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Competition of bloom-forming marine phytoplankton at low nutrient concentrations

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Abstract

Competition of three bloom-forming marine phytoplankton (diatom *Skeletonema costatum*, and dinoflagellates *Prorocentrum minimum* and *Alexandrium tamarense*) was studied through a series of multispecies cultures with different nitrate ($NaNO_3$) and phosphate (NaH_2PO_4) levels and excess silicate to interpret red tide algae succession. *S. costatum* outgrew the other two dinoflagellates in nitrate and phosphate replete cultures with 10 μmol/L Na_2SiO_3. Under nitrate limited (8.82 μmol/L $NaNO_3$) conditions, the growth of *S. costatum* was also dominant when phosphate concentrations were from 3.6 to 108 μmol/L. Cell density of the two dinoflagellates only increased slightly, to less than 400 and 600 cells/mL, respectively. Cell density of *S. costatum* decreased with time before day 12, and then increased to 4000 cells/mL (1.5 mg/L dry biomass) at $NaNO_3$ concentrations between 88.2 and 882 μmol/L with limited phosphate (0.36 μmol/L NaH_2PO_4) levels. In addition, *P. minimum* grew well with a maximal cell density of 1690–2100 cells/mL (0.5–0.6 mg/L dry biomass). Although *S. costatum* initially grew fast, its cell density decreased quickly with time later in the growth phase and the two dinoflagellates were dominant under the nitrate-limited and high nitrate conditions with limited phosphate. These results indicated that the diatom was a poor competitor compared to the two dinoflagellates under limited phosphate; however, it grew well under limited nitrate when growth of the dinoflagellates was near detection limits.

Key words: *Alexandrium tamarense*; nitrate; phosphate; *Prorocentrum minimum*; red tides; *Skeletonema costatum*

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Introduction

Species composition in natural phytoplankton assemblages is largely determined by mechanisms such as resource competition, selective grazing, and selection on the life cycle properties of individual species (Riegman et al., 1996). Accordingly, different species might be involved in algal blooms in different regions. Although algal blooms are controlled by many biological, physical, and chemical processes, a strong relationship exists between algal blooms and nutrient levels (Hodgkiss and Ho, 1997; Riegman, 1998; Granéli et al., 1999; Findlay et al., 2006; Wang et al., 2009). Numerous studies have shown that nutrients affect the growth of bloom-forming marine phytoplankton, and nitrogen and phosphorus concentrations are especially relevant (Riegman et al., 1996; Hodgkiss and Ho, 1997; Riegman, 1998; Guisande et al., 2002; Frangópulos et al., 2004; Shi et al., 2005).

Diatoms, which account for approximately 25% of global primary production, are generally the dominant species under non-limiting nutrient (silicate replete) conditions (Egge and Aksnes, 1992; Egge, 1998). Dinoflagellates rate next to diatoms under most marine conditions but with distinct periodicity (Chaudhary, 2001). Blooms dominated by diatoms occur in most locations; however, harmful dinoflagellate blooms have become increasingly common in recent years (Smayda, 1990). Decreases in the Si:N ratio (Officer and Ryther, 1980; Egge and Aksnes, 1992) and increases in the N:P ratio (Egge, 1998) due to the reduction in anthropogenic supplies of phosphorus (Egge, 1998) and silicate (Tréguer and Pondaven, 2000) have probably been responsible for this tendency. Diatoms are poor competitors at low phosphate concentrations (Egge, 1998), and phosphate limitation increases toxin production by toxic dinoflagellates (Maestrini et al., 2000; Guisande et al., 2002; Frangópulos et al., 2004). Toxin production is considered a compensatory strategy to minimize the competitive disadvantages of dinoflagellates under nutrient limitation (Frangópulos et al., 2004). To date, however, very little research has been conducted on the competition of diatoms and dinoflagellates under limited nutrient

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conditions (Labrya et al., 2008; Ou et al., 2008).

Skeletonema costatum (Greville) Cleve, a diatom, and *Prorocentrum minimum* (Pavillard) Schiller and *Alexandrium tamarense* (Lebour) Balech & Tangen, two dinoflagellates, are major causative organisms of red tide. *A. tamarense* can produce saxitoxins, which are the suite of compounds associated with paralytic shellfish poisoning (PSP) in humans (Hallegraeff, 1993). In this study, growth competition of the three algae in nutrient replete and nitrogen- or phosphorus-deficient media in multispecies cultures were observed to elucidate red tide algae succession in different nutrient conditions. A possible explanation of the succession is given, based on the comparison of nutrient physiological characteristics of the three algae. We make an attempt to relate recent increases in the frequency of harmful dinoflagellate blooms in natural waters to the changes in availability of limiting nutrients.

