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Photosynthetic physiology and growth as a function of colony size in the cyanobacterium *Nostoc sphaeroides*

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Algal size can affect the rate of metabolism and of growth. Different sized colonies of *Nostoc sphaeroides* were used with the aim of determining the effects of colony size on photosynthetic physiology and growth. Small colonies showed higher maximum photosynthetic rates per unit chlorophyll, higher light saturation point, and higher photosynthetic efficiency (α) than large colonies. Furthermore, small colonies had a higher affinity for DIC and higher DIC-saturated photosynthetic rates. In addition, small colonies showed higher photosynthetic rates from 5–45°C than large colonies. There was a greater decrease in *Fv*/*Fm* after exposure to high irradiance and less recovery in darkness for large colonies than for small colonies. Relative growth rate decreased with increasing colony size. Small colonies had less chl *a* and mass per unit surface area. The results indicate that small colonies can harvest light and acquire DIC more efficiently and have higher maximum photosynthetic rates and growth rates than large colonies.

Key words: colony size, DIC, growth rate, light, *Nostoc sphaeroides*, photoinhibition, photosynthesis, temperature

Introduction

Nostoc sphaeroides is an edible blue-green alga (cyanobacterium) found in water-filled paddies in some mountain areas of Hubei province in middle China (Li, 2000). *N. sphaeroides* reproduces by forming hormogonia or small colonies on the surface of large colonies (Li, 2000). From late autumn to early spring, local residents fill paddies with water, encouraging the growth of *N. sphaeroides*. Local residents also called it 'Tian-xian-mi' (the rice that comes from heavenly immortals) or 'Ge-xian-mi' according to some legends. *N. sphaeroides* has been used as food and a herb in China for more than 1600 years. Recent studies showed that *N. sphaeroides* has the potential to be a healthy food (Huang, 1997; Liu, 2000).

Nostoc sphaeroides can fix atmospheric nitrogen and may release the bound nitrogen and, like other *Nostoc* species (Dodds *et al.*, 1995), can increase nitrogen input to rice paddies. Desiccated *N. sphaeroides* can fully or partly recover metabolism upon rehydration; the time required for recovery is correlated with the length of the storage period. *N. sphaeroides* can also tolerate salt-stress (Li, 2000; Li & Gao, 2003). These properties make *N. sphaeroides* a good biofertilizer for rice paddies.

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The relationship between algal cell size and metabolic rate has been discussed extensively (Banse, 1976; Taquchi, 1976; Finkel & Irwin, 2000; Finkel, 2001; Gillooly *et al.*, 2001; Raven & Kübler, 2002). Small algae usually have a thinner diffusion boundary layer, a smaller 'package effect' and higher surface to volume ratios (Raven & Kübler, 2002). They consequently have higher metabolic rates with higher capacity of solutes influx/efflux and less self-shading. Small algae also have higher specific growth rates (Sunda & Huntsman, 1997; Raven, 1999). However, these studies were mainly restricted to unicellular algae or very small colony-forming planktonic algae (up to several millimeters).

Benthic colony-forming algae can be larger than planktonic algae (Raven & Kübler, 2002). In larger benthic colonies, diffusion boundary layers (DBL), which increase with increasing colony radius (Ploug *et al.*, 1999*a,b*), are thicker and may more severely decrease the nutrient uptake rate and retard the diffusion of CO_2 and O_2. In addition, more pigments occur per unit surface area in large colonies, so that the 'package effect' may be more severe in them than in the largest unicellular algae (Raven & Kübler, 2002). Steep gradients of O_2 concentration, photosynthetic rate and light were found in colonies of *N. parmelioides* (Dodds, 1989*b*). Photosynthesis- irradiance characteristics

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for cells at different depths in an algal colony may be varied. In some benthic algal communities, affinity for light (α) was lower while light saturation point (I_k) was higher for surface cells than for the interior cells (Dodds *et al*., 1999). For whole algal communities, light saturated photosynthetic rate and α (both expressed on a chlorophyll basis) decreased with increasing biomass and colony thickness (Enríquez *et al*. 1996; Dodds *et al*., 1999).

