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Cell proliferation of *Phaeocystis globasa*, a red tide causative marine microalga in various phosphorus(P)- and nitrogen(N)-replete conditions

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Abstract: *Phaeocystis globosa*, a prevalent bloom-forming microalgal species was grown in various phosphorus(P)-replete and nitrogen(N)-replete conditions, 1P1N(f/2 medium): P=5×10^{-3} g·L^{-1}, N=75×10^{-3} g·L^{-1}; 3P1N: P=15×10^{-3} g·L^{-1}, N=75×10^{-3} g·L^{-1}; 1P3N: P=5×10^{-3} g·L^{-1}, N=225×10^{-3} g·L^{-1} and 3P3N: P=15×10^{-3} g·L^{-1}, N=225×10^{-3} g·L^{-1}, and its cell growth was measured by using chlorophyll fluorescence determination and cell-counting methods. The results showed that growth curves of *Phaeocystis globosa* exposed each nutrient conditions exhibited as "S-shaped" curves through out the experiment, indicating the microalgal cells experienced three growth stages, namely slow-growth stage, fast-grow stage and stationary-growth stage. Chlorophyll fluorescence was affected obviously by the P and/or N concentrations. Significantly higher chlorophyll fluorescence was observed in the 3P1N, 1P3N and 3P3N (above 900 μg·L^{-1}) as compared with than that in 1P1N(only 850 μg·L^{-1}), but there were no significant differences in the chlorophyll fluorescence among 3P1N, 1P3N and 3P3N conditions. Besides, remarkably higher specific growth rate was found in 3P3N and 3P1N conditions(both above 0.77 d^{-1}) than 1P1N and 1P3N conditions(only 0.70 d^{-1} and 0.69 d^{-1} respectively). Finally, changes in the cell density of *Phaeocystis globosa* exposed to different phosphorus-replete and nitrogen-replete conditions in the termination of experiment were consistent with the changes in chlorophyll fluorescence, with the relatively higher cell density in 3P1N, 1P3N and 3P3N conditions than 1P1N. Our results demonstrate that high concentration of P or/and N in the water is a major factor responsible for the fast growth of microalgal cells, and that measuring the chlorophyll fluorescence in microalgal cells is a quick, simple, sensitive and reliable method, hence it should be utilized in the predicting and managing red tides.

Key words: *Phaeocystis globosa*; cell proliferation; phosphorus; nitrogen

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Red tides have drawn great attention because of their association with killing of fishes, poisoning of marine wildlife, harming of human health, and degradation of ecosystem along the coastal areas[1-3]. Strong evidence is also presented that the frequency, intensity and distribution of red tides have been increasing dramatically in recent years[4]. *Phaeocystis* is a genus of marine phytoplankton with a worldwide distribution, with species sharing a polymorphic life cycle including free-living single cells and colony-forming cells[5]. In China, a dense bloom of *Phaeocystis globasa* was first recorded in the coastal waters of southeast China in 1997; after then frequent *Phaeocystis globasa*-forming red tides continued to be recorded in 1999, 2000, 2003, 2004 and 2005[6]. A great number of studies have proved that *Phaeocystis* is a prodigious producer of DMSP, acrylic acid, and the volatile DMS, the latter being a salient greenhouse gas in the global sulfur cycle and a precursor for atmospheric particles and cloud condensation nuclei. Besides, *Phaeocystis* colonies have high C/N and C/P ratios, therefore they can remove more dissolved inorganic carbon per unit of nutrients assimilated and drive the "biological pump" to remove atmospheric CO_2 more efficiently than other phytoplankton. Besides, various negative effects of bloom of *Phaeocystis* on higher trophic levels of commercial interests, like fishing, aquafarming and tourism have been reported[7-9]. On the other hand, although a great deal of work has been done in terms of red tides, their outbreak mechanisms

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are very complex and have not been fully understood up to now. The outbreak of red tides is believed to be associated with some complex ecological and oceanographical processes, and it is also affected by a variety of environmental factors[10]. Among them, water temperature, salinity and light are thought to be the most basic factors for the survival and reproduction of red tide organisms[11-13]. Phosphorus(P) and nitrogen(N) are two essential macronutrients required in plant growth and development. Some recent studies also have shown that excessive P or/and N enrichments via industrial and agricultural inputs in to the aquatic environments play a particularly important role in the cell proliferation of microalgae, resulting in massive occurrence of red tides along the coastal water[14-15]. In the present study, we evaluate the cell growth of *P. globosa* grown in various P or/and N concentrations by measuring chlorophyll fluorescence of microalgal colonies and counting the microalgal cell number. These findings will be important for understanding the mechanisms behind massive occurrence of red tide, and for developing some useful methods for predicting and managing the frequent red tide events.

