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Promotion of hexadecyltrimethyleamine bromide to the damage of *Alexandrium* sp. LC3 by cupric glutamate

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Abstract: The effect of hexadecyltrimethyleamine bromide (HDTMAB) on the removal of *Alexandrium* sp. LC3 under cupric glutamate stress was investigated. Toxic effect of cupric glutamate on *Alexandrium* sp. LC3 was significantly promoted in the presence of HDTMAB, especially at 3.0 cmc of HDTMAB. It was found that the sulfhydryl group content of the cell decreased, while the malonaldehyde content and membrane permeability increased when *Alexandrium* sp. LC3 was treated with HDTMAB and cupric glutamate complex, compared with cupric glutamate alone. The data suggest that HDTMAB might stimulate the damage of *Alexandrium* sp. LC3 by enhancing the membrane permeability.
Keywords: HDTMAB; cupric glutamate; *Alexandrium* sp. LC3; cell membrane permeability

Introduction

Recently, red tides have occurred frequently all over the world, which resulting in both the economy loss and the environment pollution (Zhou *et al.*, 2001; Miao *et al.*, 2002; Sun *et al.*, 2004a). How to control red tides effectively has gained more and more attention. *Alexandrium* sp. LC3 is one of the most harmful red tide algae in the world (Ferrier *et al.*, 2002); about thirty dinoflagellates in this genus can release many types of natural toxins, which can kill marine animals and threaten humans. Therefore, it is necessary to prevent of the harmful bloom of *Alexandrium* sp. LC3.

Currently, spread clay and chemicals are employed widely for eliminating harmful algal blooms (HABs) (Yu *et al.*, 1993, 1999; Zhao *et al.*, 2001; Miao *et al.*, 2002; Ferrier *et al.*, 2002). However, these methods are not feasible on account of the cost and recontamination. The complex compound of copper and amino acid is one type of highly efficient bactericide (Kang *et al.*, 1996), with bactericidal activity mainly due to the copper ion. The surfactant is a surface-active compound capable of reducing surface and interfacial tension at the interfaces between liquids, solids and gases (Banat *et al.*, 2000), and is applied safely in the medicine and food industry as a bactericide (Yang *et al.*, 2000; Qin *et al.*, 2004; Glover *et al.*, 1999). Surfactants have been used to limit the algae of red tide directly. *Heterosigma akashiwo* was removed by a quaternary ammonium compound composed of only one long chain alkyl (Cao and Yu, 2003). Recently, the biosurfactant sophorolipid was proposed to mitigate harmful algal blooms (Baek *et al.*, 2003), but the high cost of biosurfactants restricts the potential for its application in HAB mitigation. Some synthetic surfactants with comparative advantages of biosurfactants but relatively low cost for HAB mitigation were screened (Sun *et al.*, 2004a, b). However using surfactant to promote extinction of red tide organisms by cupric glutamate has not previously been reported.

In this study, hexadecyltrimethyleamiue bromide (HDTMAB), a cationic organic surfactant, associated with cupric glutamate, was used to eliminate red tide by detecting the lipid peroxidation level of cells, the plasma membrane permeability and the change of sulfhydryl group content. The purpose of this study was to investigate the effect and mechanism of HDTMAB promotion of eliminating the algae by cupric glutamate and the potential application of cupric glutamate and surfactant for red tide prevention as a novel algicide and accelerant, respectively.

1 Materials and methods

1.1 Strains and cultivation

Alexandrium sp. LC3 used in this study was provided by Fishery College of China Ocean University. This algae was incubated at 21℃ in f/2 medium under the light intensity of 35—45 μE/(m^2·s) with a cycle of 12 h light and 12 h dark. The algal culture was shaken three times everyday.

1.2 Reagents

Cupric glutamate used in this study was synthesized ourselves. HDTMAB was purchased from AiBi Chemical Industry, Co., China.

1.3 Detection of cell numbers

Alexandrium sp. LC3 cells were counted with a hemocytometer under a microscope.

