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Abstract A survey on the distribution of *Nostoc sphaeroides*, an edible cyanobacterium, growing in mountain paddy fields of Zouma Town in China was carried out in May 2012. The physical and chemical properties of paddy field soil and water were analyzed. We found that in a paddy field in which *N. sphaeroides* historically did not grow when the water was acidic (pH 6.30) and showed the lowest conductivity, and the soil contained the lowest content of total phosphorus (TP) and available phosphorus (AP) compared to other fields where *N. sphaeroides* did grow. The nitrogen (N) contents in both water and soil samples were sufficient compared with the high *N. sphaeroides* yield paddy fields. These results suggest that an acidic water environment, as well as low TP and especially low AP, was the main limiting factors for the distribution of *N. sphaeroides*. In addition, comparison of the biochemical composition of field-harvested, short-term-cultivated, and long-term-cultivated *N. sphaeroides* showed that the contents of photosynthetic pigments (chlorophyll *a* and carotenoids) and soluble protein including phycobiliproteins increased gradually from wild habitat to long-term indoor cultivation *N. sphaeroides*, while both total and soluble carbohydrates showed a decreasing trend during indoor cultivation.

Keywords Nitrogen · *Nostoc sphaeroides* · Cyanobacterium paddy field · Phosphorus · Photosynthetic pigment · Protein

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Introduction

Nostoc sphaeroides Kützing is an edible cyanobacterium (blue-green alga) which mainly grows in mountain paddy fields of Zouma Town, Hefeng County (Hubei Province) in China (Li 2000). *N. sphaeroides* is also named as Ge-Xian-Mi in Chinese and has been used as a traditional food and herbal medicine because of its nutraceutical and pharmacological benefits since the Eastern Jin Dynasty (317–420 AD) as recorded in the Chinese medicinal works *Compendium of Materia Medica* (Li 1596). It has been historically suggested that *N. sphaeroides* has medicinal qualities and could be used in the treatment of a variety of diseases including hypertension, inflammation, night blindness, burns, anxiety, and chronic fatigue (Qiu et al. 2002; Rasmussen et al. 2008).

N. sphaeroides is an N-fixing and filamentous cyanobacterium which can form macroscopic or microscopic colonies in both aquatic and terrestrial environments (Qiu et al. 2002). The success of *Nostoc* in terrestrial habitats is related mainly to its ability to remain desiccated for months or years and fully recover metabolic activity within hours to days after rehydration (Dodds et al. 1995). The ability in fixing atmospheric N_2 also may provide an advantage in N-limited ecosystems (Schindler 1974; Conley et al. 2009) and

