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营养胁迫下球形棕囊藻(*Phaeocystis globosa* Scherffel)的生长行为及溶血活性

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摘要:近年来,我国广东沿海连续出现大面积球形棕囊藻(*Phaeocystis globosa* Scherffel)赤潮,产生溶血毒素等有害物质,给当地的海洋养殖业造成重大的经济损失。研究不同的生长时期及半连续培养时不同营养盐胁迫下,球形棕囊藻溶血毒素的产生行为。结果显示,批量培养的球形棕囊藻处于生长平稳期末时,溶血活性最大((21 ± 1)units/L);半连续培养时,营养盐限制对球形棕囊藻的生长有明显的抑制作用,其中 Fe^{3+} 及 N 盐限制影响最为明显。同时,营养盐限制也可促进棕囊藻溶血毒素的合成,其中 Fe^{3+} 和-Mn^{2+} 的限制性时球形棕囊藻溶血活性显著增强。这些结果表明,球形棕囊藻溶血毒素的产生与藻细胞的生长可能受不同机制的调节,溶血毒素的合成可能是环境胁迫下棕囊藻维持生存的一种策略。

关键词:溶血物质;球形棕囊藻;营养限制

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Growth and hemolytic activities of *Phaeocystis globosa* Scherffel at different mutrients condition

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Abstract: In this experiment, the productions of hemolytic substances of *Phaeocystis globosa* Scherffel at various stages of growth and under different nutrient-limited conditions in semi-continuous cultures were studied. The results showed that the hemolytic activity was highest in stationary phase, but did not decrease as cell entered into senescent phase; the hemolytic activity varied significantly among different treatments. Significantly higher hemolytic activities were detected in N- and Fe-limited cultures compared to those under non-limited conditions. However, hemolytic activity of culture under P-limited condition (N:P = 150:1) was the lowest, only 87.5 HU, lower than those under the other conditions. The average hemolytic activities per cell of cultures under N- limited and Fe-limited conditions were significantly higher than those under other nutrient limitation and non-limited conditions, whereas there was only a few differences between other three treatments. These suggested that growth and toxicity of *Phaeocystis globosa* were regulated by different factors, and that the toxin production might be related to cellular physiological stress, regulated by the availability of nutrients in *Phaeocystis globosa*.

Key words: *Phaeocystis globosa*; hemolytic activity; allelopathy

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During the period from the autumn of 1997 to the spring of 1998, a massive bloom of *Phaeocystis* first occurred in the coastal waters of East and South China sea, which caused a great impact on the local aquiculture industry. Since then, this harmful algal bloom (HAB) had frequently occurred in this area. *Phaeocystis* had morphological and physiological features that differed from those previously described for either *Phaeocystis globosa* Scherffel or *Phaeocystis pouchetii* Lagerheim[1, 2]. Furthermore, the sequence comparison of *Phaeocystis* 18S rDNA clearly showed that it was remarkably similar to several isolates of isolated *P. globosa*, and hence it was identified as *P. globosa* rather than *P. pouchetii* or another species documented by Chen *et al*[3].

There were few reports on hemolytic activity of *P. globosa* in the other waters. He *et al*. confirmed that *P. globosa* produced hemolytic toxin responsible for the toxicity of fish in the sea and identified them as a mixture of glycolipids, which was similar to that of *Prymnesium parvum*[4]. Among the other prymnesiophytes, some species, such as *Chrysochromulina* and *Prymnesium*, were known to produce hemolytic toxins that caused lysis of vertebrate red blood cells[5-9].

Many literatures showed that biosynthesis of HAB toxins were closely related to algal cells growth and their life cycle[10, 11]. The synthesis of paralytic shellfish poisoning (PSP) in synchronized cells was confirmed in early G phase and stop as the cells entered S phase in *Alexandrium fundyense*[12]. In the benthic dinoflagellate *Prorocentrum lima*, dinophysistoxin-4 (DTX_4) synthesis was initiated in G_1 phase and persisted into S phase, whereas okadaic acid (OA) and dinophysistoxin-1 (DTX_1) production occurred later during S and G_2 phases[13]. These results suggested that toxin synthesis was coupled to cell cycle events. On the other hand, the latest studies found the dissoluble nitrate and phosphor clearly fluctuated during the occurrence of *P. globosa* red tide[14]. Additionally, the concentrations of Fe^{3+} and Mn^{2+} also varied regularly with the appearance of the red tide[15]. However, little was known about the changes in hemolytic activity at different stage of growth and production of hemolytic toxins under different nutrient conditions in *P. globosa*. In this paper, the productions of hemolytic substances of *Phaeocystis globosa* Scherffel at various stages of growth and under different nutrient-limited conditions in semi-continuous cultures were studied to gain more information about toxin production in *P. globosa*.

