of the culture medium was 8.6 ± 0.2.

Effects of fresh tissue or dry powder of the two macroalgae on the growth of *P. donghaiense*

The growth of *P. donghaiense* was significantly suppressed (Tukey's, p <0.01) in the presence of fresh thalli of the two macroalgae. The cells of *P. donghaiense* gradually began to die at low macroalga density and all died at concentrations higher than 1.25 gfw L^{-1} when co-cultured with the two macroalgae (Figure 1a and b).

Addition of the macroalga dry powders significantly suppressed the growth of *P. donghaiense* (p <0.05) in the 10-day coexistence assays (Figure 2a and b). At the highest concentrations (1.2 pertusa and 2.4 $gdwL^{-1}$ for *U. pertusa* and *U. prolifera*, respectively), *P. donghaiense* cell density gradually decreased, sometimes even to zero.

Fresh tissue of *U. pertusa* was more inhibitory to the growth of *P. donghaiense* as compared to *U. prolifera*. The microalgae was observed to be completed and inhibited due to addition of high concentrations of the two macroalgae powders. The EC_{50} values for fresh tissue of *U. pertusa* and *U. prolifera* were 0.9 and 1.25 gfw L^{-1}, while the EC_{50} values for dry powder of *U. pertusa* and *U. prolifera* were 0.18 and 0.2 gdw L^{-1}, respectively.

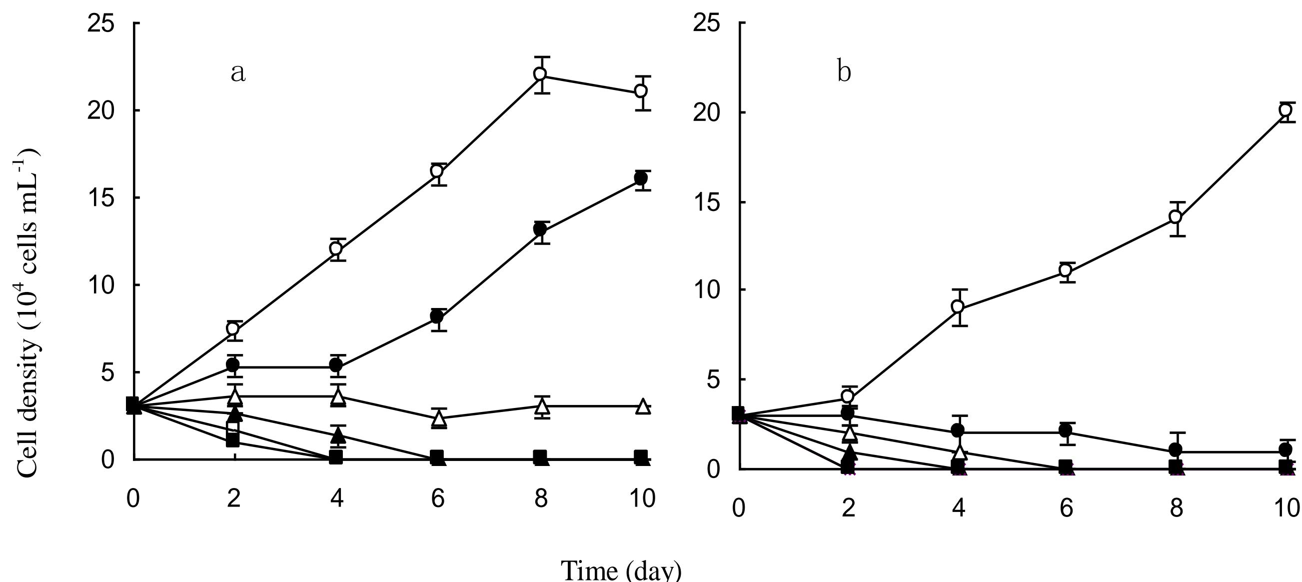

Figure 1. Growth curves of *P. donghaiense* added with different initial concentrations (○, 0; ●, 0.625; ∆, 1.25; ▲, 2.5; □, 5; ■, 10 gfw l^{-1}) of fresh tissue of *U. pertusa* (a) or *U. prolifera* (b) in coexistence assays. Data are means ±SD (n = 3). Vertical lines show the standard deviation.