Nostoc sphaeroides can grow to several centimeters in diameter. For different sized colonies, there are different physical and physical-chemical properties. Besides light, dissolved inorganic carbon and temperature are key environmental factors. Their effects on the physiology and growth of *N. sphaeroides* may also vary with colony size, but have rarely been investigated. The aim of this study was to describe how photosynthetic and growth responses to light, dissolved inorganic carbon and temperature in colonies of *N. sphaeroides* changed with colony size. The results may provide useful data for the mass cultivation of *N. sphaeroides*.

Materials and methods

Nostoc sphaeroides was obtained from the FACHB-collection of the Institute of Hydrobiology, Chinese Academic of Sciences. Small colonies, about 0.03–0.04 mm in diameter, developed from induced hormogonia were used as inocula. The initial chlorophyll *a* concentration was about 2.8 μg per litre of culture medium. Specimens were cultivated in BG110 medium (Stanier *et al*., 1971) in a CO_2 chamber (E7, Conviron, Canada) at 25°C, with constant light (approximately 96 μmol m^{-2} s^{-1} from cool-white fluorescent tubes), and aerated. *N. sphaeroides*, with a regular spherical form, were used in the following experiments.

Photosynthetic oxygen evolution of different sized colonies of *N. sphaeroides* was measured at 25°C in a Clark-type oxygen electrode chamber (Rank Brothers, England), using a halogen light source, and irradiances up to 950 μmol m^{-2} s^{-1}. Irradiance was measured with a quantum sensor (LI-185B, Li-Cor Inc.). Oxygen evolution was measured for at least 5 min at each irradiance. Photosynthetic irradiance response (P-E) curves were plotted from $P = P_m \cdot tanh\ (\alpha \cdot I/P_m) + R_d$ (Jassby & Platt, 1976), where P = measured photosynthetic rate at irradiance E, P_m = maximal photosynthetic rate, α = slope of light-limited part of P-E curves, and R_d = dark respiration rate. P_m was obtained by non-linear curve fitting of the data to Jassby's formula using Microcal Origin (Version 5.0, Microcal Software Inc.). R_d and α were obtained from linear regressions of the irradiance-limited part of the P-E curves. The irradiance at the onset of the saturated photosynthesis (E_k) was calculated as the point where the maximal photosynthetic rate intercepts the initial slope (α) of P-E curve ($E_k = (P_m + R_d)/\alpha$). The light compensation point was calculated as: $E_c = -R_d/\alpha$.

Photosynthetic oxygen evolution as a function of dissolved inorganic carbon (DIC) was measured at 25°C and 650 μmol m^{-2} s^{-1}. DIC-free medium was obtained by aerating acidified medium (by adding HCl to the medium, pH < 5) with CO_2-free N_2 for about 1 h (Gao, 1999). pH was then adjusted to 8 with Bis-Tris Propane (Sigma). Fresh samples were used for each measurement. Different concentrations of DIC (0.01 to 1.2 mM) were obtained by adding $NaHCO_3$ to the DIC-free medium. Parameters for the photosynthetic responses to DIC were obtained by fitting photosynthetic rates at various DIC concentrations to the Michaelis-Menten formula: $v = V_{max} \cdot [S]/(K_{0.5}\ (DIC) + [S])$ using Microcal Origin, where v = photosynthetic rate, V_{max} = DIC saturated photosynthetic rate, $[S]$ = concentration of DIC, and $K_{0.5}\ (DIC)$ = DIC concentration where photosynthetic activity is the half of the maximal value. Photosynthetic rates of different sized colonies were also measured at various temperatures (5–50°C) under 650 μmol m^{-2} s^{-1}.

After each measurement, colonies were collected and stored at −10°C and analysed on the next day. Chl *a* was extracted with 100% methanol for 30 min at 60°C in the dark. The extinction coefficient used to estimate chl *a* concentration was 74.5 cm^{-1} mg^{-1} ml at 665 nm (Scherer & Zhong, 1991).

Two different sizes of colony (0.08 and 0.2 cm in diameter) were exposed to high irradiance (1300 μmol m^{-2} s^{-1}, 75 min) to investigate photoinhibition (indicated as the decrease in photochemical efficiency of photosystem II). Samples were placed in a beaker at a constant temperature of 25°C. The photochemical efficiency of *PSII* (*Fv*/*Fm*) was measured at intervals using a plant efficiency analyzer (PEA; Hansatech Instruments Ltd., England) after samples had been darkened for 15 min. Recovery from photoinhibition was followed in darkness at the same temperature.