1 Materials and methods

1.1 Organism and growth conditions

A. tamarense (33–57 μm long; 28–49 μm wide), *S. costatum* (18–65 μm long; 5–10 μm wide), and *P. minimum* (12–20 μm long; 10–14 μm wide) were obtained from the Institute of Oceanology, Chinese Academy of Sciences (China). Mixtures of the three algae (each with an initial cell density of about 200 cells/mL) were cultured at 22°C and 60 μmol/(m^2·sec) in a 12 hr:12 hr of light:dark cycle in a plant growth chamber (PGX-330A-12H, Ningbo Life Apparatus, China). The stock culture was grown to its late exponential phase at a concentration of about 10,000 cells/mL for use as inocula. Experiments were conducted in 250 mL Erlenmeyer flasks with 150 mL artificial seawater enriched with f/2 solution (f/2AW) (Harrison et al., 1980), with nitrate, phosphate, or silicate added as designated (Table 1). Silicate (Na_2SiO_3) was at 10 μmol/L in all cultures. The cultures were shaken gently 2–3 times a day, and before sampling they were shaken again to ensure homogenous cell distribution.

1.2 General analyses

Cell density was monitored on alternate days by enumeration with a phytoplankton-counting chamber (0.1 mL, Institute of Hydrobiology of the Chinese Academy of Sciences, China) and converted from an appropriate calibration curve with dry biomass (dw). After the counting of diatom, cells samples were preserved with 4% formaldehyde to count the dinoflagellates. Specific growth rates (μ, day^{-1}) were calculated during the exponential growth phase using the following Eq. (1):

$$\mu = (\ln X_t - \ln X_0)/t \quad (1)$$

where, X_0 (cells/mL) is the initial cell density and X_t (cells/mL) is the cell density after t days.

1.3 Measurement of growth kinetics of three algae

Growth kinetic parameters were measured according to Frangópulos et al. (2004) with some modification. Unialgal cultures were grown in 150 mL diluted f/2 medium in 250 mL Erlenmeyer flasks at 22°C and 12 hr:12 hr of light:dark cycles with an illumination of 60 μmol/(m^2·sec). Three replicates were used for each species. At the beginning of the experiment, aliquots from f/2AW media cultures in the exponential phase were taken to reach an initial culture concentration of 500 cells/mL for each species. The experiment was run for 35 days. The initial concentrations of $NaNO_3$ and NaH_2PO_4 were 88.2 and 36 μmol/L, respectively. On the fifth day of the experiment and subsequently every 2 days for the rest of the experiment, cell density and concentrations of NO_3^- and PO_4^{3-} were analyzed for each culture flask. Samples of 3 and 1 mL from each experimental culture flask were removed for nutrient concentrations and cell density estimates, respectively. The 3 mL samples were centrifuged and the supernatant was used to estimate soluble nutrient concentrations. The concentrations of NO_3^- and PO_4^{3-} in the media were determined by the methods of Collos et al. (1999) and Harrison (1988), respectively. At day 15 of the experiment, 5 mL from each culture flask was removed and inoculated into 145 mL of new f/2AW medium without NO_3^- added. At day 25 of the experiment, 5 mL from each culture flask was removed and inoculated into 145 mL of new f/2AW medium without PO_4^{3-} added. Therefore, the experiment was carried out in three different periods with different nutrient concentrations.

Specific growth rate (μ) between successive sampling days for each species was calculated using Eq. (1). The relationship between μ and nitrate or phosphate concentration was described by the following Eq. (2):

$$\mu = \mu_{max} N/(K_g + N) \quad (2)$$

where, μ_{max} (day^{-1}) is the maximum specific growth rate

Table 1 Macronutrient composition of media used in the multispecies culture experiment (f/2AW as the basic medium)

No.	Conditions	Description	NO_3^- (μmol/L)	PO_4^{3-} (μmol/L)	SiO_3^{2-} (μmol/L)
1	Sufficient nutrient	Low N	88.2	36	10
2		Low P	882	3.6	10
3	Limited NO_3^-	Low P limited N	8.82	3.6	10
4		Middle P limited N	8.82	36	10
5		High P limited N	8.82	108	10
6	Limited PO_4^{3-}	Low N limited P	88.2	0.36	10
7		Middle N limited P	882	0.36	10
8		High N limited P	2646	0.36	10
9	Limited NO_3^- and PO_4^{3-}	Limited N and P	8.82	0.36	10

and K_g (μmol/L) is the half saturation coefficient for N (μmol/L) nutrient concentration. Low values of K_g indicate a high relative ability of a species to use low levels of nutrients and, hence to outcompete higher K_g species under nutrient limitation. Data are represented as mean and standard deviations obtained from the duplicates. Statistical significance of the data was analyzed using a one-way analysis of variance (ANOVA).

The nitrogen and phosphorus cell yields (cells/mol NO_3^- or PO_4^{3-}) were determined by calculating the nitrate or phosphate required to obtain the maximal cell density for unialgal cultures during 35 days' cultivation.