1 Materials and Methods

Phaeocystis globosa, a frequent bloom-forming microalgal species, was obtained from the Institute of Hydrobiology, Jinan University, Guangzhou, China and routinely maintained in a plant growth chamber(CC275TL2H, Hangzhou, China) under standardized condition at the constant irradiance (5 000 lx) and temperature (22±1)℃ in a 12 h/12 h (light/dark) photoperiod cycle. Artificial seawater was previously filtered through 0.45 μm porosity filters and added with f/2 enrichment solution for microalgal cultures [16]. Algal cells in exponential growth phase were employed for the experiments. All glassware and media connected with the experiments were previously sterilized. Salinity of the artificial seawater was 30‰ and the initial pH of the culture was 6.5~7.0. Prior to the experiments, in order to minimize other environmental effects on the growth of microalgal cells, pre-culture was carried out in a shaker (Incubator Shaker Series, Innova 44, NEW BRUNSWICK SCIENTIFIC) with 80 rpm at 23 ℃ and 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ downward irradiance provided by cool-white fluorescent lamps fixed on the tope of shaker with a 12 h: 12 h dark: light cycle. The exponentially-growing cells were inoculated into identical 50 mL test tubes (Schott Duran, Germany) containing 35 mL solution and maintained in the shaker under the growth conditions as described before. Four nutrient conditions were achieved by adding different amount of NaH_2PO_4 or/and $NaNO_3$ into the growth medium, which were denoted as 1P1N (f/2 medium, as the control), 3P1N, 1P3N and 3P3N as listed in Tab.1. Chlorophyll fluorescence was measured every day by using TD-700 fluorometer(Turner Designs) during the experimental period. Maximum specific growth rate of samples determined to be in the exponential growth stage was calculated by least squares fit of a straight line to the data after they had been logarithmically transformed based on the following equation: $\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)$, where X_1 and X_2 are the chlorophyll fluorescence values at day t_1 and day t_2. In the end of experiment, a 0.5-ml algal solution was sampled and the numbers of algal cell were counted under a optical microscope (OLYMPUS CX41) after preserved with Lugol's solution. Each cell sample counting was repeated at least three times and averaged. All experiments were carried out in triplicate for each treatment, and the tubes containing microalgal cultures were shaken gently twice every day.

Table 1 Nutrient treatments different in phosphorus and nitrogen concentrations used in the experiment

Nutrient concentration	1P1N(f/2, control)	3P1N	1P3N	3P3N
Phosphorus($g \cdot L^{-1}$)	5×10^{-3}	15×10^{-3}	5×10^{-3}	15×10^{-3}
Nitrogen($g \cdot L^{-1}$)	75×10^{-3}	75×10^{-3}	225×10^{-3}	225×10^{-3}

2 Results

The changes in growth curves of *Phaeocystis globosa* cultured in various nutrient circumstances were illustrated in Fig.1. In the beginning of the experiment, *Phaeocystis globosa* grew quite slowly, while dramatical fast growth of *Phaeocystis globosa* cells could be observed from the 2nd day to the 5th day, after when the chlorophyll fluorescence of *Phaeocystis globosa* showed a relatively stable increase to the temination of the experiment, with the growth curves exhibiting "S-shaped" curves throught out the experiment. However, it was evident that chlorophyll fluorescence of *Phaeocystis globosa* grown in the 1P1N remained at the lowest level during the experiment, as compared with other three nutrient conditions.