1.4 Measurement of the content of malonaldehyde

The malonaldehyde (MDA) content of the cell was measured by the TBA method (Li and Mei, 1989). After addition of phosphate buffer

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(concentration 50 mmol/L, pH 7.0) and quartz sand, 0.5 g of harvested cells of *Alexandrium* sp. LC3 culture was triturated in ice-bath and centrifuged at 20000 × g for 20 min at 4℃. The final volume of the supernatant was made up to 5 ml. 1 ml of the supernatant with 3 ml of 27% tricarboxylic acid and 1 ml of 2% thiobarbituric acid were incubated at 95℃ for 30 min and cooled in an ice-bath immediately, then centrifuged at 1500 × g for 10 min. The absorbance of the supernatant was measured at 532 nm. After deduction of the absorbance at 600 nm, the MDA content was measured by using an extinction coefficient of 155 L/(mmol·cm).

1.5 Measurement of SH group content

The sulfhydryl group (SH group) content of the cell was measured by 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) method (Ellman, 1959). After addition of 75 μl diluted buffer, 25 μl DTNB reagent and 400 μl methanol, 25 μl of extracted algal protein was centrifuged at 3000 × g for 5 min. The absorbance of the supernatant was measured at 412 nm.

1.6 Measurement of membrane permeability

The permeability of the cell membrane was measured by the ultraviolet absorption method (Liu *et al.*, 1985). The conductivity was measured using a conductometer (DDS- Ⅱ A). The relative permeability of the cell membrane was evaluated by the ratio of relative conductivity to total conductivity.

2 Results

2.1 Inhibitory effect of cupric sulfate or cupric glutamate on *Alexandrium* sp. LC3

After incubation, 50 ml of exp. growth-phase culture of *Alexandrium* sp. LC3 (10^5 cells/ml) was inoculated into 50 ml f/2 fresh medium, and cupric sulfate and cupric glutamate were added to the medium, respectively. The final concentration of each was 1.0×10^{-3} mol/L (determined by the concentration of Cu^{2+}). As the culture without cupric sulfate or cupric glutamate was controlled, the survival rate of *Alexandrium* sp. LC3 was calculated after 7 d incubation (Fig.1). The results showed that cupric glutamate had a higher extinguishing rate than cupric sulfate, and had more extinguishing superiority to cupric sulfate with time. This suggested that somewhat larger and more frequent doses of cupric sulfate would be just as effective as cupric glutamate to remove algae cells, which was consistent with Masuda and Boyd (1993).

2.2 Inhibitory effect of various concentration of HDTMAB on *Alexandrium* sp. LC3 under cupric glutamate stress

The critical micelle concentration of HDTMAB was 0.92 mmol/L. It was associated with cupric glutamate to eliminate *Alexandrium* sp. LC3. After incubation, 50 ml of exp. growth-phase culture (10^5 cells/ml) was inoculated into f/2 fresh medium with cupric glutamate (final 0.5×10^{-3} mol/L, determined by the concentration of Cu^{2+}), 0, 0.1, 0.3, 1.0, 3.0 cmc of HDTMAB were added to the medium, respectively. As the culture without cupric glutamate or HDTMAB was controlled, the survival rate of *Alexandrium* sp. LC3 was calculated (Fig.2). The results showed HDTMAB promoted extinction increased with increasing HDTMAB concentration and time. This effect was most remarkable at 3.0 cmc, with the lowest algae survival rate. The survival rate of algae treated by 1.0 cmc HDTMAB was higher than that treated by 0.3 cmc HDTMAB. It was probably related to cellular adaptation to stress, algae cell regulated the membrane permeability under stress and inhibited the copper ion and other toxic materials entrance into the cell to some degree. Under heavy metal operation, the physiological index and enzyme activity of the cell would suffer the process of being promoted, inhibited, promoted or the other of being inhibited, promoted, inhibited, which was a familiar phenomena in environmental biology.