Colonies of similar fresh weight (0.45 g) but different diameters were transferred to glass tubes containing 100 ml BG110 medium to measure growth. Relative growth rate (*RGR*, % day^{-1}) was calculated from: $RGR = (lnW2 - lnW1)/T \cdot 100$, where *W1* represents the initial fresh weight and *W2* is the fresh weight after T days (Gao & Nakahara, 1990).

Water content was calculated as: $Wc = (Fw - Dw)/Dw \cdot 100$, where Wc = water content, Fw = fresh weight and Dw = dry weight (85°C, 24 h).

Results

P-E curves of different sized colonies of *N. sphaeroides* showed no apparent photoinhibition at the highest experimental irradiance (950 μmol m^{-2} s^{-1}; Fig. 1), which was about nine times higher than the growth irradiance. When expressed on a chlorophyll basis, both P_m and α decreased significantly with increasing colony diameter (ANOVA, $p < 0.01$). On a dry weight basis, small colonies also had higher P_m, but there was no consistent change in α with colony size (Table 1). The differences in E_k, E_c and R_d among different

sized colonies were not significant. Photosynthetic responses to DIC concentrations in different sized colonies of *N. sphaeroides* are shown in Fig. 2. V_{max} decreased significantly with increasing colony size (ANOVA, $p < 0.001$). $K_{0.5}$ *(DIC)* increased significantly (ANOVA, $p < 0.01$) with increased colony sizes (Table 2), suggesting that large colonies had a lower affinity for DIC.

Photosynthetic rates of small colonies of *N. sphaeroides* at all temperatures were higher than those of large ones (Fig. 3). *N. sphaeroides* of different sizes all reached their highest photosynthetic activity at 35°C, and died at 50°C. The larger the colonies, the smaller the changes of photosynthetic rate with temperature. The ratios of net photosynthetic rates at 35°C to those at 5°C were 8.7, 5.6 and 3.6 for colonies with diameters of 0.08, 0.2 and 0.6 cm, respectively.

Obvious photoinhibition occurred in *N. sphaeroides* under 1300 μmol m^{-2} s^{-1} (Fig. 4). *Fv/Fm* values for *N. sphaeroides* decreased significantly (ANOVA, $p < 0.001$) after exposure to 1300 μmol m^{-2} s^{-1} for only 15 min. The effects of high irradiance were more marked in large colonies, which also showed slower and less complete recovery in darkness than small colonies. Recovery of *Fv/Fm* could be divided into a quick and a slow phase. The quick phase lasted for 90 and 120 min and the rate of recovery during this phase was 0.38% min^{-1} and 0.22% $min^{-1}$for colonies of 0.08 and 0.20 cm, respectively. The recovery of *Fv/Fm* was nearly complete after 4 h in darkness for colonies of 0.08 cm but, in larger colonies (0.2 cm), recovery had reached only 78% of initial values after 20 h in darkness.

The relative growth rate of colonies of *N. sphaeroides* decreased significantly with increasing colony diameters (Fig. 5; ANOVA, $p < 0.001$). Colonies 0.08 cm in diameter had an RGR 2.3 times greater than that of colonies 0.6 cm in diameter.

The chlorophyll *a* content (on both an area and a dry weight basis) of different sized colonies of *N. sphaeroides* increased significantly (ANOVA, $p < 0.001$) with increased colony diameter (Table 3). whereas the surface area to volume ratio decreased significantly (ANOVA, $p < 0.05$) Water content in colonies with diameter of 0.15 cm was

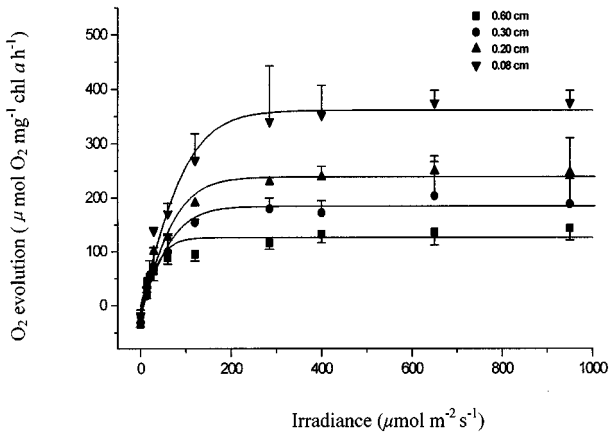

Fig. 1. Photosynthetic O_2 evolution as a function of irradiance for different sized colonies of *Nostoc sphaeroides* at 25°C and 189 μM dissolved inorganic carbon. Values are means ± SD ($n = 6$).