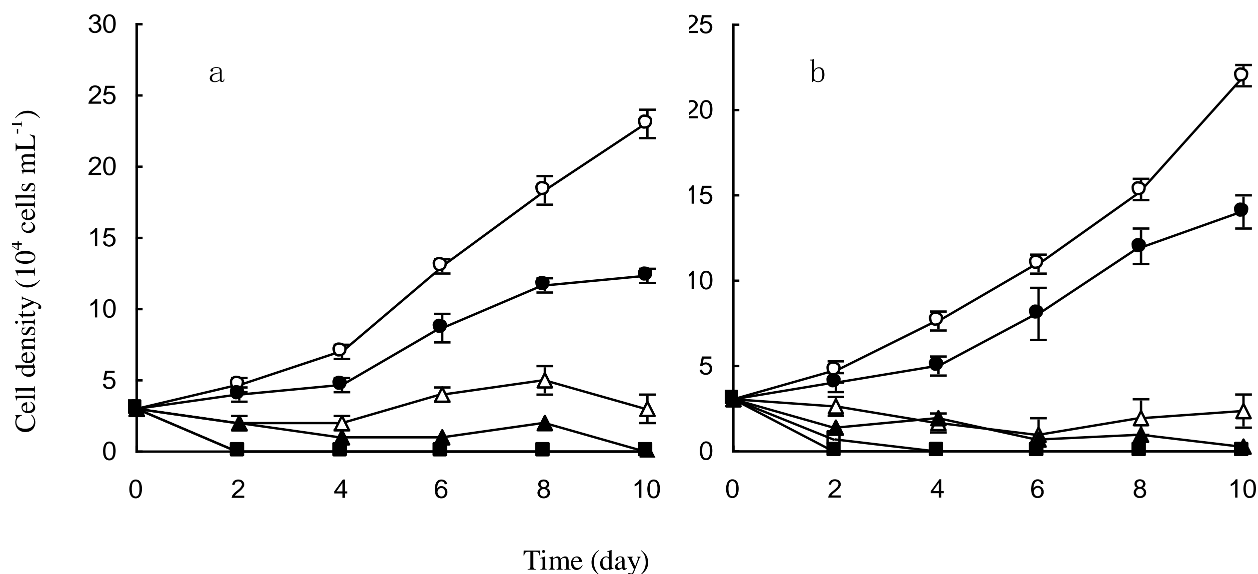

Figure 2. Growth curves of *P. donghaiense* added with different initial concentrations (○, 0; ●, 0.15; ∆, 0.3; ▲, 0.6; □ ,1.2; ■, 2.4 gdw l^{-1}) of dry powder of *U. pertusa* (a) or *U. prolifera* (b) in coexistence assays. Data are means ± SD (n = 3). Vertical lines show the standard deviation.

Effects of macroalgal culture medium filtrate on *P. donghaiense*

The growth of *P. donghaiense* was significantly reduced (Tukey's, $p < 0.05$, Figure 5A and B) when cultures were treated with an initial or semi-continuous addition of medium pre-cultured with *U. pertusa* or *U. prolifera*.

