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*Full Length Research Paper*

# Growth effects on mixed culture of *Dunaliella salina* and *Phaeodactylum tricornutum* under different inoculation densities and nitrogen concentrations

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***Dunaliella salina* and *Phaeodactylum tricornutum* are two important marine microalgae rich in bioactive substances and other high-value constituents. In this study, growth effects on mixed culture of these two microalgae were studied under different inoculation proportions (10:0, 7:3, 5:5, 3:7, 0:10) and low, medium and high nitrogen concentrations of 1.4, 14 and 140 mg/l, respectively. By evaluating cell density, $OD_{680}$, biomass, chlorophyll a and protein content in the culture, it was found that colony cell growth of *D. salina* and *P. tricornutum* was increased with the increasing of nitrogen concentrations. Additionally, mixed culture of *D. salina* and *P. tricornutum* under high and medium nitrogen concentrations increased the growth of cell colonies (especially when the inoculation proportion was 7:3) and chlorophyll a content by as much as 96.7 and 132.8%. Protein content was also increased by 1.3 and 2.8 folds when compared with that obtained with monoculture of *D. salina* and *P. tricornutum*. In contrast, when the mixed culture was done under low concentration of nitrogen, cell colonies growth was restricted due to limitation of nitrogen.**

**Key words:** *Dunaliella salina*, *Phaeodactylum tricornutum,* inoculation density, mixed-culture, nitrogen concentration.

## INTRODUCTION

Microalgae are photosynthetic biological resources that have rapid growth speeds, strong adaptability and broad diversity. As microalgae are rich in a vast array of bioactive compounds, such as carotenoid pigments, vitamins, proteins, fatty acids, sterols and polysaccharides, they have been widely used in food processing, cosmetics, pharmaceuticals, sewage treatment and so on. Presently, commercial cultivation of microalgae is mainly targeted to direct human consumption for the health food market, extractable compounds of nutritional supplements or food additives (Wikfors and Ohno, 2001). In this sense, how to increase the microalgal density and biochemical compositions has become a major problem challenging the commercial application of microalgae. A large number of studies have been carried out on many useful mciroalgal species like *Spirulina platensis, Spirulina maxima*, *Dunaliella salina*, *Phaeodactylum tricornutum*, *Platymonas subcordiformis*, *Chlorella pyrenoidosa*, *Haematococcus pluvialis* (TriPathi et al., 2002; Inbaraj et al., 2006). Concerning about microalgal culturing, there have been two main paths to be followed: one is focused on isolation and physiological characterization of species or strains to evaluate their desirable characteristics for cultivation (for example, rapid growth rate, broad tolerance of environmental conditions, high product content in algal cells); the other is on engineering of culture systems (García et al., 2007). It has been reported that biochemical composition of microalgae can be substantially altered by manipulating their culture conditions such as nutrient composition, concentrations, light intensity, photoperiod or temperature (Brown et al., 1989). Similarly, growth characteristics and biochemical composition of microalgal species could also be altered when two or more microalgal species were

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cultured together in the same culture medium (Phatarpekar et al., 2000). Very few studies deal with the mixed cultures of marine microalgae and most of the work has been carried out basically on the relationship of red tidy microalgae. Previous studies demonstrated that mixed culture of microalgae is not simply a mixture of individual monocultures; the growth characteristics, biochemical compositions, nutritional compositions and other growth factors of mixed culture can be substantially different from those of monocultures (Phatarpekar et al., 2000). Cai et al. (2005) studied the inter-specific competition among three species of red tide microalgae and the results demonstrated that cell density of one microalgae could be significantly influenced by the other when they were cultured together. There are also studies indicating that colony cell density in the mixed culture was lower or close to one single species of microalgae (Chen et al., 2003). In comparison, D'Elia et al. (1979) found that cell density in mixed culture could be higher than monoculture. Currently, there is little information on the effects of mixed culture on the growth of marine microalgae with special economic values.

The purpose of this study was to investigate the cell colonial growth of *D. salina* and *P. tricornutum* under different inoculation densities and nitrogen concentrations in mixed cultures. *D. salina* belongs to Dunaliella, Volvocales, Chlorophytaceae and Chlorophyta and *P. tricornutum* belongs to Phaeodactylum, Phaeodactylales, Pennatae and Bacillariophyta. They were selected in this study due to their similar culture conditions and growth periods in laboratory conditions, as well as their economic values. This study provides important baseline information for the development of a promising and effective way for commercial culturing of microalgae and extraction of bioactive algal products.

## MATERIALS AND METHODS

### Microalgal species and cultivation procedures

The unialgal cultures of *P. tricornutum* and *D. salina* were obtained from the Institute of Hydrobiology, Jinan University, Guangzhou, China. The microalgal cultures were previously grown in a seawater growth medium enriched with sterile f/2 medium containing nitrate, phosphate, trance metals and vitamins (Guillard, 1962). The seawater used for culture medium preparation in this study was sterilized and artificial seawater was prefiltered through a 0.45 μm pore size membrane. The stock cultures were stored in sterilized 500 ml Erlenmeyer flasks sealed with cotton plugs to allow gas exchange and inoculated in a plant growth chamber under the controlled ambient conditions of 23°C, a 12 /12 h light/dark cycle and an irradiance of approximately 90 $\mu mol/m^2 \cdot s^1$ supplied by a cool-white fluorescent light (CC275TL2H, Hangzhou). Stock cultures were grown for at least four generations prior to the inoculation of experimental cultures and the microalgal cells in the exponential growth phase were used.