2 Results

2.1 Growth competition of the three algae in N or P sufficient cultures

Figure 1 shows the growth curves of three multispecies culture algae (*S. costatum*, *P. minimum* and *A. tamarense*) with replete nitrate (Fig. 1a) or phosphate (Fig. 1b) cultures. The *S. costatum* was dominant with replete nitrate or phosphate cultures with 10 μmol/L SiO_3^{2-}, and its maximal cell density was 110,000 and 230,000 cells/mL (46.6 and 97.4 mg/L dw) respectively. Cell numbers of *P. minimum* and *A. tamarense* increased to approximately 800–1000 cells/mL (0.3–2.4 mg/L dw) under nitrate replete (882 μmol/L) low phosphate (3.6 μmol/L) conditions. In the phosphate replete (36 μmol/L) low nitrate (88.2 μmol/L) cultures, the growth of *P. minimum* tended to increase with time, while that of *A. tamarense* initially increased and then decreased with time. It was evident that the diatom *S. costatum* was a good competitor in nutrient replete conditions, and the growth of the other two

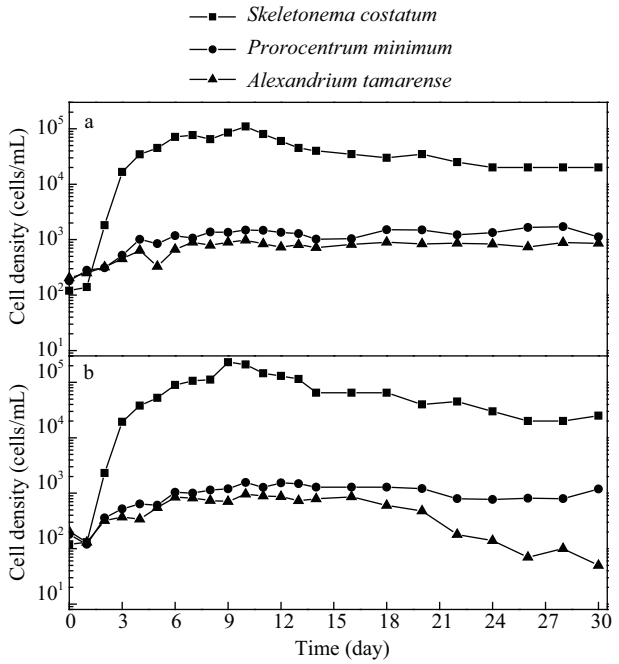

Fig. 1 Growth curves of the three species of algae cultivated in multispecies way under nitrate replete (882 μmol/L) low phosphate (3.6 μmol/L) (a) and phosphate replete (36 μmol/L) low nitrate (88.2 μmol/L) (b) levels with 10 μmol/L SiO_3^{2-}.

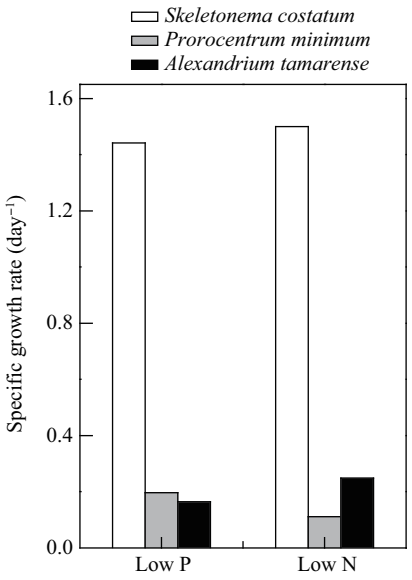

Fig. 2 Specific growth rates of the three species of algae cultivated in multispecies way under low phosphate (Low P) and low nitrate (Low N) with 10 μmol/L SiO_3^{2-}.

dinoflagellates was restricted in the multispecies culture system.

The specific growth rate of the three multispecies culture algae under nitrate or phosphate replete conditions is shown in Fig. 2. The specific growth rates of *S. costatum* and *A. tamarense* in phosphate replete cultures were higher than those in nitrate replete cultures, and it was the contrary in *P. minimum*.

2.2 Growth characteristics of the three algae in N or P limited cultures

The growth curves of the three multispecies culture algae under limited nitrate or phosphate conditions are shown in Fig. 3. In the case of nitrate limited conditions, *S. costatum* was dominant, while the dinoflagellates were favored under limited phosphate conditions. When nitrate concentration was limited in the media (8.82 μmol/L) and the phosphate concentration ranged from 0.36 to 108 μmol/L, cell density of the three algae increased to differing degrees with time. Cell density of *S. costatum* reached a maximum on day 7 (8920 cells/mL, 3.8 mg/L dw) when PO_4^{3-} was 0.36 μmol/L, and then it decreased markedly with time (Fig. 3o), while the maximal cell density of *P. minimum* and *A. tamarense* were as high as 1000 cells/mL (0.3 and 2.8 mg/L dw, respectively). When the phosphate concentrations ranged from 3.6 to 108 μmol/L, the cell number of *S. costatum* was in the order of 10^4 cells/mL (23.6–36.2 mg/L dw) after 10 days (Fig. 3a, b, c); however that of the two dinoflagellates increased only slightly, to no more than 400 and 600 cells/mL (0.1 and 1.6 mg/L dw), respectively.