Effects of macroalgal extracts on the growth of *P. donghaiense*

The growth of *P. donghaiense* was significantly suppressed (Tukey's, $p < 0.05$) by the addition of the methanol extracts to the assay culture at various concentrations (Figure 3a and b), and the inhibition increased

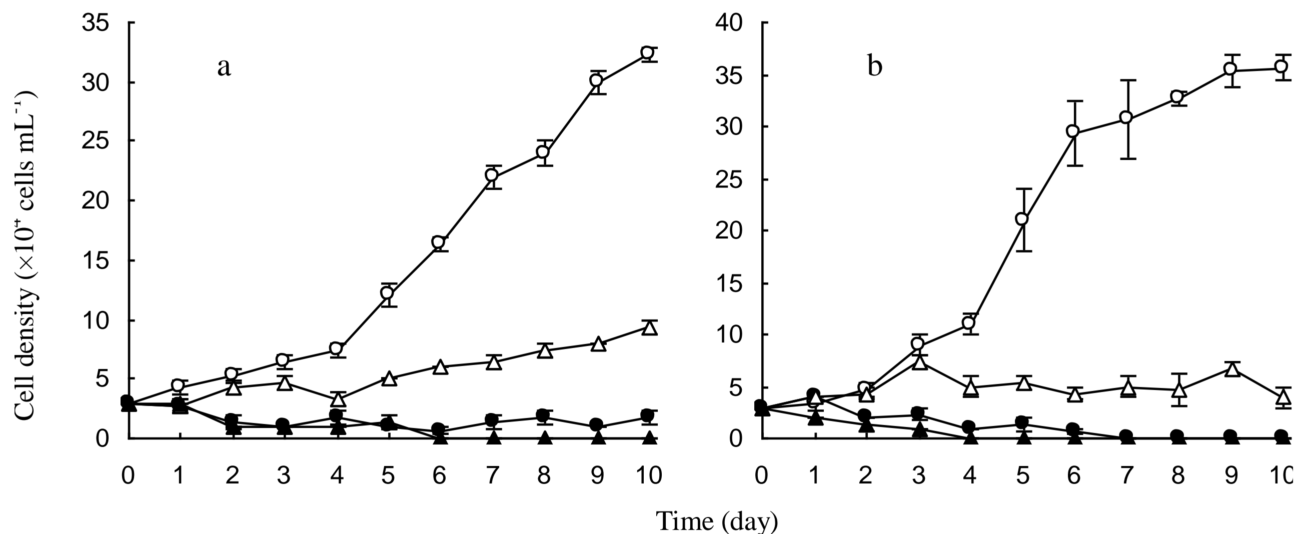

Figure 3. Growth curves of *P. donghaiense* with initial (●) or semi-continuous (▲) addition of culture medium filtrate of *U. pertusa* (a) or *U. prolifera* (b) (○ initial addition; Δ semi-continuous addition). The data are means ± SD (n = 3). Vertical lines show the standard deviation.