### Experimental design

Mixed culture of *P. tricornutum* and *D. salina* was established by using different cell inoculation densities (D10:P0, D7:P3, D5:P5, D3:P7 and D0:P10). Each treatment was carried out in triplicate.

Maximum environmental capacities of *P. tricornutum* and *D. salina* were compared by growth curve fitting using logistic equation (Volterra, 1926). It is determined that consumption of one *D. salina* cell equals to that of 7.6 *P. tricornutum* cells. Using the cell density of *D. salina* as the standard, the initial total cell density of the mixed cultures was set at $5.60 \times 10^5$ cell/ml. Three nitrogen concentrations were achieved by adding different concentrations of $NaNO_3$ to the mixed cultures, with a low concentration of 1.4 mg/l (LN), a medium concentration of 14 mg/l (MN) and a high concentration of 140 mg/l (HN).

### Determination of cell density and biomass

Daily optical density of algal cultures was measured at a wave length of 680 nm using UV-spectrophotometer (TU-1900, Beijing) every two days during the entire cultural period to monitor the cell growth. Cell density was monitored and recorded with a hemocytometer (XB.K.25, Shanghai) under optical microscope (Olympus CX41, Japan) at the same time of the day on the $1^{st}$, $9^{th}$ and $17^{th}$ day at the HN and MN conditions and the $1^{st}$ and $11^{th}$ day at the LN till the increase was less than or equaled to 5%. Additionally, cell biomass was determined using an electronic balance (Sartorius BS210S, Germany) in the lab after drying the filters with microalgal pellet for 72 h at constant 90°C (ZBY149-83, Shanghai).

### Measurement of biochemical compositions

Chlorophyll a and protein content in this study were assayed on the $9^{th}$ and $17^{th}$ day at HN and MN and $11^{th}$ day at LN, respectively. Chlorophyll a was assayed using a method described by Cong et al. (2007). In each assay, 5 ml of algal suspension was transferred into a centrifuge tube. After centrifugation under relative centrifugal force of 5000 *g* for 5 min and removal of the supernatant, 90% acetone was added into the centrifuge tube to reach an extraction volume of 5 ml. The centrifuge tube was then placed in dark for 24 h and followed by centrifugation for 15 min under the relative centrifugal force of 5000 *g*. Finally, the supernatant in the centrifuge tube was transferred into a glass cuvette with a 1 cm path length and the absorbance of the solution was measured at 630, 647, 664 and 750 nm against 90% acetone reference. The chlorophyll a concentration (Chl a) was thus, computed using the following equation:

$$\text{Chl a } (\mu g/L) = [11.85(D_{664} - D_{750}) - 1.54(D_{647} - D_{750}) - 0.08(D_{630} - D_{750})] \cdot V_E/(V_S \delta)$$

Where, $V_E$ is for extraction volume in centrifuge tube, ml; $V_S$ is for water volume, L; δ is for cuvette optical path, cm.

Protein content was assayed by a method of UV absorption described by Li (2000). A certain volume of microalgal suspension in the culture was centrifuged for 10 min under a relative centrifugal force of 5000 *g*. The algal cells was transferred to a volumetric flask afterwards and 10 ml of distilled water was added to the flask to re-disperse the cells. The microalgal suspension in the flask was then transferred into a test tube to freeze and thaw three times. Use a ultrasonic cell crusher (JY96-II, NingBo) to improve the effect of cell crushing. Finally, the microalgal suspension was centrifuged for 10 min under a relative centrifugal force of 5000 *g*. The absorbance of the supernatant was measured at 260 and 280 nm against distilled water. The protein content was calculated as follows:

Protein content (mg/ml) = $1.55A_{280} - 0.76A_{260}$



**Data analysis**

All the data were analyzed by computer software of excel 2003 (Microsoft Company) and SPSS11.5 (SPSS Inc.). SPSS11.5 was used to determine the significant differences in biomass and biochemical composition between the cultures. Significances in all statistical tests were judged at p levels of 0.01 and 0.05.

## RESULTS

### Growth of cell density and biomass

Figure 1 shows the growth curves of *D. salina* and *P. tricornutum* under the monoculture and mixed culture conditions. The growth curves were depicted with the measurement of daily optical densities. Comparatively speaking, in every case, a lag-phase, a logarithmic phase, a stationary phase can be distinguished. Growth trends were similar in both HN and MN conditions and the growth rate was noticeably higher in HN and MN than LN culture. $OD_{680}$ of *D. salina* monoculture increased rapidly on day 3 without any apparent lag phase in HN and MN, then entered into stationary period on day 11 and day 15, respectively; while in case of LN, it was the 3rd day that correspond to stationary period. $OD_{680}$ of *P. tricornutum* followed the similar trend with *D. salina* at HN and LN. For MN, the increase in daily optical density of *P. tricornutum* was higher than that of *D. salina* between day 11 and day 17; whereas mixed culture continued to increase gradually during the corresponding period.