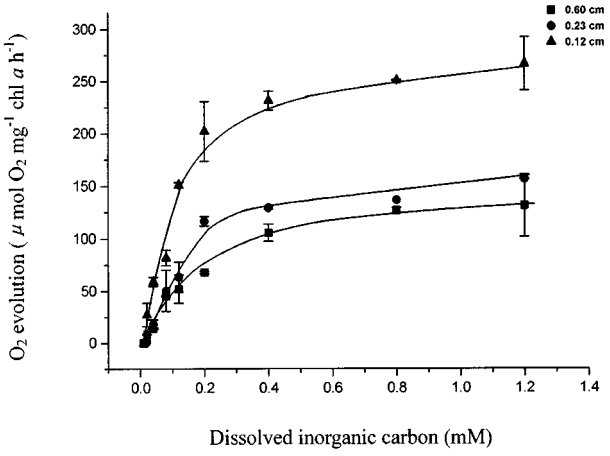

Fig. 2. Photosynthetic O_2 evolution as a function of dissolved inorganic carbon (DIC) for different sized colonies of *Nostoc sphaeroides* at 25°C and 650 μmol m^{-2} s^{-1}. Values are means ± SD ($n = 6$).

Table 1. Parameters of photosynthesis-irradiance curves for *Nostoc sphaeroides* colonies of different sizes

Colony size (Φ cm)	P_m (chl)	α (chl)	E_k	R_d	E_c	P_m (dw)	α (dw)
0.67	147.3 ± 9.5^{c}	3.4 ± 0.4^{c}	51.4 ± 7.8	23.8 ± 8.8	7.3 ± 3.5	1189 ± 76^{c}	27 ± 3^{ab}
0.30	189.4 ± 19.3^{c}	3.5 ± 0.8^{bc}	61.1 ± 9.9	20.3 ± 1.7	6.0 ± 1.5	1287 ± 131^{c}	24 ± 5^{b}
0.20	243.2 ± 22.1^{b}	5.0 ± 0.3^{ab}	56.6 ± 6.3	35.7 ± 8.5	7.1 ± 1.2	1737 ± 158^{b}	35 ± 2^{a}
0.08	374.1 ± 16.0^{a}	5.4 ± 0.1^{a}	73.0 ± 3.0	21.3 ± 6.6	4.0 ± 1.3	2101 ± 89^{a}	30 ± 1^{ab}

Within each column, values with different superscript letters are significantly different at $p = 0.05$ (Tukey's test). Values are means ± SD ($n = 6$) derived from P-E curves of Fig.1. Units: P_m (μmol O_2 mg^{-1} chl *a* h^{-1}) or (μmol O_2 g^{-1} dw h^{-1}); α (μmol O_2 mg^{-1} chl *a* h^{-1}) (μmol m^{-2} s^{-1})$^{-1}$ or (μmol O_2 g^{-1} dw h^{-1}) (μmol m^{-2} s^{-1})$^{-1}$; E_k and E_c (μmol m^{-2} s^{-1}); R_d (μmol O_2 mg^{-1} chl *a* h^{-1}).

Table 2. Parameters of the response of photosynthesis to dissolved inorganic carbon for *Nostoc sphaeroides* colonies of different sizes

Colony size (Φ,cm)	$K_{0.5}(DIC)$ (μM)	$K_{0.5}(CO_2)$ (μM)	V_{max} (μmol O_2 mg^{-1} chl *a* h^{-1})
0.60	260 ± 30^a	5.7 ± 0.7^a	165.0 ± 8.3^b
0.23	196 ± 51^b	4.3 ± 1.1^b	185.0 ± 16.5^b
0.12	147 ± 28^b	3.2 ± 0.6^b	306.6 ± 18.7^a

Within each column, values with different superscript letters are significantly different at $p = 0.05$ (Tukey's test). Values are means $\pm$ SD ($n = 6$) calculated from data in Fig. 2.