The growth trends of the three algae under phosphate limited (0.36 μmol/L) conditions were different from those of nitrate limited conditions. Like the 8.82 μmol/L NO_3^- concentration conditions, the cell density of *S. costatum* reached a maximum at day 6 (14,300 cells/mL, 6.1 mg/L dw) under high nitrate (2646 μmol/L) phosphate limited

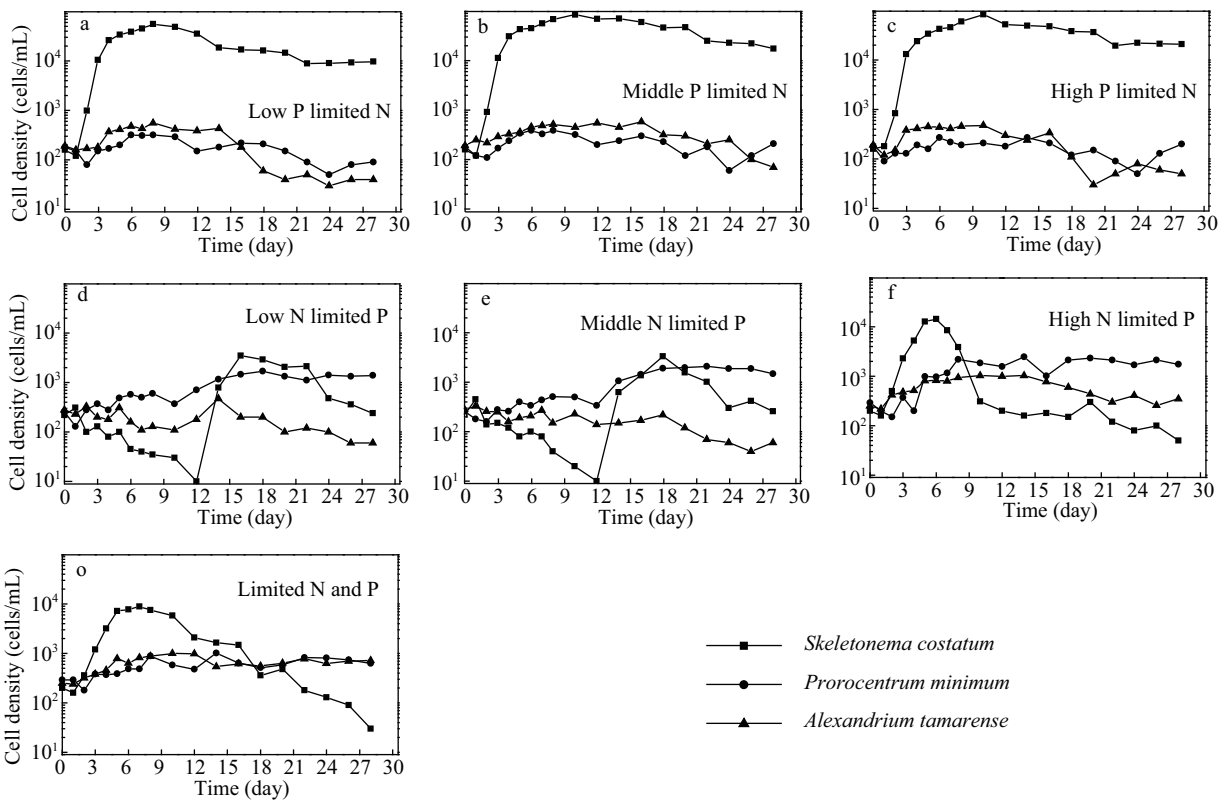

Fig. 3 Growth curves of the three species of algae cultivated in multispecies way with 10 μmol/L SiO_3^{2-} under limited nitrate or/and phosphate levels.

conditions, and then it decreased markedly with time (Fig. 3f); while the cell number of the two dinoflagellates tended to increase, with the maximal cell density of *P. minimum* and *A. tamarense* being 2470 cells/mL (0.8 mg/L dw) and 1040 cells/mL (2.9 mg/L dw) respectively. When NO_3^- concentrations ranged from 88.2 to 882 μmol/L, the cell density of *P. minimum* increased with time to a maximum of 1690–2100 cells/mL (0.5–0.6 mg/L dw), while the cell density of *A. tamarense* did not increase and the growth of *S. costatum* decreased before day 12, and then increased to no more than 4000 cells/mL (1.5 mg/L dw) (Fig. 3d, e).