Higher $OD_{680}$ occurred in mixed culture than those in monoculture at HN and MN conditions. In HN condition, no significant differences were observed in the first three days between mixed culture and monoculture. However, remarkably higher $OD_{680}$ was observed from the 5th day onward to the end of the experiment, especially on the 15th day under the treatment of D5:P5. $OD_{680}$ reached to 45% ($p < 0.01$) and 14.2% ($p < 0.05$) higher than those of *D. salina* and *P. tricornutum* monoculture. At MN condition, higher $OD_{680}$ was observed in mixed culture too, especially for the treatment of D5:P5. However, there was not distinctive predominance of $OD_{680}$ in mixed culture when the nitrogen concentration was low ($p > 0.05$).

Microalgal cell densities under different nitrogen concentrations were counted and recorded for three times (1st, 9th and 17th day) in HN and MN conditions and for twice in LN condition(1st and 11th day) during the experiments. The results are presented in Figure 2. Remarkably, higher cell densities were observed at medium and high nitrogen concentrations than those at low nitrogen concentration. In HN condition, the cell densities were increased by more than $5.33 \times 10^6$ cell/ml at the end of the experiment, while at LN condition, they remained below $1.8 \times 10^6$ cell/ml. In addition, substantial differences in microalgal cell density were found when the cells were grown in different inoculation densities at HN and MN conditions. This phenomenon was particularly noticeable at the treatment of D7:P3, in which cell densities were increased by 17.3% ($p < 0.05$) and 18.8% ($p < 0.05$) at MN, 38% ($p < 0.01$) and 28.4% ($p < 0.01$) at HN when compared with those in monocultures. As for D5:P5 and D3:P7 treatments, cell densities in D5:P5 were close to those in D3:P7 at MN, but were higher in HN culture; however, these differences were not significant ($p > 0.05$). Within the system of low nitrogen concentration, cell densities of mixed culture had no noticeable effect in comparison to monoculture and the monocultured *P. tricornutum* had higher cell density at the end of the experiment.

Colonial biomass of mixed culture and monoculture of *D. salina* and *P. tricornutum* and was manifestly affected by nitrogen concentrations as shown in Figure 3, with approximately 0.59-0.74 g/ at LN, 0.88-1.15 g/ at MN and 1.06-1.32 g/l at HN at the end of the experiment, suggesting that higher nitrogen concentration could promote the growth and final yield of marine microalgae. The maximum biomass at MN (0.847 g/l) of *D. salina* monoculture was found on day 9 and raised to 0.883 g/l on day 17. While in HN, the value raised from 0.857 to 1.075 g/l. As for *P. tricornutum* monoculture, it increased from 0.945 to 1.033 at MN and 0.77 to 1.06 g/l at HN. However, there were only 0.74 and 0.69 g/l accumulated biomass at LN for these two microalgae monoculture.

In the case of mixed culture, similar increase in biomass was observed, with higher biomass at HN and MN conditions. For example, in MN, increased biomass of the treatment of D7:P3 at the end of experiment was 26% ($p < 0.05$) and 7.8% ($p < 0.05$) higher in comparison to monoculture, 4.7% ($p > 0.05$) higher than D5:P5 and 3.6% lower than D3:P7 ($p > 0.05$). For HN, biomass from day 9 to the end of the experiment (1.32 g/l D7:P3; 1.18 g/l D5:P5 and 1.16 g/l D3:P7, respectively) was higher ($p < 0.05$) in mixed culture than monoculture (1.07 g/l, 1.06, respectively) during the corresponding phases too.

### Changes in some chemical compositions across the experiment

Chemical compositions of cell colonies including protein and chlorophyll a content were determined at different nitrogen concentrations. Protein is considered to be very important for algal cell division, while chlorophyll a is the main pigment transforming the light energy into chemical energy, which represents the photosynthetic ability (Sun et al., 2004). The results obtained in this study indicated that there were significant differences in the chlorophyll a and protein contents under different inoculation densities and nitrogen concentrations. As illustrated in Figures 4 and 5, chlorophyll a and protein content increased with the increase of N concentrations. Monocultured cells of *D. salina* in HN and MN were 6.94 and 4.53 folds ($p < 0.01$) increase in chlorophyll a content than those in LN;

chlorophyll a content in *P. tricornutum* was 20.08 and 12.12 folds ($p < 0.01$) higher than that in low nitrogen.