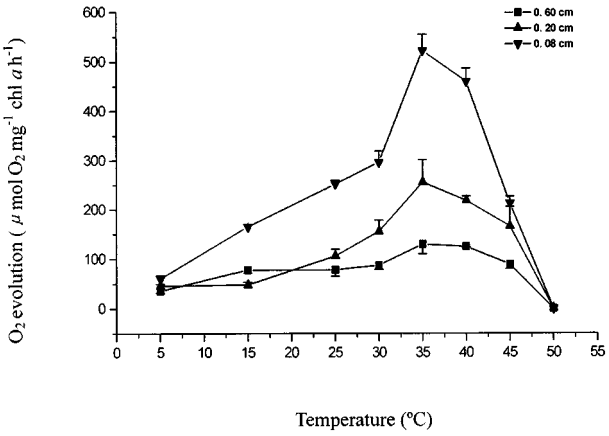

Fig. 3. Photosynthetic O_2 evolution as a function of temperature for different sized colonies of *Nostoc sphaeroides* at 650 μmol m^{-2} s^{-1} and 189 μM dissolved inorganic carbon. Values are means $\pm$ SD ($n = 9$).

significantly lower than that in larger colonies ($p < 0.05$, Tukey's test).

Discussion

The changes of P_m and α with colony size (Table 1) in this study are consistent with the results of Enríquez *et al.* (1996) and Dodds *et al.* (1999). Both of these papers reported a negative relationship between both P_m and α (on the basis of carbon/chlorophyll) with assemblage thickness/biomass (mg chl m^{-2}) in periphyton communities. Lower chl *a* contents per unit surface area in small colonies (Table 3) avoid packaging and self-shading among chl *a* molecules. This means that individual chl *a* molecules in small colonies have more chance of being excited by light energy at a given irradiance, and this can lead to a higher α (Taquchi, 1976; Enríquez *et al.*, 1994; Rodrigues *et al.*, 2000).

In this study, P-E measurements were carried out in BG110 medium without additional inorganic carbon. The DIC concentration was only 189 μM,

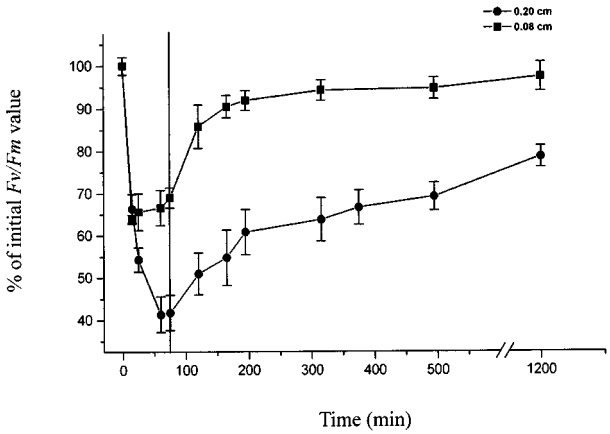

Fig. 4. *Fv*/*Fm* during and after exposure to 1300 μmol m^{-2} s^{-1} in different sized colonies of *Nostoc sphaeroides*. The initial *Fv*/*Fm* values were 0.49 and 0.50 for colonies with diameters of 0.08 and 0.20 cm, respectively. The vertical line indicates the beginning of dark recovery. Values are means $\pm$ SD ($n = 10$).

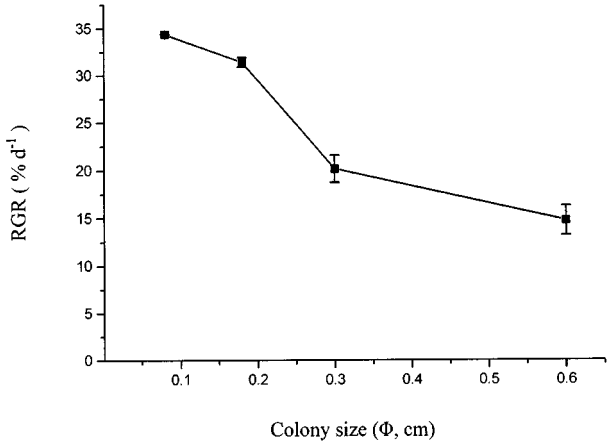

Fig. 5. Relative growth rate of different sized colonies of *Nostoc sphaeroides* after cultivation for 10 days at 25°C and 96 μmol m^{-2} s^{-1}. Values are means ($\pm$ SD) of three independent cultures.