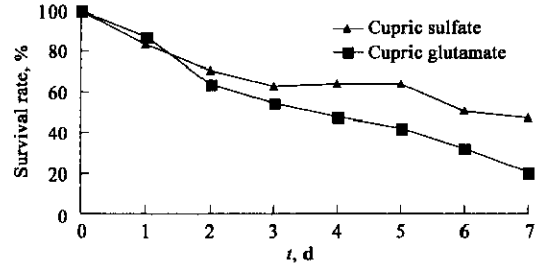

Fig.1 Effect on the extinction rate of *Alexandrium* sp. LC3 by cupric sulfate or cupric glutamate

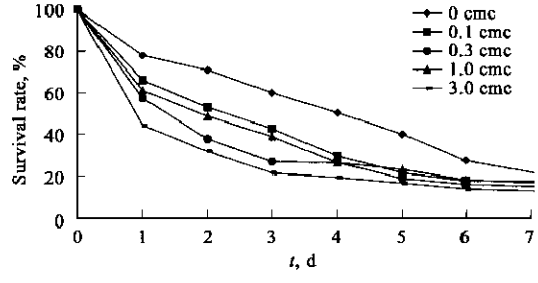

Fig.2 Effect on the extinction rate of *Alexandrium* sp. LC3 by cupric glutamate and different concentration of HDTMAB

2.3 Effect of various concentrations of HDTMAB on the membrane peroxidatic level of *Alexandrium* sp. LC3 under complex operation

Malonaldehyde, which can oxidate SH groups and cross-link lipids, nucleic acids, saccharides and proteins, is one of the main products of the peroxidation of the cell membrane (Zeng *et al.*, 1991). The cell membrane peroxidatic level can be evaluated by the content of MDA. HDTMAB was associated with cupric glutamate (final 0.5×10^{-3} mol/L) to

extinguish *Alexandrium* sp. LC3. As the culture without cupric glutamate or HDTMAB was regarded as a control, the content of MDA was measured after 6 d (Fig.3). The MDA content of algae treated with cupric glutamate was remarkably higher than that of the control, which showed that cupric glutamate notably promoted the cell membrane peroxidatic level notably. The MDA content of each group under complex operation was always higher than that of algae culture with only cupric glutamate operation. Algae cultured with 3.0 cmc HDTMAB under complex operation had the highest MDA content, which was 3.65 and 11.45 times that of the 0 cmc HDTMAB treatment group and control group respectively. The cell membrane peroxidatic level could be promoted by addition of HDTMAB.

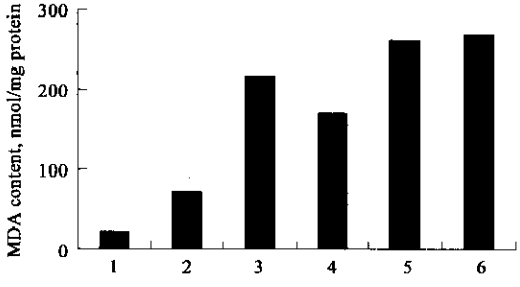

Fig.3 Effect on the MDA content in *Alexandrium* sp. LC3 by different concentration of HDTMAB under complex operation
1: controlled group; 2: 0 cmc; 3: 0.1 cmc; 4: 0.3 cmc; 5: 1.0 cmc; 6: 3.0 cmc

2.4 Effect of various concentration of HDTMAB on the SH group content of *Alexandrium* sp. LC3 under complex operation

After 6 d incubation, the SH group content in *Alexandrium* sp. LC3 with various concentrations of HDTMAB under cupric glutamate stress was measured (Fig.4). Compared with that of controlled group, the SH group content lessened dramatically. The SH group content of each group under complex operation was always lower than that of algae culture with only cupric glutamate operation and decreased slightly with increased HDTMAB concentration. Algae cultured with 3.0 cmc HDTMAB under complex operation had the lowest SH group content, which was 66% and 56% of that of 0 cmc HDTMAB treatment group and controlled group respectively. It was important for the SH group to maintain the normal conformation of the protein and the membrane, and was usually oxidated by MDA. As MDA accumulated in the cell, SH group content decreased as it was oxidated. Therefore, the SH group content was negatively correlated with that of MDA, with a correlation coefficient of -0.9419.