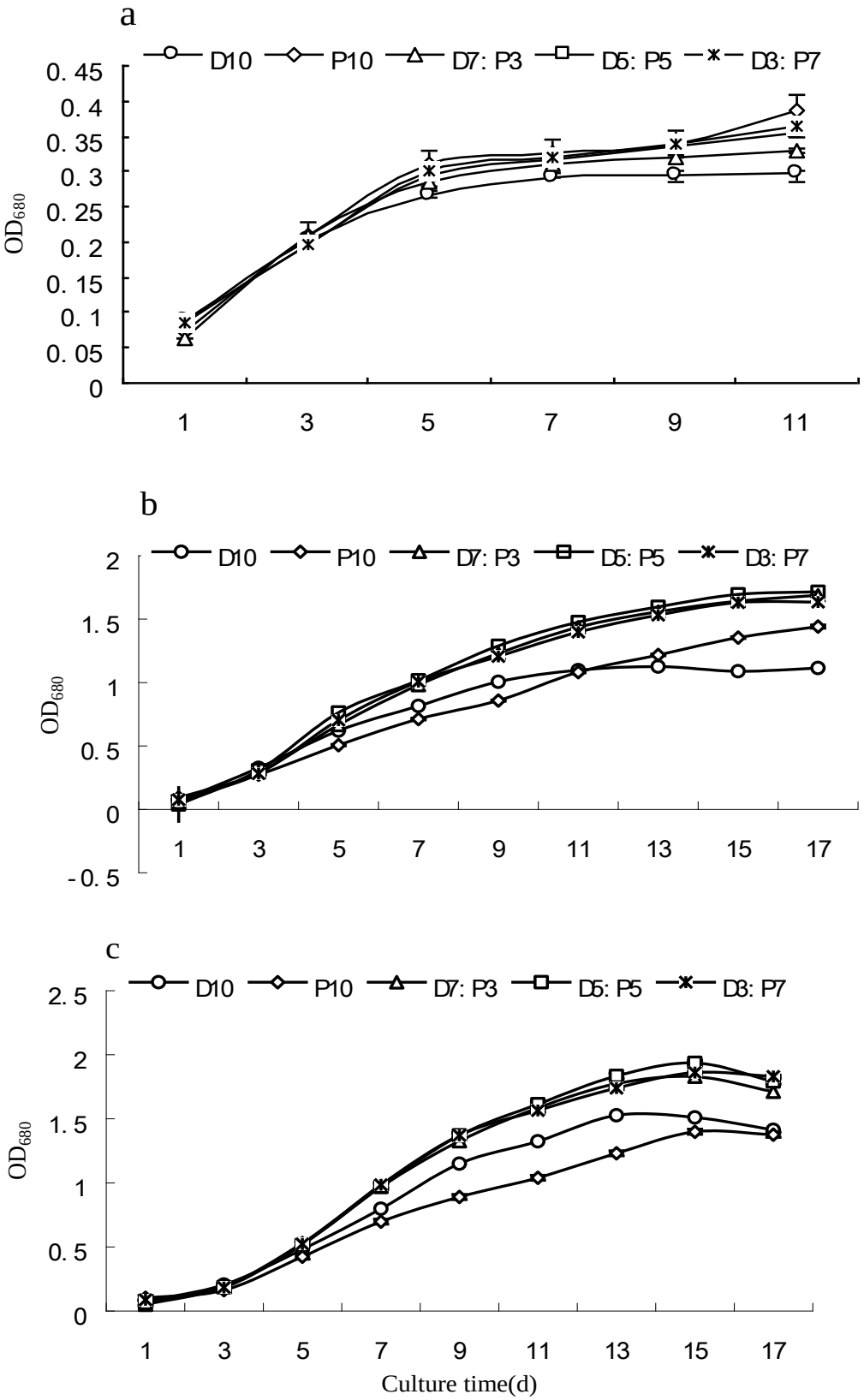


**Figure 1.** Value of $OD_{680}$ of *D. salina* (D) and *P. tricornutum* (P) under different inoculation density and nitrogen concentration. A: 1.4 mg/l; B: 14 mg/l; C: 140