which was unlikely to have been saturating for photosynthesis, since it was lower than the $K_{0.5}$ *(DIC)* for *N. sphaeroides* colonies with diameters of 0.23 (196 μM) and 0.60 cm (260 μM) and only slightly higher than that for 0.08 cm colonies (Table 2). Consequently, the P_m- values obtained (Table 1) were for light saturation, which are determined by carbon procurement (Taquchi, 1976; Henley, 1993; Lambers *et al.*, 1998).

Cyanobacteria can take up both CO_2 and HCO_3^- as their photosynthetic carbon source and have a CO_2 concentration mechanism (CCM; Salon *et al.*, 1996; Sültemeyer *et al.*, 1998). The active transport of inorganic carbon into the cell cytosol is regarded as an important component of a functional CCM, and this may lead to a higher affinity for inorganic carbon (Sültemeyer *et al.*,

Table 3. Chlorophyll *a*, water content and surface area/volume ratio (s/v) for *Nostoc sphaeroides* colonies of different sizes

Colony size (Φ,cm)	chl *a* (μg cm^{-2})	chl *a* [mg g^{-1}(dw)]	Water content (%)	s/v (cm^{-1})
0.67	13.9 ± 3.4^{a}	8.1 ± 1.7^{ab}	6500^{b}	8.96^{c}
0.45	11.4 ± 1.2^{a}	8.2 ± 0.9^{a}	7160^{ab}	13.3^{bc}
0.30	6.0 ± 0.3^{b}	6.8 ± 0.3^{ab}	7630^{a}	20.0^{bc}
0.23	5.8 ± 0.4^{b}	7.1 ± 0.5^{ab}	7000^{ab}	26.7^{ab}
0.15	5.0 ± 0.4^{b}	5.6 ± 0.4^{b}	2610^{c}	40.0^{a}

Within each column, values with different superscript letters are significantly different at $p = 0.05$ (Tukey's test). Chl *a* contents are means $\pm$ SD ($n = 3$); water content and s/v are means of two measurements.

1998). So anything that influences the diffusion and transport of DIC or enzymatic assimilation of DIC will also affect P_m and affinity for DIC. The higher surface to volume ratios of small colonies (Table 3) allow more cells to come into direct contact with the culture medium. Thinner sheaths, thinner diffusion boundary layers (DBL), and less slime result in fewer barriers for diffusion of DIC, O_2 and other nutrients in small colonies (Chang, 1980; Ploug *et al*., 1999*b*). Consequently, DIC and other nutrients can be supplied to surface and inner cells in small colonies more effectively under high light levels, and this may result in a higher P_m and higher affinity for DIC. In addition, the efflux of O_2 can be retarded by the sheath, slime and DBL (Prufert-Bebout *et al*., 1993; Ploug *et al*., 1999*a*; Potts, 2000), so that higher O_2 partial pressure may exist around the surface and interior cells in large colonies as a result of the greater resistance to O_2 diffusion, especially in high light environments. Higher O_2 concentrations compete with CO_2 for Rubisco and lower both P_m and the affinity for DIC in large colonies.

There was no evidence of photoinhibition at high irradiances (up to 950 μmol m^{-2} s^{-1}) in the P-E measurements (Fig. 1) but substantial inhibition of *Fv*/*Fm* was observed in *N. sphaeroides* during longer exposures to 1300 μmol m^{-2} s^{-1} (Fig. 4). Rodrigues *et al*. (2000) also reported that *Laminaria abyssalis* and *Laminaria digitata* did not show photoinhibiton at 1600 μmol m^{-2} s^{-1} in P-E measurements, but significant inhibition of photosynthetic O_2 evolution and *Fv*/*Fm* was observed after exposure to 1000 μmol m^{-2} s^{-1} for 15 min. Absence of photoinhibition in P-E measurements does not mean that photoinhibition does not occur (Henley, 1993; Hill, 1996): irradiances which are supersaturating for surface cells may be below saturation for deeper-layer cells, so that stimulation of deeper-layer cells may compensate for inhibition of surface cells (Hill, 1996; Dodds *et al*., 1999); photoinhibition is time-dependent, and the short exposures used for P-E measurements may be too short to observe photoinhibition; *Fv*/*Fm* is a more sensitive indicator of photoinhibition than P_m (Henley, 1993).