Phosphate concentrations did not affect the specific growth rate of the two dinoflagellates grown under nitrate limited conditions (Fig. 4a). The specific growth rate of *S. costatum* was almost the same under different phos-

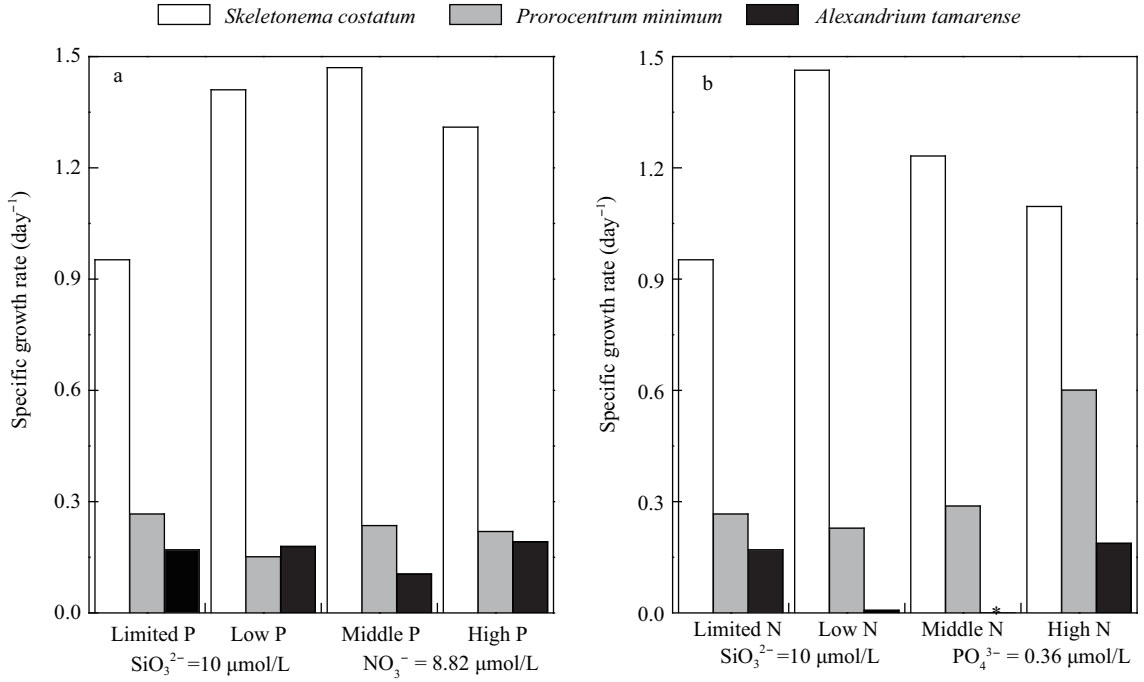

Fig. 4 Specific growth rates of the three species of algae cultivated in multispecies way under limited nitrate (a) and limited phosphate (b) levels with 10 μmol/L SiO_3^{2-} (* indicates that no growth was detected).

phate levels except for the 0.36 μmol/L PO_4^{3-}. Under phosphate limited conditions, the specific growth rates of *A. tamarense* were the same when NO_3^- was 8.82 and 2646 μmol/L, and they were zero when NO_3^- was 88.2 and 882 μmol/L. Specific growth rates of *P. minimum* increased with increasing NO_3^- concentration from 88.2 to 2646 μmol/L, but those of *S. costatum* decreased (Fig. 4b).

2.3 Changes of nutrient concentration in the medium

Figures 5 and 6 indicate the changes of NO_3^- and PO_4^{3-} concentrations in multispecies cultures. Nitrate and phosphate concentrations in the N or P sufficient cultures decreased rapidly before day 12 (Fig. 5). At day 24 the nitrate concentration remained at 800 μmol/L in the N sufficient culture while the phosphate concentration decreased to almost zero (Fig. 5a), and in P sufficient culture the nitrate and phosphate concentrations were approximately 35 and 6 μmol/L, respectively (Fig. 5b). It was evident that both nitrogen and phosphorus were replete in N or P sufficient cultures.

In N-limited cultures (8.80 μmol/L NO_3^-) the nitrate concentration decreased quickly with time before day 6 and decreased to almost zero at day 9, with phosphate concentration ranging from 3.6 to 108 μmol/L (Fig. 6a1). Likewise, the concentration of phosphate decreased quickly with time before day 6, and in particular phosphate was depleted for low P condition while it remained at 17 and 90 μmol/L for middle and high P, respectively (Fig. 6b1). In P-limited cultures (0.36 μmol/L PO_4^{3-}) the nitrate concentration decreased slightly and was as high as 84, 848 and 2304 μmol/L for low, middle and high N conditions, respectively, at day 28 (Fig. 6a2); whereas the concentration of phosphate decreased sharply to zero at day 12 (Fig. 6b2). In N and P-limited cultures the nitrate concentration decreased quickly to less than 4 μmol/L at day 6 and was then maintained at this level (Fig. 6a1), while the concentration of phosphate decreased sharply to zero at day 3 (Fig. 6b1). It was suggested that N or P was rather deficient for cell growth in N or P-limited cultures.