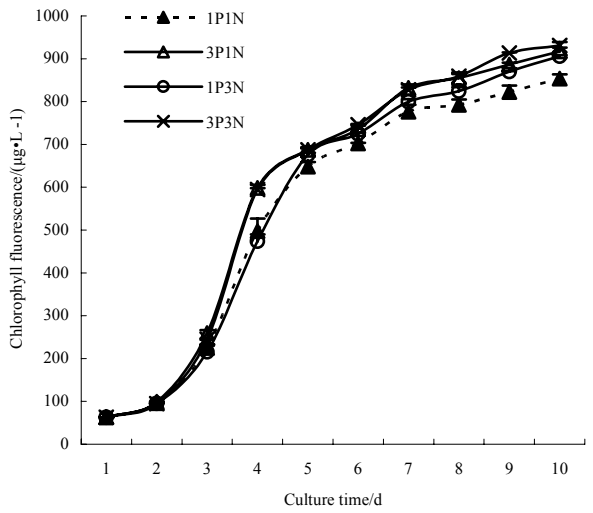

Fig. 1 Growth curves of *Phaeocystis globosa* under different nutrient conditions over time. Each point indicates the mean of three replicates with standard error

For example, in the last day of the experiment, the chlorophyll fluorescence in 1P1N was only about 850 $\mu g \cdot L^{-1}$, but that in 3P1N, 1P3N and 3P3N reached to above 900 $\mu g \cdot L^{-1}$, which was significantly higher than that in 1P1N. On the other hand, *Phaeocystis globosa* grown in 3P3N and 3P1N showed the highest chlorophyll fluorescence, follwed by that in 1P3N condition across the experiment period. But in the termination of experiment, there were no significant differences among the chlorophyll fluorescence values of *Phaeocystis globosa* exposed to 3P1N, 1P3N and 3P3N conditions.

As shown in Fig. 2, specific growth rate of *Phaeocystis globosa* exposed to different nutrient conditions varied significantly. It was obvious that relatively higher specific growth rate of *Phaeocystis globosa* was found in the nutrient conditions of 3P1N and 3P3N, both amounting to approximately 0.77 d^{-1}, but the specific growth rate of *Phaeocystis globosa* grown in the 1P1N and 1P3N conditions only came to about 0.70 d^{-1} and 0.69 d^{-1} respectively.

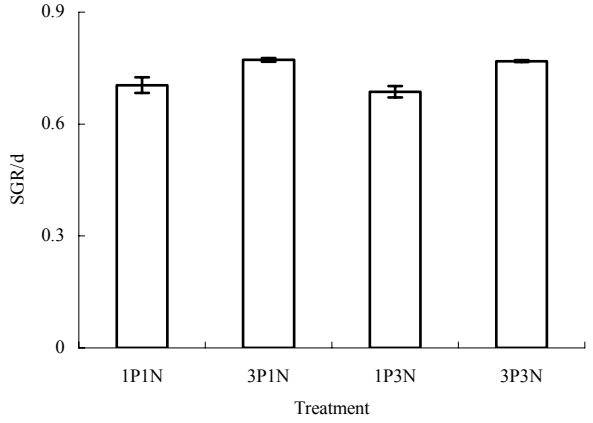

Fig. 2 Specific growth rate of *Phaeocystis globosa* under different nutrient conditions during the exponential growth stage. Each point indicates the mean of three replicates with standard error

The differences in the cell density of *Phaeocystis globosa* cultured in various nutrient conditions were presented in Fig. 3. Phosphorus and nitrogen nutrient had significant effects on the cell density. When the marine microalga *Phaeocystis globosa* was grown in the f/2 medium(1P1N), the cell density in the end of experiment only accounted for 180×10^4 cell·mL^{-1}; however, the cell densities for the *Phaeocystis globosa* in the 3P1N, 1P3N and 3P3N conditions were increased to 220×10^4 cell·mL^{-1}, 216×10^4 cell·mL^{-1} and 237×10^4 cell·mL^{-1}, respectively, significantly than the cell denisty of *Phaeocystis globosa* in 1P1N. But there were no statistical differences among the cell densities of *Phaeocystis globosa* grown under the 3P1N, 1P3N and 3P3N conditions.