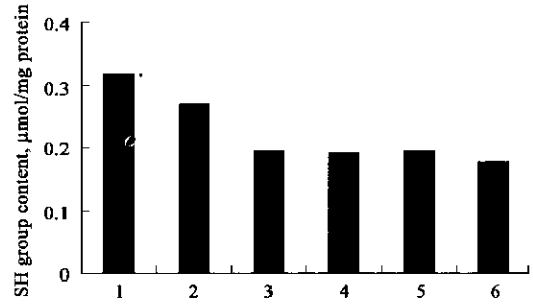

Fig.4 Effect on the SH group content in *Alexandrium* sp. LC3 by different concentration of HDTMAB under complex operation
1: controlled group; 2: 0 cmc; 3: 0.1 cmc; 4: 0.3 cmc; 5: 1.0 cmc; 6: 3.0 cmc

2.5 Effect of various concentrations of HDTMAB on the cell membrane permeability of *Alexandrium* sp. LC3 under complex operation

The permeability of the cell membrane could be used as a physiological criterion to evaluate the response of algal cells under stress. As the selective permeability of the plasma membrane altered under stress, the salt or organic compound leaked from the cell to the peripheral space and led to the increase of peripheral space conductivity. Therefore, the degree of the damage of the plasma membrane was determined by the change of conductivity of the peripheral space (Fig.5). The enhancement of the membrane permeability of these groups with increase of HDTMAB concentration indicated aggravation of cell damage. This was consistent with that reported by Glover *et al.* (1999). Under complex operation, the algae cultured with 3.0 cmc HDTMAB had the lowest SH group content and highest MDA content and membrane permeability, which was 127% of that of the 0 cmc HDTMAB treatment group. The membrane permeability was positively correlated with the content of MDA while negatively correlated with that of SH group, with correlation coefficients of 0.8768 and -0.7837, respectively.

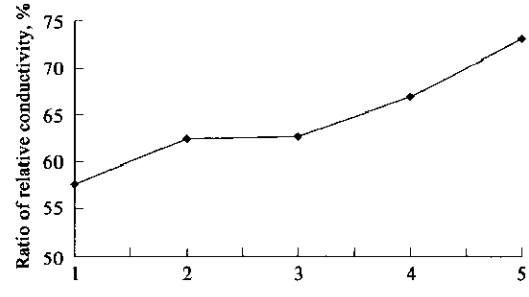

Fig.5 Effect on membrane permeability of *Alexandrium* sp. LC3 by different concentration of HDTMAB under complex operation
1: 0 cmc; 2: 0.1 cmc; 3: 0.3 cmc; 4: 1.0 cmc; 5: 3.0 cmc

3 Discussion

Cupric sulfate used to control the red tide algae was ever a widely accepted method worldwide. Although cupric sulfate can effectively eliminate red tide algae, it harms other organisms and the marine ecological environment due to the extremely high cupric concentration when used directly.

Now, spread clay used to control the red tide algae is a widely accepted method worldwide (Yu *et al.*, 1999; Anderson, 1997; Sengco *et al.*, 2001). Over the past 25 years, clays have been investigated in several countries as a means of removing harmful algae from the water column. Red tide is controlled through the flocculation of clay particle to the red tide organism. Although clay was considered as a promising and attractive direct control option, clay is not available for some locations with HAB problems, and high transportation cost would quickly render this method uneconomical (Sun *et al.*, 2004b). Furthermore, this is limited in the long run. It is highly desirable to find alternative options.