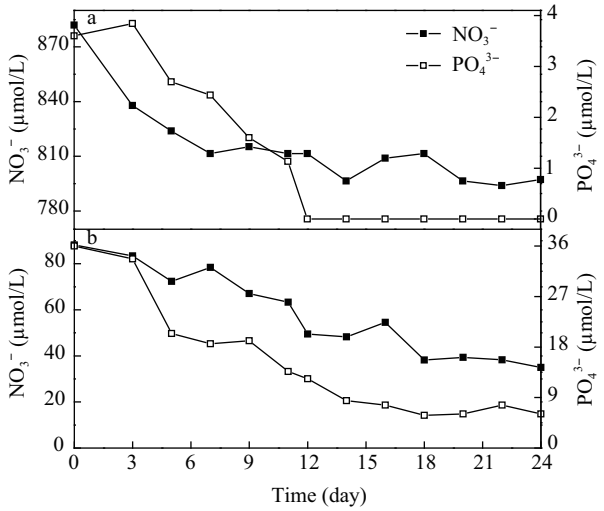

Fig. 5 Concentration change of NO_3^- and PO_4^{3-} in media of multispecies cultured under low phosphate (a) and low nitrate (b) levels with 10 μmol/L SiO_3^{2-}.

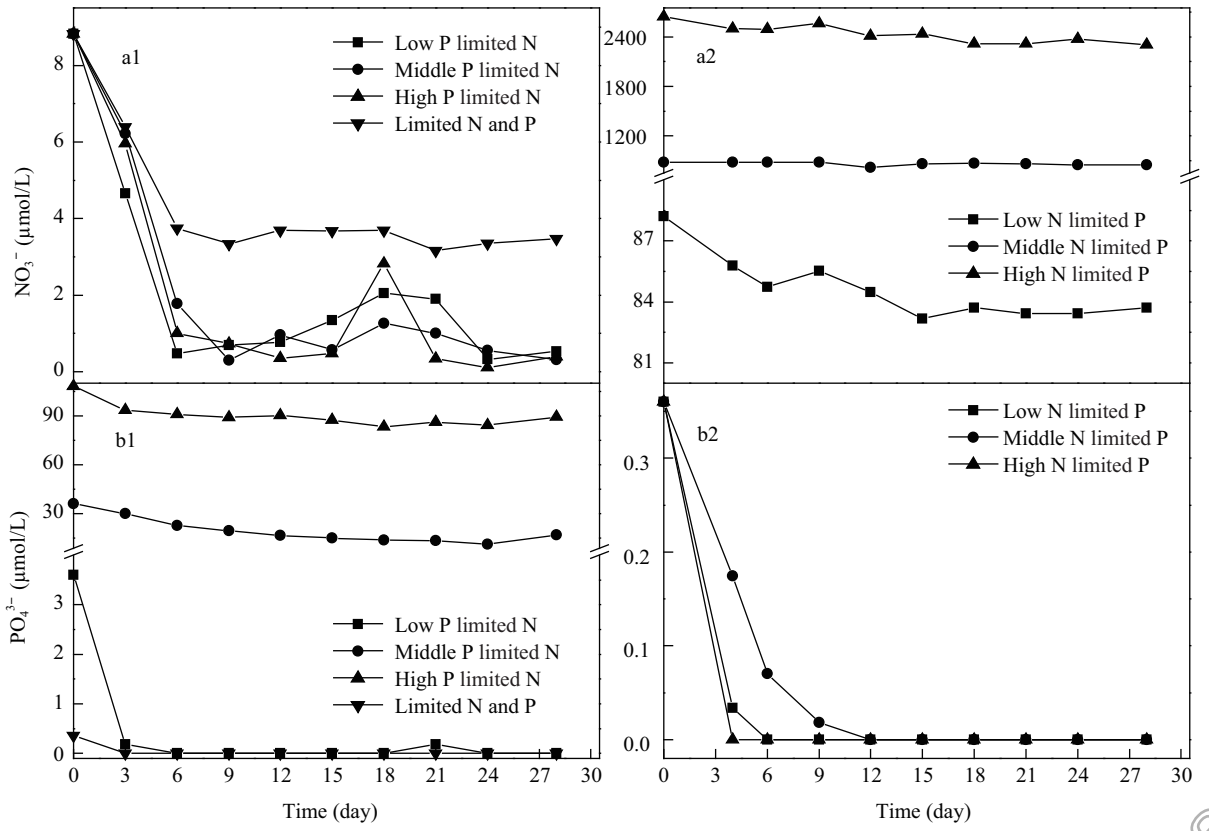

Fig. 6 Concentration change of NO_3^- (a) and PO_4^{3-} (b) in media of multispecies cultured under limited nitrate with different phosphate levels (1) and limited phosphate with different nitrate levels (2).