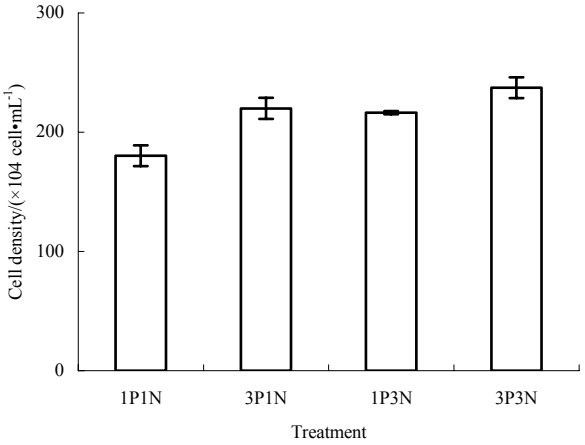

Fig. 3 Cell density of *Phaeocystis globosa* under different nutrient conditions in the termination of experiment. Each point indicates the mean of three replicates with standard error

3 Discussions

Some species of marine microalgae proliferate or assemble so quickly under some given conditions that they can give brownish yellow or reddish color to the entire body of water depending on the algae involved, which is best known as red tides [17]. It has been well documented that red tides have increased in the frequency, intensity and geographic distribution during the last decades[18]. Let's take China for example. In 2001, 77-time red tide events were recorded and the polluted area was over 15 000 km^2, which was an increase of 49 times and 5 000 km^2 as compared with

those in 2000. The geographic distribution of red tide outbreak covered the East Sea, Bohai Sea and Yellow Sea of China[19]. On the other hand, frequent occurrences of red tides have been reported to destroy the natural marine ecosystems, affect the socio-economical activities and inflict significantly negative impacts on the human healthy[20]. As it is known that low light penetration, high respiration or massive decay of red tide organisms as a consequence of overproliferation or overassemblage will lead to great oxygen depletion in the water body, thus causing deleterious decrease of fish catch or increased mortality of benthic organisms. In addition, unpleasant appearance and taste of the waterbody will result from the devastating red tides, leading to the losses of recreational resources. What is worse, some red tide-causative microalgae have the potential to produce toxins, and the toxic and lethal substances produced by such microalgae can bring about mass death of aquatic animals, like some fishes, shrimps, crabs and so on; in some cases, it can also bring about the poisoning of human beings. For instance, a red tide occurred from the mid-March to mid-April, 1998 in the northern coastal waters of the South China Sea, including Hong Kong. By 17 April, about 1 month after the blooming, nearly all the corners of the coastal waters of Hong Kong had been invaded and affected, causing about 2500 tonnes of fishes killed, with a direct economic loss of HK$ 250 million; while in the Mainland waters, this HABs had killed more than 260 tonnes fish by 17 April, resulting in a direct economic loss of about 40 million Yuan RMB[21]. Some field and laborotory studies have been conducted on the red tides in these decades, and some progress has been achieved, indicating that the occurrence of red tides is closely related to the combination of physical, chemical and biological factors, but researches on the mechanisms underlying the massive outbreak of red tide are still in an early stage and a great amount of relevant work is still needed to be continued and enhanced.

Phosphorus and nitrogen are two essential nutrients required by plant growth and development. The unceasing input of these two nutrients into the river and other aquatic environments has become the first primary factor responsible for the eutrophication of waters, and they are shown to be playing a very crucial role in the fast growth and division of microalgal cells. In the present study, the f/2 medium, also described as 1P1N, was used as the control. In fact, low nutrient levels of P or N have been proved to restrict the growth of microalgal cells significantly as compared with the f/2 medium, which is believed to meet the basic nutrient demand for most of microalgal growth. Our results also indicated that 1P1N can satisfy the growth and proliferation of *Phaeocystis globosa* during the whole experimental period, and three obvious growth stages, namely slow-growth stage, exponential-growth stage and steady-growth stage were finished. But in other three nutrient conditions, 1P3N, 3P1N and 3P3N, containing much more P or/and N, *Phaeocystis globosa* still showed an increasing tendency in cell growth, suggesting that higher P or/and N concentrations could still promot the cell growth to a certain degree. For example, in the end of experiment, the chlorophyll fluorescence of *Phaeocystis globosa* grown in the 1P1N(f/2 medium) was only about 850 $\mu g \cdot L^{-1}$, but the chlorophyll fluorescence of *Phaeocystis globosa* grown in the higher nutrient conditions, 3P1N, 1P3N and 3P3N peaked at above 900 $\mu g \cdot L^{-1}$, and the similar increasing trend towards increasing nutrient conditions was also confirmed by the cell density countings, which were significantly lower in the 1P1N(f/2 medium) in the comparision with other thress higher nutrient conditions. However, it needs to point out that the ratio of P and N has also been reported to be a key factor in controlling the microalgal growth, and next step some experiments will be continued with much more P or/and N concentrations/ratios in order to go deeper in to the role of P or/and N in the cell growth and proliferation of microalgae.