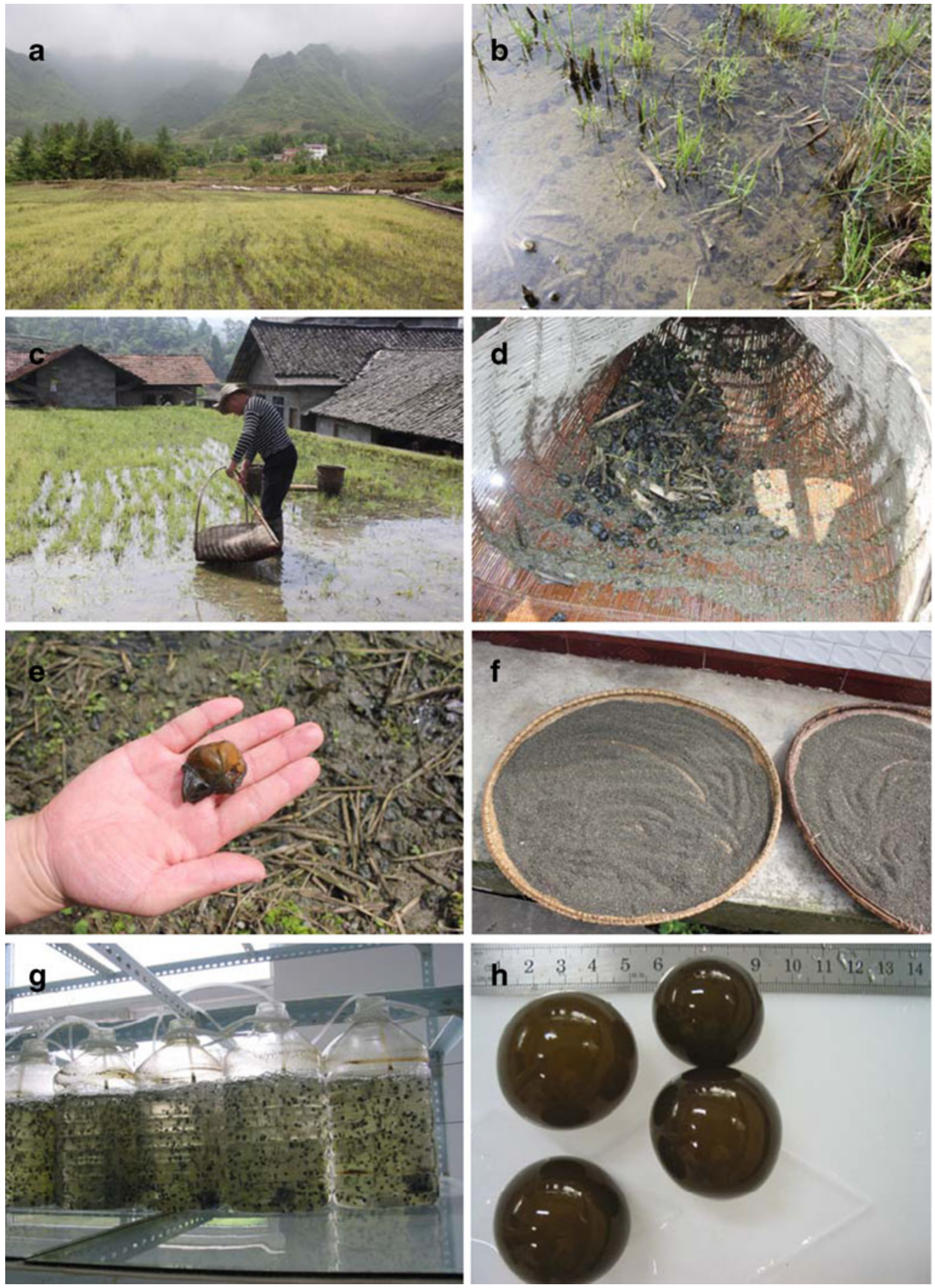

Fig. 1 The edible blue-green alga, *N. sphaeroides*, in wild habitat (Zouma town) and indoor cultivation (authors' laboratory). a Wild habitat. b Growing in paddy field. c Harvesting with "hapa" called by local residents. d Collected colonies in various sizes. e Schistic colony in wild habitat. f Air-dried on bamboo box. g Indoor cultivations. h Pearl-shaped colonies in lab

contribute significantly to soil fertility in many terrestrial systems, including paddy fields (Rother and Whitton 1989; Sinha and Häder 1996).

Mature colonies of lab-cultured *N. sphaeroides* are dark green and pearl shaped (Fig. 1g, h), and are developed from hormogonia with gas vesicles (Li 2000; Gao and Ai 2004). Macroscopic colonies form during winter in paddy fields in Zouma town (Fig. 1a–d), which are usually irrigated after the rice harvest in November (Qiu and Liu. 2004). *N. sphaeroides* can grow to several centimeters in diameter (Fig. 1e, f), and different sized colonies have different physiological and biochemical characteristics (Li and Gao 2004; Deng et al. 2008b).

Unfortunately, the habitats of *N. sphaeroides* have been radically damaged or altered as the result of human activities, such as changes in farming patterns, fertilizer abuse, and widespread use of herbicides and pesticides over recent decades in China and this has led to sharp reduction in the production of *N. sphaeroides* (Li 2000; Qiu et al. 2002; Liu 2004). Nowadays, *N. sphaeroides* has become rarer in most parts of China where it had been well-known years before.

However, its price keeps increasing up to about 3,000 RMB kg^{-1} dry weight currently. In recent years, local government has tried to restore the habitat and production in response to the heavily reduced resources, and also looked towards possible artificial indoor cultivation driven by market demand (Fig. 1g, h). However, the specific reasons for the reduced distribution and productivity are unclear and there are no effective methods in restoration. Differences have been found in taste and morphology between indoor cultivations and wild *N. sphaeroides* (Li 2000; Deng et al. 2008b); however, fewer studies on changes in biochemical composition of the cultured biomass have been reported for the indoor-cultivated periods.

In this study, a local survey was carried out by the authors in May 2012. Physical and chemical properties of water and soil were determined. Differences in biochemical composition were comparatively studied between lab-cultivated and field-harvested *N. sphaeroides*. Our aims were to determine the limiting factors for *N. sphaeroides* growth so as to provide theoretical and practical support for production–restoration of wild habitat, and to provide guiding information on later indoor *N. sphaeroides* cultivation.

Materials and methods

Zouma (29°49′N, 110°25′E), a mountainous town of Hefeng County, is located in the western parts of Hubei Province (Fig. 2) about 960 m above sea level. The annual mean temperature is about 12.2 °C, with a minimum of −14.5 °C in winter and a maximum of 35 °C in summer. The annual average duration of sunshine is 1,342 h, with frost-free period of 220 days. There are about 800 ha paddy fields suitable for *N. sphaeroides* growth (Liu 2004). Four sampling sites representing four different types of paddy fields were studied high: high-yield field (15 g dry-weight m^{-2}); low: low-yield field (less than 1 g dry-weight m^{-2}); ever: once grown but now not present; never: historically unsuitable for growth of *N. sphaeroides*.

Test organism and culture conditions

N. sphaeroides colonies were collected from the paddy fields of Zouma Town in December 2010, November 2011, and May 2012. Trichomes of *N. sphaeroides* for further cultivation were obtained from samples collected in December 2010 and November 2011, according to the method described by Deng et al. (2008a) with modifications. Cultures derived from the sample collected in 2011 were designated as the short-term indoor cultivation group, while those derived from the sample collected in 2010 was designated as the long-term indoor cultivation group.