2.4 Growth kinetics parameters of three algae

Maximum growth rate (μ_{max}) and the half saturation coefficient (K_g) of the three algae for PO_4^{3-} and NO_3^- are shown in Table 2, and they are significantly different ($p < 0.05$). The μ_{max} indicates the intrinsic growth rate of the algae. *S. costatum* had the highest intrinsic growth rate among the three species. The K_g is the nutrient (NO_3^- or PO_4^{3-}) concentration at which the μ is one-half of μ_{max}, representing the competitive ability of a phytoplankton species under nutrient limitation. Nitrate and phosphate required to give one-half of μ_{max} were 35.55 and 1.52 μmol/L in *A. tamarense*, which were 1.7 and 1.1 times and 3.6 and 8.9 times higher than *P. minimum* and *S. costatum*, respectively.

Table 2 Growth kinetics parameters of the three species of algae for PO_4^{3-} and NO_3^-

Species	μ_{max} (day^{-1})	K_g (μmol/L)	
		NO_3^-	PO_4^{3-}
S. costatum	1.68 ± 0.23	9.84 ± 1.42	0.17 ± 0.03
P. minimum	0.51 ± 0.10	20.52 ± 3.56	1.39 ± 0.08
A. tamarense	0.39 ± 0.07	35.55 ± 2.17	1.52 ± 0.12

μ_{max}: maximum growth rate; K_g: half saturation coefficient.

2.5 Nitrogen and phosphorus cell yields in unialgal culture

Differences in nitrogen and phosphorus cell yields in unialgal cultures were observed among the three species (Fig. 7). Nitrogen and phosphorus cell yields for *S. costatum* were 0.908 cells/pmol NO_3^- and 10 cells/pmol PO_4^{3-}, which were 3.7 and 2.0-fold those for *P. minimum* and were 18.2 and 10.0-fold for *A. tamarense*, respectively. The difference in nitrogen and phosphorus cell yields indicated that the nitrogen and phosphorus cell-quotas were different among the three species. The required NO_3^- and PO_4^{3-} per cell for *S. costatum*, *P. minimum* and *A. tamarense* were approximately 1 and 0.1 pmol, 4 and 0.2 pmol, and 20 and 1.0 pmol according to nitrogen and phosphorus cell yields. It was evident that compared with phosphorus cell-quotas, nitrogen cell-quotas varied more significantly among the three algae.

3 Discussion

At nutrient concentrations typical of conditions at the onset of high-latitude phytoplankton spring blooms and blooms in upwelling regimes (nitrate and phosphate concentrations of 15–35 μmol/L and 1–2 μmol/L, respectively), the ratio of the maximum potential NO_3^-:PO_4^{3-} fluxes is 38:1 (Riebesell et al., 1993). Hodgkiss and Ho (1997) suggested that when dissolved N levels are greater than 0.1 mg/L (7 μmol/L) and dissolved P levels are greater than 0.02 mg/L (0.6 μmol/L), red tide occurrences are highly probable. Diatom blooms are more likely when the silicate concentration is higher than 2 μmol/L according to mesocosm experiments (Egge, 1998). In the present study, the nutrient levels at low N (88.2 μmol/L NO_3^- and 36 μmol/L PO_4^{3-}, N:P = 2.5:1) and low P (882 μmol/L NO_3^- and 3.6 μmol/L PO_4^{3-}, N:P = 245:1) with 10 μmol/L silicate were high enough for red tide blooms. Diatoms have high intrinsic growth rates and, in general, dominate under non-limiting conditions (Egge, 1998). Accordingly, *S. costatum* was dominant in the low N and low P conditions. It was evident that with sufficient nutrients, the diatom dominated and was likely to develop into a bloom. In addition, the results showed that growth tendencies of the three species were different under different nutrient (N and P) concentrations. Compared with low N condition, low P gave rise to much lower specific growth rates and cell densities in *S. costatum*, and the growth of the two dinoflagellates was better under low P conditions rather than low N conditions.

Increased nutrient loading is conducive to red tide blooms. The disequilibrium of N and P input in different regions not only results in the alteration of the N:P ratio but also changes the composition of the phytoplankton community (Riegman, 1998). Algae exhibit different competition abilities under limiting nutrient conditions. Low values of K_g indicate a high relative ability of the species to use low levels of nutrients and, hence to outcompete higher K_g species under nutrient limitation (Tilman, 1976). Growth kinetics parameters of the three algae in this study were rather different. Compared with that of the diatom *S. costatum*, K_g of the two dinoflagellates *P. minimum* and *A. tamarense* was much higher. Therefore, in theory,