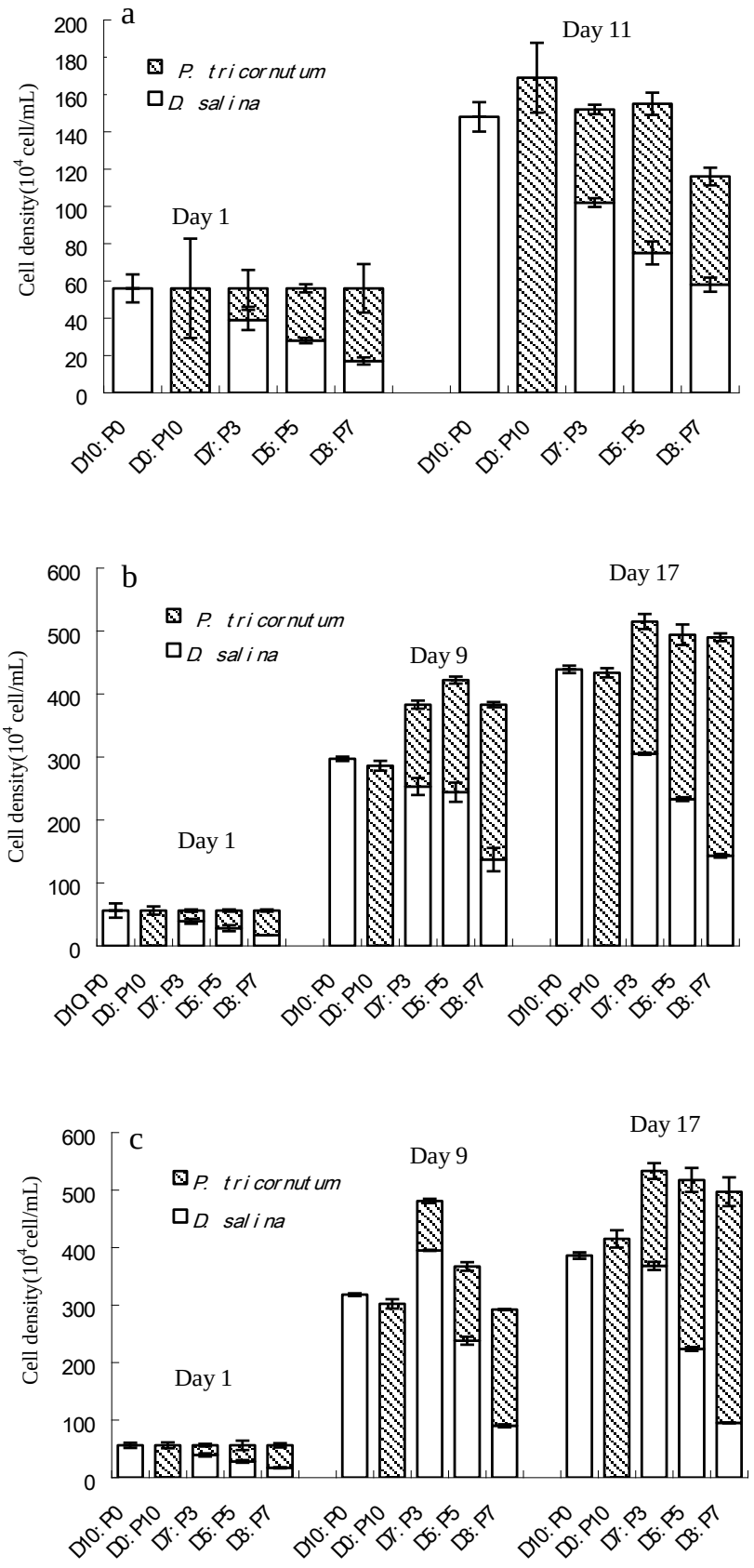


**Figure 2.** Microalgal cell density of *D. salina* (D) and *P. tricornutum* (P) at different sampling time under various inoculation density and nitrogen

concentration. A: 1.4 mg/l; B: 14 mg/l; C: 140 mg/l.



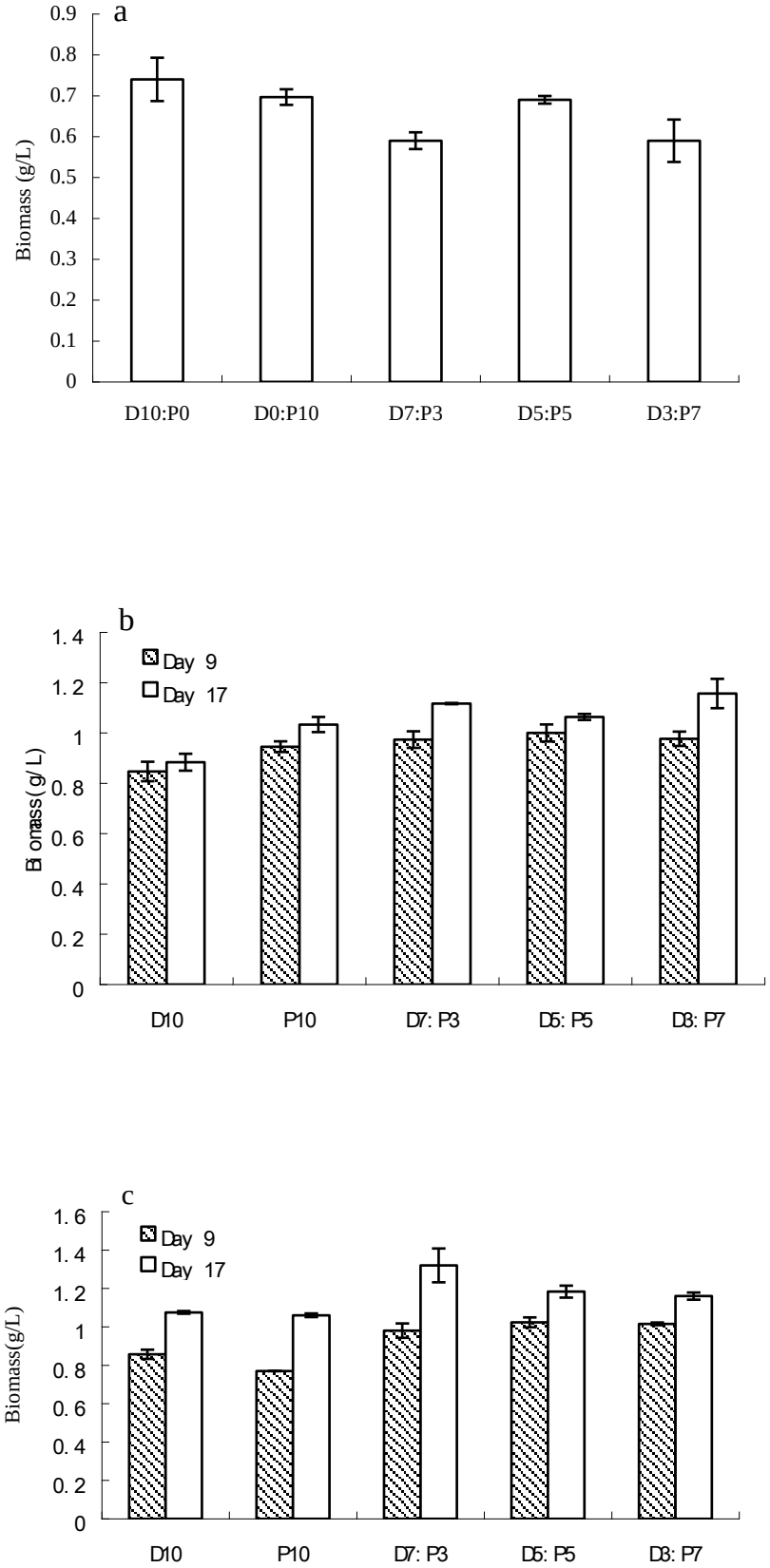


**Figure 3.** Microalgal biomass of *D. salina* (D) and *P. tricornutum* (P) at different sampling time under various inoculation density and nitrogen