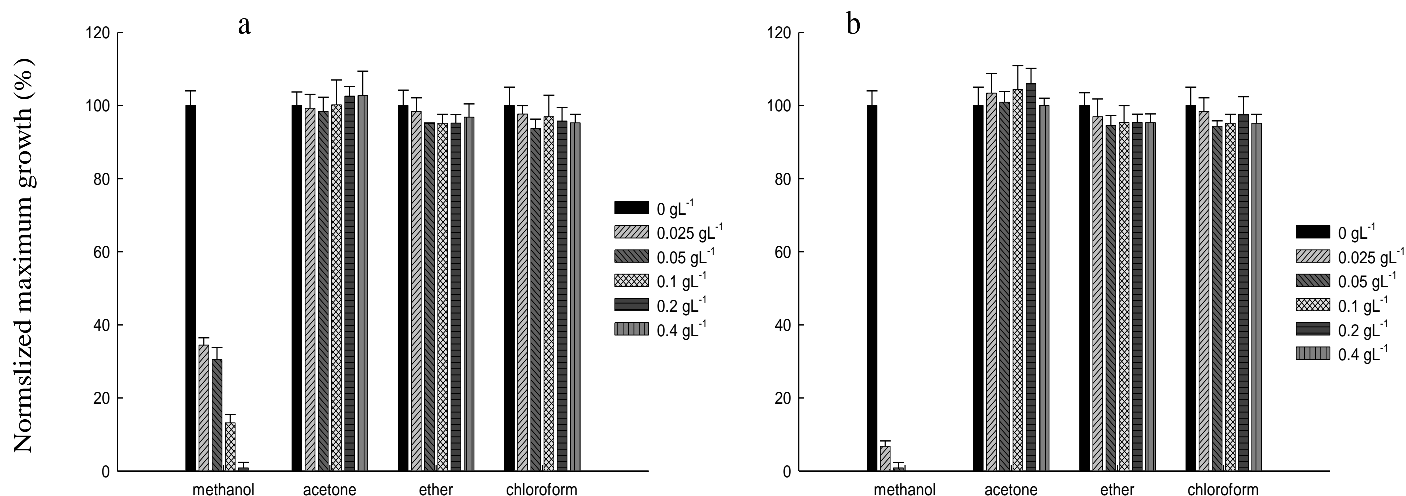

Figure 4. Normalized maximum growth of *P. donghaiense* (C) in the presence of different concentrations (gLl^{-1}) of 4 organic solvent extracts of *U. pertusa* (a) or *U. prolifera* (b). Data represent average values (n=3) with corresponding error bars (1SE).

with increase in the amount of the extracts added. At concentrations of 0.1, 0.2 and 0.4 gL^{-1} for *U. prolifera* and 0.4 gL^{-1} for *U. pertusa*, the cells of *P. donghaiense* started to die at the beginning of the assays and were all dead by the end of the experiments. The EC_{50} values for methanol extract of *U. pertusa* and *U. prolifera* were 0.029 and 0.02 gL^{-1}, respectively. On other hand, the growth of *P. donghaiense* was not significantly reduced (Tukey's, p >0.05) by the addition of acetone, ether or chloroform extracts of *U. pertusa* or *U. prolifera* at the concentrations tested (Figure 4a and b).

Effects of allelopathic bioassays

Allelopathic bioassays indicated that the compounds in the petroleum ether extracts of *U. pertusa* and *U. prolifera* had the strongest algicidal effect on *P. donghaiense* at concentrations as low a 20and 17 mgL^{-1}, respectively (Figure 5a and b). No algicidal effect was observed for

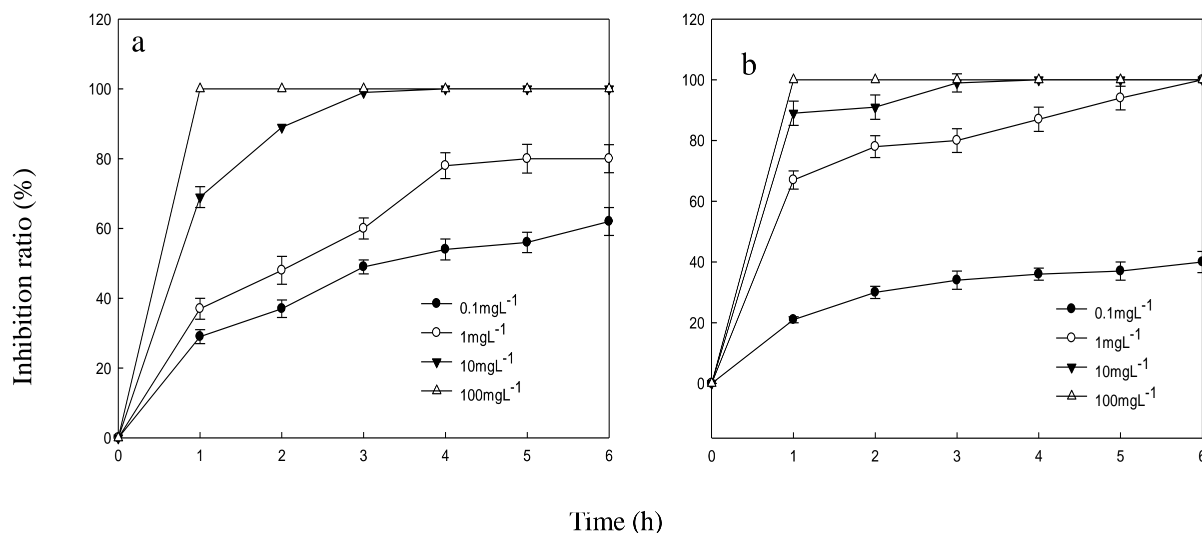

Figure 5. Motility inhibition effect of the petroleum ether phase of extract from *U. pertusa* or *U. prolifera* (b) on microalgae. The control was considered as having no motility inhibition effects.