1 Materials and methods

1.1 Sampling and culture of algae

Phaeocystis globosa Scherffel, strain Shantou 97 was kindly provided by Dr. S. H. Lu at Science and Engineering College of Jinan University in Guangzhou, China. The strain was maintained in our laboratory in artificial seawater supplemented with f/2 medium[16]. In the present study, *P. globosa* Scherffel was grown in 16 2L- Erlenmeyer flasks each containing 1.5L f/2 medium. All media used were prepared by filtration through 0.22μm Millipore filters. Each flask filled with 1.5L growth medium was inoculated with 50ml exponentially growing *P. globosa* Scherffel cells at 2.55×10^6 cells/ml. Cultures were grown at (22 ± 1)℃ under Photosynthetic Active Radiation (PAR) of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (cool-white fluorescent tubes) with 12 h light/dark cycle for 15 days. Cells were counted under an inverted microscope and samples were taken each day.

1.2 Semi-continuous cultures of algae

According to the method developed by Johansson and Granéli[16], after the batch cultures reached high cell densities, 0.8 L of sample was taken each day and replaced with an equal volume of fresh medium. Three N: P supply ratios (of mass) and 1/10 concentration of Fe^{3+} and Mn^{2+} of f/2 medium were used everyday in the replacing media (three replicates of each) (Table 1). The other nutrients were added to all cultures at the levels corresponding to medium f/2[17]. Vitamins (B biotin and thiamin) were added following the method of Schöne[18].

The inoculated medium was used to estimate the cell density. 10 days after semi-continuous cultures, the cells were

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individually collected for extracting of hemolytic substances.

1.3 Extraction of hemolytic substances

Cultures were centrifuged at 3000 rpm for 10 min. to harvest cells, which were re-suspended in 50 ml of the mixed solution of menthol, chloroform and water (13:7:5, v/v) and agitated vigorously ultrasonically (600M, 30min) at 4℃. The chloroform fractions were fully evaporated to dryness (25℃, 0.1 MPa) in a rotatary evaporator, and then re-dissolved in 70% methanol. The extract from a 1 L culture was dissolved in 1 ml of 70% methanol.

Table 1 Inorganic nutrient concentrations (mg/L) in the daily added new media in semi-continuous cultures of *P. globosa*

Daily added concentration	N: P ratio				
	1.5:1	15:1	15:1(-Fe)	15:1(-Mn)	150:1
$NaNO_3$	7.5	75	75	75	75
$NaH_2PO_4 \cdot 6H_2O$	5	5	5	5	0.5
$FeCl_3 \cdot H_2O$	—	—	0.316	—	—
$MnCl_2 \cdot H_2O$	—	—	—	0.018	—

"—" means that their concentrations keep the same as f/2 medium

1.4 Hemolytic test

The hemolytic substances from *P. globosa* were identified as a mixture of glycolipids, and the hemolytic action resembled that of digitonin, a non-ionic surfactant, so the hemolytic activity of the extract from *P. globosa* was estimated as a digitonin equivalent. The hemolytic test was performed according to the method developed by Simonsen and Moestrup[19], with the following modifications. 1ml of algal extract was added to 4 ml of 0.5% rabbit blood in isotonic phosphate buffer (pH 7.2). After a 30 min incubation at 37℃, the hemolytic activity was detected spectrophotometrically by measuring the absorbance at 540 nm (10 mm cuvette). Tests were performed in triplicate, and 70% methanol was used as control.

Rabbit blood was drawn out of white rabbits obtained from the animal laboratory of Jinan University and used within 3 days. The blood was centrifuged (1000 r/min, 10min), and the serum and upper layer of white blood cells (buffy coat) was removed. Erythrocytes were washed 3 times with phosphate-buffered saline (PBS; pH 7.2) and brought to final concentration (v/v) of 0.5% in isotonic citrate buffer (NaCl 73 mmol/L, sodium citrate 42 mmol/L, glucose 114 mmol/L).

2 Results

2.1 Cells growth

Cell growth of *Phaeocystis glosbosa* in batch culture was demonstrated in Fig. 3. After three days' culture, algal cells entered its exponential phase, and then the cells abruptly increased from 2.92×10^5 cells/ml to 1.63×10^6 cells/ml, lasted about 8 days after that the culture got into its stationary phase of growth, then in about 12 days, the growth went to decline.