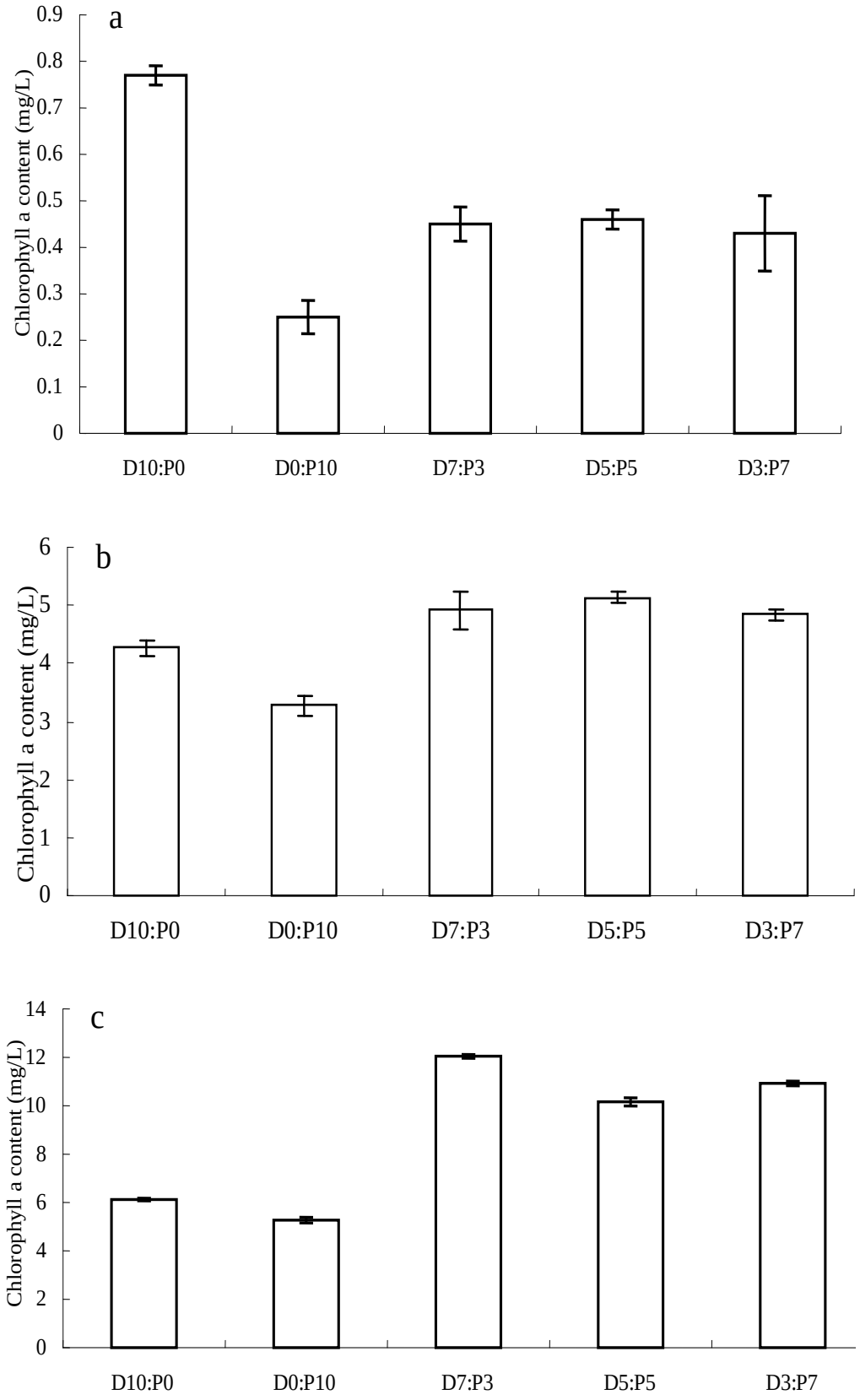


**Figure 4.** Microalgal chlorophyll a content of *D. salina* (D) and *P. tricornutum* (P) under different

inoculation density and nitrogen concentration. A: 1.4 mg/l; B: 14 mg/l; C: 140 mg/l.