There is no doubt that finding some new techniques to quickly predict the cell growth or proliferation is necessary for the better controlling and managing red tides, thus reducing the harmful effects resulting from red tide events on the environment and society. Actually, the most traditional methods are to count the cell number of microalgal cells in a certain volume water with the help of microcopy and hemocytometer, or to measure the optical density of microalgal culture by using spectrophotometer. These traditional methods are generally time-consuming and troublesome[22]. In our study, chlorophyll fluorescence of microalgal

colonies was used to predict the growth status of microalgal cells with the help of fluorometer. The finding provided an evidence that the alternation of microalgal cell densities caused by P/N nutrient variation was consistent with that presented by chlorophyll fluorescence of colonies of *Phaeocystis globosa*, both suggesting an increasing tendency of cell growth towards nutrient richness. Therefore, it can be concluded that P or/and N are external factors eliciting frequent blooming of *Phaeocystis globosa* along the coastal areas, and that measurement of chlorophyll fluorescence of microalgal populations could be considered into predicting the occurrence of over-proliferation of *Phaeocystis globosa* cells in water body.

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海洋赤潮藻球形棕囊藻在氮磷富营养下的细胞增殖

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摘要：利用常见海洋赤潮微藻球形棕囊藻（*Phaeocystis globosa*）为试验研究材料，以f/2海洋微藻营养液为对照（1P1N：磷质量浓度为5×10^{-3} g·L^{-1}，氮质量浓度为75×10^{-3} g·L^{-1}），设置3组富磷和富氮营养处理（3P1N：磷质量浓度为15×10^{-3} g·L^{-1}，氮质量浓度为75×10^{-3} g·L^{-1}；1P3N：磷质量浓度为5×10^{-3} g·L^{-1}，氮质量浓度为225×10^{-3} g·L^{-1}；3P3N：磷质量浓度为15×10^{-3} g·L^{-1}，氮质量浓度为225×10^{-3} g·L^{-1}），利用细胞记数和叶绿素荧光测定等方法研究了藻细胞在不同富磷和富氮条件的增殖情况。结果显示，不同浓度磷和氮营养下的藻体荧光值变化在试验周期内均呈现“S”型曲线，表明藻细胞的生长经历缓慢期，快速期和平缓期3个阶段；同时，不同的富磷和富氮营养条件对球形棕囊藻的叶绿素荧光值有一定的影响，其中在对照1P1N下的藻体荧光值最低，在试验结束时（第10天）只有850 μg·L^{-1}，而在3P1N, 1P3N和3P3N条件下的藻体荧光值均达到900 μg·L^{-1}以上，显著高于1P1N下的藻体荧光值，表明富磷和富氮营养可以促进藻细胞的生长增殖，但在试验设置的不同富磷和富氮营养下的藻体荧光值之间没有显著的差异。就不同磷和氮营养条件下的藻最大比生长速率而言，3P3N和3P1N条件下的最大，均达到0.77 d^{-1}，明显高于1P1N和1P3N条件下的藻最大比生长速率（分别只有0.70 d^{-1}和0.69 d^{-1}）。此外，试验结束时细胞密度的变化趋势与藻体荧光值相似，富磷和富氮营养条件下的细胞密度显著高于1P1N下的细胞密度，而富磷和富氮营养条件下的细胞密度间也不存在显著的差异。研究结果揭示，水体中的高磷和高氮营养浓度是导致藻细胞大量快速增殖的一个主要因素，而利用叶绿素荧光来测定藻细胞增殖是一种快速、简便，灵敏和可靠的方法，可在今后赤潮监测过程中多加利用，以能及时、准确地预测预报赤潮爆发，从而减少其对环境和经济的影响。

关键词：球形棕囊藻（*Phaeocystis globosa*）；细胞增殖；磷；氮