the butanol and distilled water extracts. The main components of the allelopathicals in the petroleum ether extract and ethyl acetate extract dissolved out within 20 min, indicating that these allelochemicals are relatively high polar.

DISCUSSION

Rice (1984) initially defined allelopathy as the effects of one plant including microorganisms on another plants through the release of a chemical compound(s) into the environments and its/their activity. This definition considered both stimulating and inhibiting effects, depending on the concentration of the compound (s). Now, only the suppression of growth of a plant by a toxin released from a nearby plant is considered as allelopathy. Allelopathy has been found to play an important role in algal bloom sequences in freshwater ecosystems (Gross, 2003). However, it is very difficult to study allelopathic effects among aquatic organisms under natural conditions because many other factors, ranging from nutrient and light competition, temperature to pH changes, can totally mask an allelopathic effect. It is therefore essential at least at the initial phase of the research to identify particular set of allelopathic interactions among aquatic organisms under a controlled system.

In the present study, in order to differentiate allelopathy from the factors described above, the experiments were conducted under stable non-competitive environmental conditions, to prevent avoid potential nutrient and light competition, as well as the possible effects of bacteria. Schmidt and Hansen (2001) analyzed the effects of pH on the immobilization of *Heterocapa triquetra* cells by *Chrysochromulina polylepis* and found that pH had a remarkable effect on *H. triquetra*. He found that the macroalgae could change the pH of the culture medium in which it was grown, making it unsuitable for microalgae to grow, generating an allelopathy-like effect. In our coexistence assays, the pH of the culture medium was measured at the beginning and the end of the experiments. No evidence was found that the change in pH of the culture medium was associated with the growth inhibition of the two microalgae. Therefore, the secretion of substances by *U. pertusa* or *U. prolifera*, was more likely responsible for allelopathic effects observed in *P. donghaiense* during the experiments.

Nakai et al. (1999) showed that the growth inhibition of cyan bacteria by the macrophyte *Miriophyllum spicatumn* needed a continuous supply of some unstable, growth-inhibitory allelopathic compounds. They also found that the initial addition of culture solution of *M. spicatumn* did not inhibit the growth of *Microcyctis aerugimosa*, whereas quasi-continuous addition did show the inhibitory effects on the growth.

In an analysis of growth reduction of *Scenedesmus acutus* by *Chara aspera*, Van Donk and van de Bund (2002) found it occurred only when *C. aspera* was actually present in the medium during the experiment period, and when the algae were inoculated into medium in which *C. aspera* was cultured but removed before the experiment, no inhibitory effect was detected. In the present study, the results from the macroalgal culture medium filtrate assays demonstrated that the filtrates (initial or semi-continuous addition) were inhibitory to *P. donghaiense*, indicating that *P. donghaiense* was very sensitive to potential allelopathic substances. Sanna et al. (2004) reported that the sensitivity of an organism might also depend on the nature and chemical properties of the allelochemicals to which it is exposed. It could be expected

that the same organism would respond differently to filtrates from different algae.

In the present study, methanol extracts from *U. pertusa* and *U. prolifera*, were found to have strong inhibitory effects on *P. donghaiense*, whereas acetone, ether and chloroform extracts from the same macroalgae had no effect at the concentrations tested, suggesting that the extracted allelochemicals did not exist in extracts other than methanol extracts. There are reports indicating that bloom-forming green macroalgae could produce chemical defenses against herbivores (van Alstyne et al., 2001) and their extracts have allelopathic properties (Alamsjah et al., 2008). In the present study, we have demonstrated that the bloom-forming green macroalgae *U. pertusa* and *U. prolifera*, have allelopathic effects on the red tide microalga *P. donghaiense*.