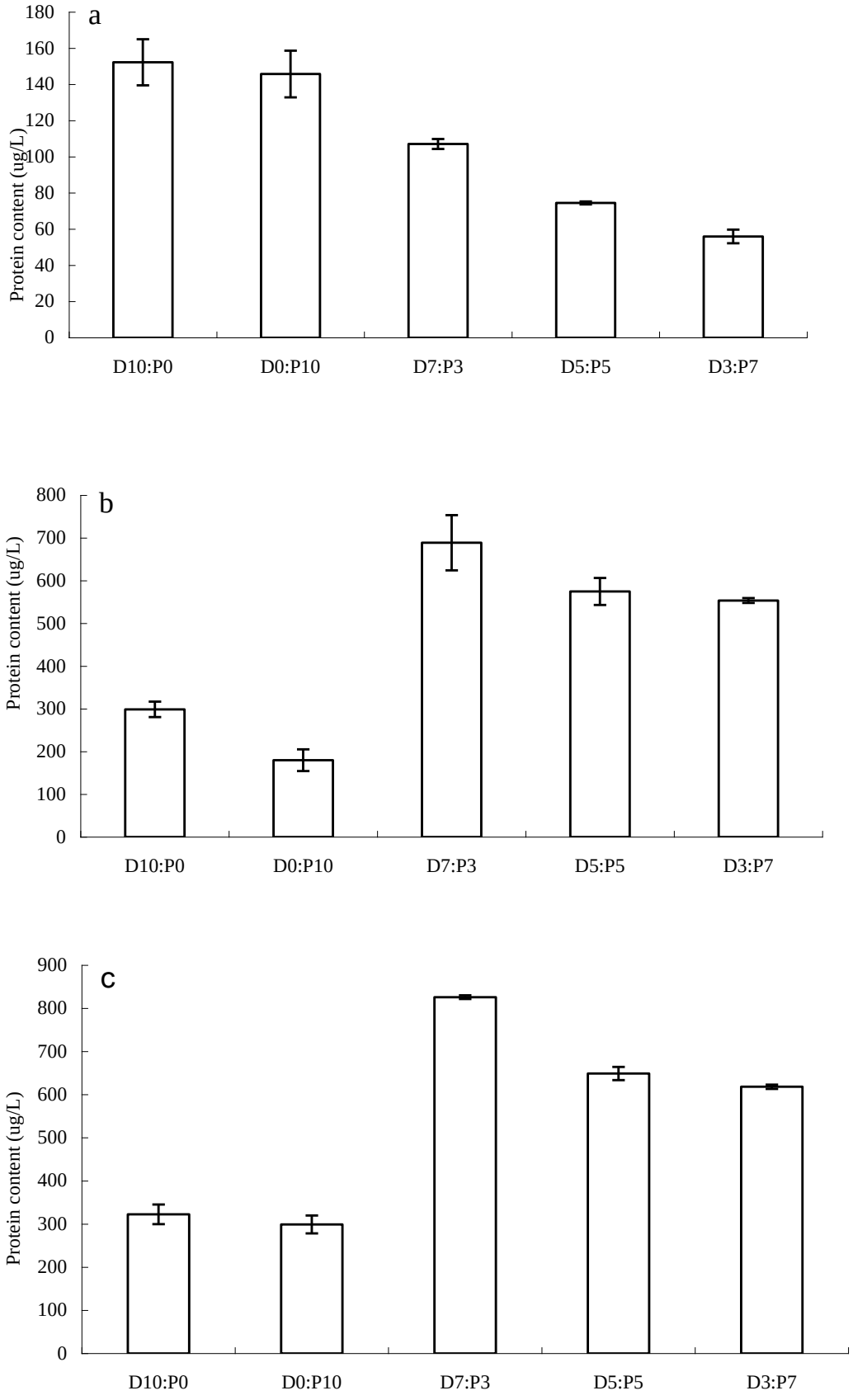


**Figure 5.** Microalgal protein content of *D. salina* (D) and *P. tricornutum* (P) under different

inoculation density and nitrogen concentration. A: 1.4 mg/l; B: 14 mg/l; C: 140 mg/l.



Additionally, the increasing content of chlorophyll a in mixed culture was obvious during cultivations of all microorganisms. This was particularly evident at the cultivations of D5:P5 at MN and D7:P3 at HN, where the contents were exceeded by 20.6 and 56.7% ($p < 0.01$) in MN, 96.7 and 132.8% ($p < 0.01$) in the latter case in comparison to monoculture. However, averaged chlorophyll a content at 0.45 mg/l of mixed culture was close to that in the monocultures in low nitrogen concentration.

Substantial variation in protein content was also found at different nitrogen concentrations. *D. salina* monoculture showed 1.12 fold and 96.3% ($p < 0.01$) increase in HN and MN conditions than in LN, and *P. tricornutum* increased by 1.05 folds ($p < 0.01$) and 23.5% ($p < 0.05$). Besides, increase in protein concentration was monitored during the cultivation of D7:P3 group. Protein contents of around 826 µg/l in HN, 689 µg/l in MN and 145 µg/l in LN were detected, which exhibited a similar trend as chlorophyll a.