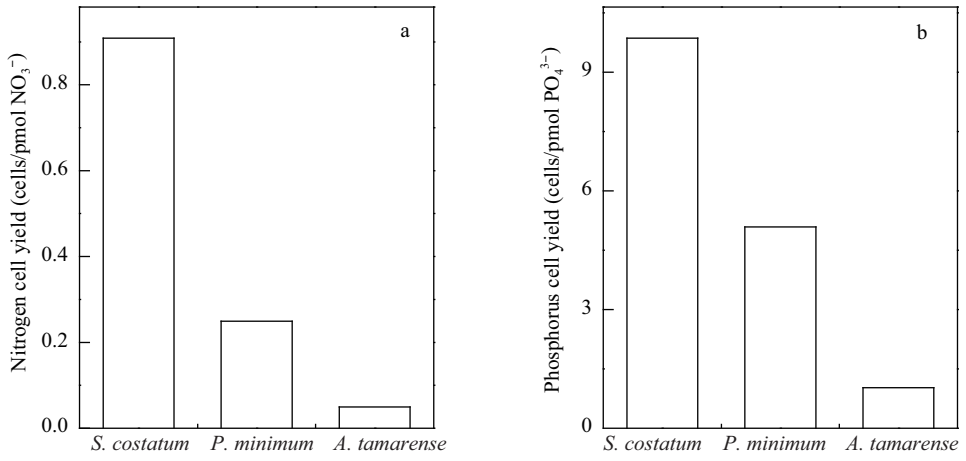

Fig. 7 Nitrogen (a) and phosphorus (b) cell yields under unialgal culture.

S. costatum should better compete under nutrient limitation and its blooms would occur when other factors permited. However, our results showed that the diatom was not a dominant species under P-limited conditions.

Some experimental data indicate that diatom growth is more limited than growth of flagellates at low phosphate concentration (Riegman et al., 1996; Egge, 1998), as diatoms have a high phosphorus demand or a lower uptake affinity for phosphate compared to flagellates (Egge, 1998). Our results showed that *S. costatum* was a poor competitor compared to *P. minimum* and *A. tamarense* under phosphate limited conditions regardless of different nitrate levels. The high intrinsic growth rate in diatoms allows them to outcompete other species with low growth rates. Sufficient energy is necessary for the fast growing diatoms; however, limited phosphate availability restricts the amount of energy acquired by cells. Therefore, *S. costatum* was deprived of its fast growing ability at phosphate limited levels, while the growth rate of *P. minimum* and *A. tamarense* was hardly affected. As *S. costatum* has low nitrate demands, it grew well under limited nitrate conditions, while *P. minimum* and *A. tamarense*, two dinoflagellates requiring rather large amount of nitrate, possess very low cell yields. In the present study, the nitrate limited conditions caused the cell yields of the two dinoflagellates to decease dramatically, although it had little effect on their specific growth rates. Lomas and Glibert (2000) indicated that *S. costatum* had a greater capacity to take up and reduce NO_3^- relative to growth N demands than the flagellated species. Moreover, diatoms contain large numbers of internal vacuoles for nitrate storage (Eppley and Coatsworth, 1968), and once phosphorus is available, diatom blooms might form. Eppley et al. (1971) also suggested that the degree of light/dark differences in nitrogen assimilation differs among species and could be relevant for competition among species for growth-limiting nutrients and hence for species succession. The *S. costatum* diatom assimilated nitrate and ammonium primarily during the day and grew well when irradiance was fairly high (Eppley et al., 1971). In the present study, irradiance seemed favorable for nitrate assimilation in *S. costatum*. Conversely, the two dinoflagellates appeared relatively unsuccessful in nitrate limited conditions, which might result from their sluggish responses to irradiance (Eppley et al., 1968). Cell-quotas of nitrogen and phosphorus for *S. costatum* were the lowest, namely the required NO_3^- and PO_4^{3-} for *S. costatum* were 1/4–1/20 and 1/2–1/10 that of the two dinoflagellates, respectively, which might also explain why diatoms and dinoflagellates showed different responses to the nitrogen and phosphorus limited conditions.

Recently, harmful dinoflagellate blooms have become more and more frequent, which has been attributed, in part, to eutrophication (Smayda, 1990). Removal of nutrients, especially phosphate, from human nutrient inputs into the sea increases the N:P ratio and increases the possibility of P-limitation and of phosphorus limited growth in diatoms (Skjoldal et al., 1993). In addition, silicate input into the ocean has decreased in modern times (Tréguer and Pondaven, 2000). Consequently, under eutrophic conditions non-siliceous phytoplankton species can reach enhanced biomass levels while diatom growth remains limited by the availability of silicate (van Bennekom et al., 1975). Furthermore, elevated atmospheric CO_2 due to the increased industrial combustion of fossil fuels has decreased the pH values of seawater, hence decreasing the inorganic carbon utilization ability of *S. costatum* (Rost et al., 2003). However, the strategy of dinoflagellates to accommodate changing CO_2 concentrations is different from diatoms (Nimer et al., 1997). In natural environments, diatoms might be poor competitors due to the decreased phosphorus and silicate, together with the increasing atmospheric CO_2. In addition, the influences of other nutrients such as zinc on the frequency of harmful dinoflagellate blooms should also be considered as diatoms and dinoflagellates show different sensitivity to zinc concentration changes (Hu et al., 2003a, 2003b).

Acknowledgments

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