Cupric glutamate, which is a complex compound of copper and amino acid, has been used as a high efficient bactericide in protection of plants. However, no such study has been reported in the control of harmful algae. In this study, cupric glutamate had an excellent ability to extinguish *Alexandrium* sp. LC3 and was superior to cupric sulfate, with increasing superiority with time due to slow release of copper ions. The toxicity of copper to algae is not related to total concentration of copper, but with the concentration of free copper ions (Verweij *et al.*, 1992). The affinity of the copper ion with the SH group was more than that with the copper-amino acid complex compound, which induced the dissociation of the complex compound due to the existence of SH groups in the cell membrane, so that the copper ions were released from cupric glutamate gradually to be combination with SH group, then the function of SH group in cell membrane and enzymes was lost and the membrane permeability increased. In contrast to the controlled group, the SH group content of the algal cell treated by 0.5×10^{-3} mol/L cupric glutamate decreased by 15.8%, which was a joint result of incessant binding with copper ions and oxidization by MDA. Enhancement of membrane permeability stimulated more copper ion entry into the plasma membrane.

Furthermore, the complex compound of metal and amino acid could stimulate the uptake of trace elements by fish and shrimp (Nelson, 1991) while it inhibited the growth of red tide algae. Trace elements, such as Fe, Cu, Zn, and so on, play an important physiological role as prosthetic groups or activators of enzymes and constituted organic compounds with special functions in tissues and organs. In the alkaline midgut of fish and shrimp, the digestion and absorption of trace element with a form of inorganic salt was low and the total bioavailability was lower than 20% due to its low solubility in alkaline conditions. By dint of the absorption pathway of amino acids, more trace elements were taken up by fish and shrimp and stimulated their growth (Zhao and Lu, 1997).

The surfactant has been applied widely in the medicine and food industry as a bactericide. It was previously reported (Cao and Yu, 2003) that HDTMAB could be regarded as an algaecide to directly eliminate the red tide algae effectively. In this study, algal cell extinction by cupric glutamate was obviously promoted by HDTMAB added to the medium. Compared to the cupric glutamate only, the SH group content of the HDTMAB and cupric glutamate complex operation group decreased, while the MDA content and the membrane permeability increased. HDTMAB promoted the killing efficiency and the algae cell had the highest damage while cultured in 3.0 cmc HDTMAB medium. While treated with 3.0 cmc HDTMAB, the cell death rate was about 110% of that due to treatment with cupric glutamate alone; the SH group content decreased 34%, and the MDA content and membrane permeability increased 265% and 27%, respectively. With increasing HDTMAB concentration, MDA accumulated in the cell, the cell membrane peroxidatic level increased and SH group content decreased as was oxdized, which led to the plasma membrane being seriously damaged and enhancement of the membrane permeability, arose mass extravasation of intracellular components. By the conjugation of the hydrophobic headecanoic group with membrane lipid and hydrophilic cation with membrane protein, HDTMAB conjugated with and inserted or penetrated into the membrane (Luo and Sun, 1998). The separation of membrane protein and membrane lipid caused by the micelle concentration of HDTMAB made enhanced membrane permeability and damaged membrane function.

Furthermore, compared to other surfactants with phenyl cycle, alkanes and multiple side chains, HDTMAB could be decomposed by more than 90% in one week due to its long linear chain structure. It would not bring secondary pollution to the marine environment.

4 Conclusions

Cupric glutamate used in this study assaulted the *Alexandrium* sp. LC3 cell membrane and affected the integrity and function of the membrane, then eliminated algal cells effectively. The extinction was promoted by addition of HDTMAB. The promotion was due to enhanced cell membrane permeability by

HDTMAB.

The above results indicated the promoting mechanisms were that: (1) the cell membrane was destroyed by cupric glutamate and that HDTMAB promoted this process; (2) HDTMAB induced denaturation and disintegration of intracellular protein, and then inhibited cell growth. This research indicates the potential of HDTMAB as an accelerant in controlling harmful algal blooms in the future due to its efficiency in promoting cupric glutamate mitigation of *Alexandrium* sp. LC3 cells.

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