## DISCUSSION

Marine microalgae have important applications in many fields. Previous studies on the culture mode of microalgae were mainly focused on red tidy algal species and two potential competition modes on the formation of red tides were identified. One is in nutrition level, while the other lies in the changes of the mutual relationships of microalgae, which is firstly caused by the direct contracts of cells or the substance secreted by microalgal mutual interaction (Honjo, 1994; Uchida et al., 1999). Chen et al. (2007) conducted an investigation on the interaction among different microalgae by monoculture, bialgal culture and mixed culture and found that mixed culture played an important role in the reproduction of microalgae; it promoted the propagation of *Skeletonema costatum*, but inhibited the growth of the other two microalgae. Similarly, Soong (1980) found that “hormone analogue” produced by *chlorella* was capable of stimulating the cell growth of yeast in the process of beer yeast fermentation. There were also well-documented studies showing that growth effect of microalgae in monoculture was different from that of mixed culture. Microalgae in mixed culture could be either promoted or prohibited (Zhou et al., 2006). The results obtained in this study demonstrated that mixed culture of *D. salina* and *P. tricornutum* at high nitrogen concentrations and the inoculation proportion especially proportion D7:P3 significantly increased the total biomass and biochemical composition. For example, under HN, monocultured cells of *D. salina* showed 45.2% ($p < 0.01$) increase in biomass, while 6.94 ($p < 0.01$) and 1.12 folds increase in chlorophyll a and protein content than those in LN. Cells of *P. tricornutum* were increased by 52% ($p < 0.01$), 20.08 ($p < 0.01$) and 1.05 folds ($p < 0.01$) in biomass, chlorophyll a and protein content, respectively, as comparison to those in low nitrogen. Additionally, when the two microalgal species were inoculated at a proportion of D7:P3 at HN, the biomass of mixed culture at the end of the experiment was improved by 22.8% ($p < 0.05$) and 24.5% ($p < 0.05$) than that of monoculture, while the chlorophyll a content was increased by 96.7% ($p < 0.01$) and 132.8% ($p < 0.01$) and the protein content was increased by 1.57 ($p < 0.01$) and 1.77 ($p < 0.01$) folds when compared with those of *D. salina* and *P. tricornutum* monoculture. *D. salina* is a unicellular Chlorophyta microalga of Chlorophyceae class and Volvocales order. The main morphological characteristic of *D. salina* that distinguishes this microalga from other Volvocales is the absence of a polysaccharide cell wall. And for this reason, *D. salina* could be easily digested by humans and animals. Besides, they are with high efficiency of conversion of light, energy, nutrients into biomass, so they can accumulate more dry weight as well as carotene and chlorophyll a under appropriate cultivation conditions. Additionally, when *D. salina* was grown in higher nitrogen, presence of high N imposes a stress on the anaplerotic metabolism (Giordano, 2001). Main anaplerotic enzyme in green microalgae of phosphoenolpyruvate carboxylase (PEPC) is also hypothesized to play an important role in the mechanism of adaptation to high N (Huppe et al., 1994).

Improving the maximum cell density is a crucial but difficult facet for commercial application of marine microalgae. At present, many studies have been conducted to increase the output of microalgal cells by a variety of physical, chemical and biological means (Garrido, 2008). In agricultural practices, it has long been realized that production of crops could be improved by the positive interactions between/among the same or different species (Zhu et al., 2009). As marine microalgae are photosynthetic organisms, it is reasonable to assume that they may have similar positive biological association with each other. Phatarpekar et al. (2000) compared the growth performance, biochemical and nutritive values of *Isochrysis galbana* and *Chaetoceros calcitrans* in mixed culture with those in monoculture under laboratory condition and found that cell density in mixed culture was lower or equal to that in monoculture, but cellular concentrations of chlorophyll a, protein, carbohydrate, lipid and particulate organic carbon were significantly higher in mixed culture; the calculated dietary energy content was also significantly higher in mixed culture when compared with monocultures during all the growth phases. Microalgal cellular chlorophyll a content at mixed culture of proportion D5:P5 was detected to be increased by 20.6% ($p < 0.05$) and 56.7% ($p < 0.01$) and protein content at a proportion of D7:P3 was increased by 1.3 ($p < 0.01$) and 2.8 ($p < 0.01$) folds, respectively at MN condition, which exhibited the similar trends as Phatarpekar et al. (2000). Moreover, potential ability in improving colonial cell biomass of mixed culture was detected in a recent research of growth effect on *P.*

*subcordiformis* and *D. salina* in mixed cultured and monoculture (Cai and Duan, 2008).

Biotic or abiotic factors are considered to have different effects on the growth of microalgal colony in aquatic system. Previous studies have indicated that nutrients such as nitrogen or phosphorus are essential for the growth of phytoplankton, which could restrict energy transformation, carbon fixation and so on (Herzig et al., 1989). Suitable nitrogen concentration could promote microalgal growth, while limitation of nitrogen inhibited the growth. The findings outlined here indicate a similar trend. In this study, three nitrogen levels were set up and the results demonstrated that colonial cell growth in mixed culture and monoculture under different nitrogen levels was different. Total cell density increased with the increasing of nitrogen concentrations. This could be explained as follows: Firstly, *D. salina* has flagellum, microalgae swimming could enhance the utilization of resources and lights, while at low nitrogen, nutrition limitation might cause resource competitions; secondly, when two microalgal species were mixed together, certain substances released by one species of microalgae may moderate the growth of the other as found in previous studies; thirdly, main anaplerotic enzyme of phosphoenolpyruvate carboxylase (PEPC) in green microalgae is hypothesized to play an important role in the adaptation of high N as said earlier. Fogg (1955) on the first time discovered that marine microalgae could continuously release metabolites to the environment which could restrict or promotes the growth of other microalgae. Underlying mechanisms are still needed to be investigated by using physiological, biochemical and molecular analysis. Besides, additionally, future studies should be carried out to consider the different characteristics of microalgae and environmental factor optimization to set up similar combination in practical application.

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