The allelopathic mechanisms of macroalgae on microalgae remain to be elucidated. Nagayama et al. (2003) reported the algicidal activity of phlorotannins from the brown alga *Ecklonia kurome* on red tide microalgae (*Karenia mikimotoi*, *Cochlodinium polykrioides* and *Chattonella antiqua*). They showed that phlorotannins caused cells of the microalgae to lose their motility, and almost all of *K. mikimotoi* and *C. polykrikoides* cells became round and then expanded and burst after exposure to phlorotannins.

They believed that phlorotannins might interact with microalgae proteins, leading to the algicidal actions. For example, interactions with channel proteins in cell membranes may disturb the control of osmotic pressure. Inhibition of photosynthesis may be one of prevalent allelopathic mechanisms as well. We speculated that the allelopathic mechanism by which *U. pertusa* or *U. prolifera* inhibit on *P. donghaiense* might be related to disrupted cellular control of osmotic pressure in the microalgae, because the motility and modality of the microalga cells were reduced by these allelochemicals. However, more studies are need to elucidate the models of action of these potential allelochemicals on *P.donghaiense*.

Kakisawa et al. (1988) reported that the brown alga, *Cladosiphon okamuranus* Tokida, produced allelopathic substances named 6z, 9z, 12z, 15z-octadecatetraenoic acid and 5z, 8z, 11z, 14z, 17z- octadecatetraenoic acid, two unsaturated fatty acids. They demonstrated that the two unsaturated fatty acids had strong algicidal activity on *H. akashiwo*. With the addition of these the two fatty acids, cells of *H. akashiwo* became round-shaped, expanded in size and then raputred. Suzuki et al. (1996) isolated 5z, 8z, 11z, 14z, 17z- octadecatetraenoic acid from *Neodilsea yendoana*, and found that this unsaturated fatty acid could completely kill cells of *Monostroma oxyspermum*. Their study demonstrated that unsaturated fatty acids might be parts of the components of the allelochemicals in the macroalgae, since unsaturated fatty acids are known to produce free radicals when oxidized in seawater, which may attack algae like toxins (Murata et al., 1989). Based on these results, we showed that some of unsaturated fatty acids in *U. pertusa* and *U. prolifera* might be responsible for observed allelopathy and perhaps these algae possess several other allelochemicals as well. We are presently analyzing the compounds in the petroleum ether phase and ethyl acetate phase from the two macroaglae by GC/MS. Further eludication of these compounds would help to develop strategies and algaecides for ecologically safe, highly efficient and specific biological controls of algae.

Both green and red tides are harmful to the marine ecosystems and the coastal economy, and many studies have been initiated and conducted to control the undesirable algal growth. In the present study, we have shown that the growth of *P. donghaiense* was inhibited by the fresh tissue, dry powder and methanol extract of two bloom-forming green macroalgae *U. pertusa* and *U. prolifera*. The macroalgae might secrete compounds that are kind of allelochemicals to inhibit the growth of *P. donghaiense*. These results not only provide insights into chemical interactions between macroalgae and microalgae but also suggest a novel method of utilizing bloom-forming green macroalgae to inhibit the red tide microalgae. This provides a novel and economic means to control red tide. Isolation and characterization of the allelochemicals from *U. pertusa and U. prolifera* is under way.

ACKNOWLEDGEMENTS

We thank Prof. Timothy A. Nelson for linguistic advice of the manuscript, and the colleagues in our laboratory for their kind assistance. The study was supported by the National Natural Science Foundation of China (No. 31200400), the Natural Science Foundation of Shandong Province (No. 2009ZRB01461) and the Science and Technology Project of Institutions of Higher Education of Shandong (No. J10LC13).

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