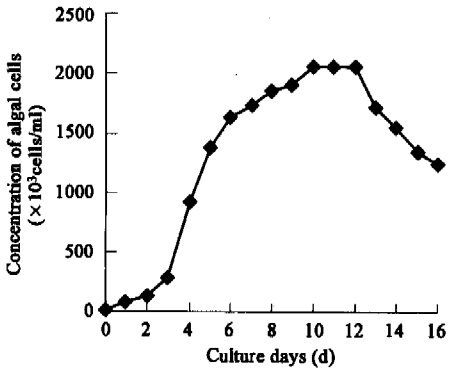

Fig.1 Cell growth of *Phaeocystis glosbosa* Scherffel in batch culture

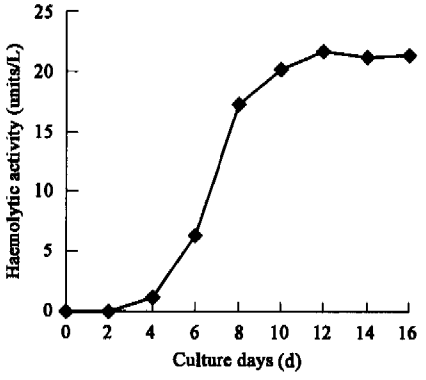

Fig.2 Hemolytic activity of *Phaeocystis glosbosa* Scherffel in batch culture

2.2 Hemolytic activity during the course of its growth stage of *P. globosa* cells

Figure 2 presented the changes in hemolytic activity from algal cells at various stages of growth. Little or no hemolytic

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activity was detected within 4 days after the inoculation when the nutrient was sufficient in cultures. Then, hemolytic activity increased apparently from day 4 to 10. After day10, the augment of hemolytic activity became slower and the maximum of hemolytic activity reached 21 ± 1 units/L in day 12.

2.3 Cell density and hemolytic activity of *P. globosa* cells at the different nutrition conditions

The growth curves of *P. globosa* under different nutrient conditions in semi-continuous cultures were observed from the beginning of culture. The new added nutrients were not enough to support the cells growth at $1.94 \times 10^6 \cdot ml^{-1}$ that was reached in the non-limited batch cultures (Fig. 1). As a result, all cultures experienced a rapid drop in cell density during the following ten days. The cells densities differed significantly among the different treatment. The cell density under Fe^{3+}-limited condition was the least in all the treatments. The cell densities of cultures under N-limited condition were significantly lower than those under P-limited and non-limited condition. These results suggested that Fe^{3+} limitation had the most significant effect on cell density, and that nitrogen limitation had more influence on cell density than phosphorus limitation.

The hemolytic activity varied significantly among different treatments. In N- and Fe^{3+}-limited cultures, significantly higher hemolytic activities were detected compared to those under non-limited condition, P- and Mn^{2+}-limited conditions. However, hemolytic activity of culture under P-limited condition (N : P = 150 : 1) was the lowest, only 87.5 HU/L, lower than that under the other conditions (Fig. 4). Their average hemolytic activities per cell of cultures under N- limited and Fe-limited conditions were also higher than those under other and non-limited conditions, whereas the activity in Fe-limited culture was higher than that in N-limited culture (Fig. 4).

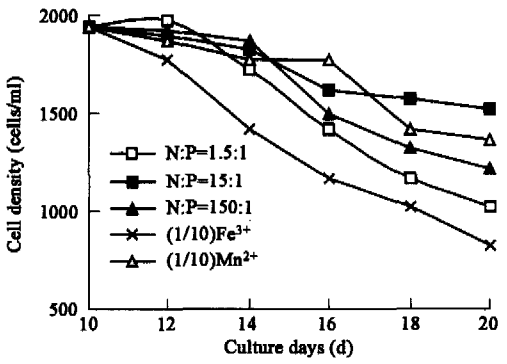

Fig.3 Cell densities of *Phaeocystis globosa* Scherffel in semi-continuous cultures under different nutritional conditions.