Both the increase in thermal dissipation of excess excitation energy and damage to *PSII* are responsible for the decrease of *Fv*/*Fm* under high light (Demmig-Adams & Adams, 1992). Photoinhibition induced by increase of thermal dissipation is regarded as a photoprotective reaction and usually decreases rapidly in the dark, but photoinhibition caused by photodamage requires more time for recovery because it involves the synthesis of new proteins. In large colonies, more severe photoinhibition and a less complete recovery indicated that more damage had been done to *PSII* of large colonies in high light. In addition, the higher P_m in small colonies of *N. sphaeroides* can dissipate more light energy and thus prevent the occurrence of photoinhibition (Henley, 1993; Lambers *et al*., 1998). Less photoinhibition in small colonies has important ecological meanings; it can protect them against the damage induced by high irradiance and maintain high photosynthesis under midday sun in the field.

Some species of *Nostoc* are able to endure extreme environmental temperatures (Dodds *et al*., 1995). One strain of *N. muscorum* can survive at 60 ± 5°C in hot springs (Greenwood & Steenbergen, 1976). Many species of *Nostoc* are the predominant biota in cold polar environments such as ice shelves, glaciers and ice-capped lakes (Vicent, 2000). This study shows that *N. sphaeroides* maintained net oxygen evolution from 5–45°C, but died at 50°C (Fig. 3). *N. sphaeroides* grows in the water-filled paddies in winter and is harvested in spring. During its growth, the environmental temperature is low and may be near or below 0°C, so that active photosynthesis at low temperatures is essential to achieve an increase in biomass. Light and the supply of CO_2 are two key factors that influence the slope of the temperature-response curve. In the experiments, photosynthesis was measured at saturating irradiances in BG110 medium, so that DIC supply was limiting. Thus the inefficient supply of CO_2 in large colonies as

discussed above is partly responsible for the low value for the initial slope.

Small colonies exhibited higher growth rates than large colonies, and similar trends were observed in marine phytoplankton (Sunda & Huntsman, 1997). *N. sphaeroides* was cultured at 96 μmol m^{-2} s^{-1}, which was above E_k, so that the diffusion of DIC and O_2 may play an important role in the growth of *N. sphaeroides*, as suggested for other *Nostoc* species (Dodds, 1989*a*). But colony size had no effect on the growth of *N. pruniforme* in the field (Dodds & Castenholz, 1987) and light seemed to be the most important factor. These measurements of the growth of *N. pruniforme* by Dodds & Castenholz (1987) were conducted at low temperatures (about 4°C) and low irradiances (mean values 0.11–0.31 J m^{-2} d^{-1}), and the growth rates ranged from 0.692–3.05 mg (g Fw)$^{-1}$ d^{-1}, compared with 148–343 mg (g Fw)$^{-1}$ d^{-1} for *N. sphaeroides* in our experiments.

In addition to its effects on the physiological responses and growth rate, colony size affects the storage of resources. Large colonies can contain more mucus, which are mainly composed of extrapolysaccharides (EPS). EPS include arabinose, galacturonic acid, ribose, xylose and glucose, and can be consumed as an energy source when there is no exogenous energy. Continued inorganic nitrogen uptake and protein synthesis in the dark, at the expense of mucus as a carbon source, was found in *Phaeocystis* colonies (Lancelot *et al.*, 1986). The storage of water is another important role of EPS (Potts, 2000) and water content was higher in larger colonies (Table 3). Furthermore, the sheath of cyanobacteria has been found to contain metal elements (Tease & Walker, 1987). We also found that the loss of water was slow in larger colonies (unpublished data). This suggests that large colonies can exist longer in some stress conditions.

Our results suggest that several parameters of the photosynthetic responses to light, inorganic carbon and temperature, as well as growth rate are functions of colony size in *N. sphaeroides*. Small colonies have higher affinities for light and DIC, and consequently higher photosynthetic rates and relative growth rates than large colonies. Small colonies have the advantage in utilizing limited environmental resources and restricting damage by high light. If we want more productivity in mass cultivation of *N. sphaeroides*, small colonies are preferred.

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