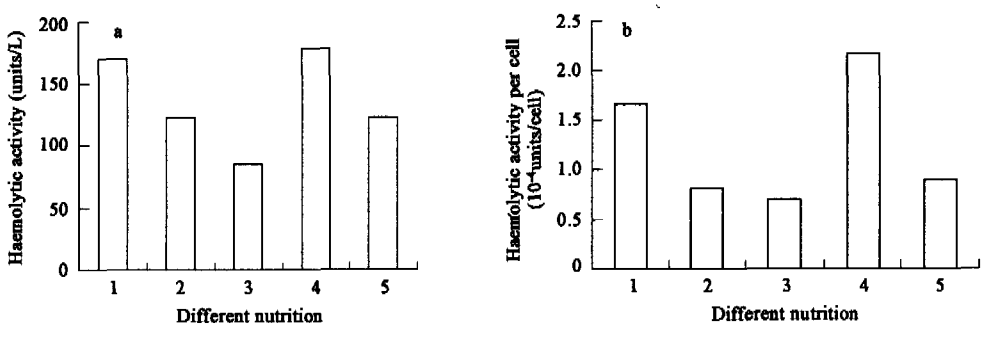

Fig.4 (Ⅰ) Hemolytic activity of *Phaeocystis globosa* cells and (Ⅱ) Hemolytic activity per *P. globosa* cell in semi-continuous cultures under different nutrient conditions. Notes: 1. N:P = 1.5:1; 2. N:P = 15:1; 3. N:P = 150:1; 4. 1/10[Fe^{3+}]; 5. 1/10[Mn^{2+}]. [Fe^{3+}] and [Mn^{2+}] refer to the concentration of Fe^{3+} and Mn^{2+} in f/2 medium.

3 Discussion

Changes of hemolytic activities were presented at various growth stages of *P. globosa* cells. The hemolytic activity was highest in stationary phase, but did not decrease as cells entered into senescent phase (Fig. 2), suggesting that growth and toxicity of *P. globosa* were regulated by different factors, and that production of hemolytic substances in *P. globosa* may be enhanced when the cells were grown in nutrient deficient condition.

Nitrogen limitation had a significant promotion on the production of hemolytic substances in *P. globosa* contrasted

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with some relevant reports that P-starvation had more, which was in agreement with previous studies showing that N-limitation could promote toxicity of *P. parvum*[17]. However, it was interesting to note that Fe limitation also stimulated the production of hemolytic substances in *P. globosa*. According to our knowledge, it was the first time that Fe-limitation had been reported.

This study had showed that there was a close relationship between environmental conditions and toxicity of *P. globosa*. High toxicity had been observed with the increase in cell density and loss in nutrients, indicating that the growth and toxicity of *P. globosa* were regulated by different factors. It could be predicted that the growth and toxin production of *P. globosa* might have different optimal requirements, and that toxicity of *P. globosa* was fully exhibited only when the growth was limited.

The relationship between the environmental conditions and phytoplankton toxicity was still cryptic although it had been studied in the last years. The influence of limited conditions of some crucial nutrients on toxicity had been presented for many phytoplanktons that produced distinct toxins in chemical structure, but the increase in toxicity under certain nutrient limited condition had been observed extensively. Several studies showed that algal toxins had a negative influence on both herbivorous zooplankton and other algae[20, 21]. The negative influence of toxins, in fact, had a function of competing organisms and grazers, so called "allelopathy", and would certainly be of value for phytoplankton. During the break of the HAB of *Phaeocystis globosa*, cell density of *P. globosa* swiftly increased in a short time and the available nutrients in environment were rapidly exhausted. Hence, it could be speculated that production of hemolytic substance in *P. globosa* gave the alga an opportunity to gain sufficient nutrient for its growth by inhibition on other aquatic or algae in the same water. The closely related *P. pouchetii* was reported recently to be poor competitor for N at both light levels[22]. Granéli and Johansson also reported the increase in the production of allelopathic substances by *Prymnesium parvum* cells grown under N- deficient condition[23]. It was reasonable to speculate that the lower ability in competing for N results in the sensitivity to N-limited condition for *P. globosa* due to the similarity in morphological and overlapping ranges between *P. pouchetii* and *P. globosa*. In fact, both species were commonly referred to as *P. globosa* until recent genetic analysis[24]. Thus, from an ecological point of view, an increased toxin production when nutrients were limited would be a great advantage, giving the algae an opportunity to proliferate where they would otherwise be incapable of competing with competitively superior species of algae. However, the influences of hemolytic toxins produced by *P. globosa* on growth of other algae had not been studied in this paper, which deserved to be addressed in the further studies.

4 Conclusions

The toxicity of *P. globosa* was closely related with algal cell growth stages. The hemolytic activity of toxic substances reached the maximum at the end of stationary phase, but lower at early exponential phase. Moreover, the hemolytic activity of *P. globosa* was also related with different nutrient conditions. Significantly higher hemolytic activities were detected under nitrogen (N) and iron (Fe)-limited conditions compared to those under non-limited condition as well as P- and Mn-limited conditions in semi-continuous culture. The increase in hemolytic activity was probably related to physiological stress from nutrition limitation, which may explain the difference in hemolytic activity in *P. globosa* at different coastal areas. According to the results, we considered that an increased toxin production in nutrients starvation would give the algae an opportunity to proliferate under specific conditions that limited cell growth.

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