After being rinsed with distilled water five times, the *N. sphaeroides* colonies were sterilized with 75 % alcohol for several seconds, and then rinsed again with distilled water. The sterilized *N. sphaeroides* colonies were ground using a sterilized glass homogenizer and the homogenate was centrifuged for 10 min at 7,200×*g* with a Hitachi High-Speed Refrigerated Centrifuge. Free trichomes were resuspended in 8 mL BG_{11} liquid medium (fixed nitrogen-free BG_{11} medium, Rippka et al. 1979) and transferred into 250-mL conical flasks with about 150 mL BG_{11} medium. The trichomes were grown under continuous illumination with low light intensity of 10 μmol photons s^{-1} m^{-2} photosynthetically active radiation (PAR) provided by 40-W Philips cool white fluorescent lamps at 25±1 °C.

The cultures were inoculated into 10-L serum bottles with 9 L BG_{11} growth medium when trichomes started to form macrocolonies ranging in size from 0.3 to 0.6 mm in diameter. Diameters of the colonies were measured with a scale ruler in minimum range to 0.1 mm. The cultures were aerated with filter-sterilized ambient air and subjected to continuous illumination with PAR of 50 μmol photons s^{-1} m^{-2} at 25±1 °C. The medium was renewed every ten days. *N. sphaeroides* colonies ranging in size from 3 to 4 mm in diameter were selected for the determination of parameters as described below.

Water sample collection and analysis

Two types of water samples, water in paddy field and water for irrigation, were collected between 9:00 am to 11:00 am on a sunny day in May 2012. The samples were stored in 500-mL polyethylene bottles at 4 °C, and analyzed as soon as possible. Water temperature (WT), dissolved oxygen (DO), and pH were determined from 9:00 am to 11:00 am in situ using a portable dissolved oxygen meter (YSI 550A, YSI Inc., USA) and a portable pH meter (YSI pH60). The conductivity was measured using an advanced conductivity meter (Orion 150A+, USA). Dissoluble total phosphate (DTP), orthophosphate (PO_4^{3-}), dissoluble total nitrogen (DTN), nitrate (NO_3^-–N), and ammonium (NH_4^+–N) were analyzed according to Chinese National Standard methods (4th ed.) for water quality analysis.

Soil sample collection and analysis

The paddy soil was collected from the surface layer (0–5 cm) and under layer (5–10 cm) of the four different types of paddy fields in May 2012. Samples were air-dried at room temperature to constant weight, and ground to powder with a mortar and pestle (Qin et al. 2013). The powder was sieved using an 80-mesh screen and kept in a dry place before determining the following parameters as described below (Ruban et al. 1999).

Powdered samples (0.5 g) were placed into screw-capped centrifuge tubes (50 mL) with 30 mL distilled water. Different forms of nitrogen (N) were extracted on a shaker at 150 rpm for 12 h. The mixture in the tubes was filtered with cellulose acetate membranes (0.45 μm pore sizes, 47 mm diameter). The filtrate was used for the determination of DTN, NO_3^-–N, nitrite (NO_2^-–N), and NH_4^+–N using the method described in Chinese National Standard methods (4th ed.) for water quality analysis. For phosphorus (P) determination, procedure of extraction, and measurement of total phosphorus (TP) and available phosphorus (AP), the method described by Bao (2000) was used. Three replicates were analyzed for each group.

Relative water content

Relative water content (RWC) was calculated according to the equation: RWC (%)=100 %×(W_f–W_d)/W_f, where W_f and W_d are the fresh and dry weight of *N. sphaeroides* colonies, respectively. Fresh weight (W_f) was determined using the method of Gao and Ai (2004). *N. sphaeroides* colonies were washed several times with distilled water and the water adhering to the surface of colonies was absorbed with tissue paper. Dry weight (W_d) was determined by freeze-drying the colonies to constant weight.

Photosynthetic pigments determination

Chlorophyll *a* (Chl *a*) and carotenoids were measured spectrophotometrically after extraction with 95 % ethanol in darkness for 24 h at 4 °C as described by Wintermans and de Mots (1965).

Phycobiliproteins (PBPs), including phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE), were determined by the method described by Myers and Kratz (1955). They were extracted in phosphate-buffered saline (PBS; 0.05 mol L^{-1}, pH 7.8) via sonication on ice. The absorbance of the extract was determined at 652, 615, and 562 nm.

Soluble and total carbohydrate measurement

For soluble carbohydrate (SC) measurement, *N. sphaeroides* colonies were suspended in distilled water and then extracted in boiling water for 30 min. The solution was cooled to room temperature and centrifuged for 10 min at (24,000×*g*). SC was determined by the enthrone sulfuric acid spectrophotometric method described by Brink et al. (1960). Total carbohydrate (TC) was extracted and measured by the method of Qin et al. (2012).

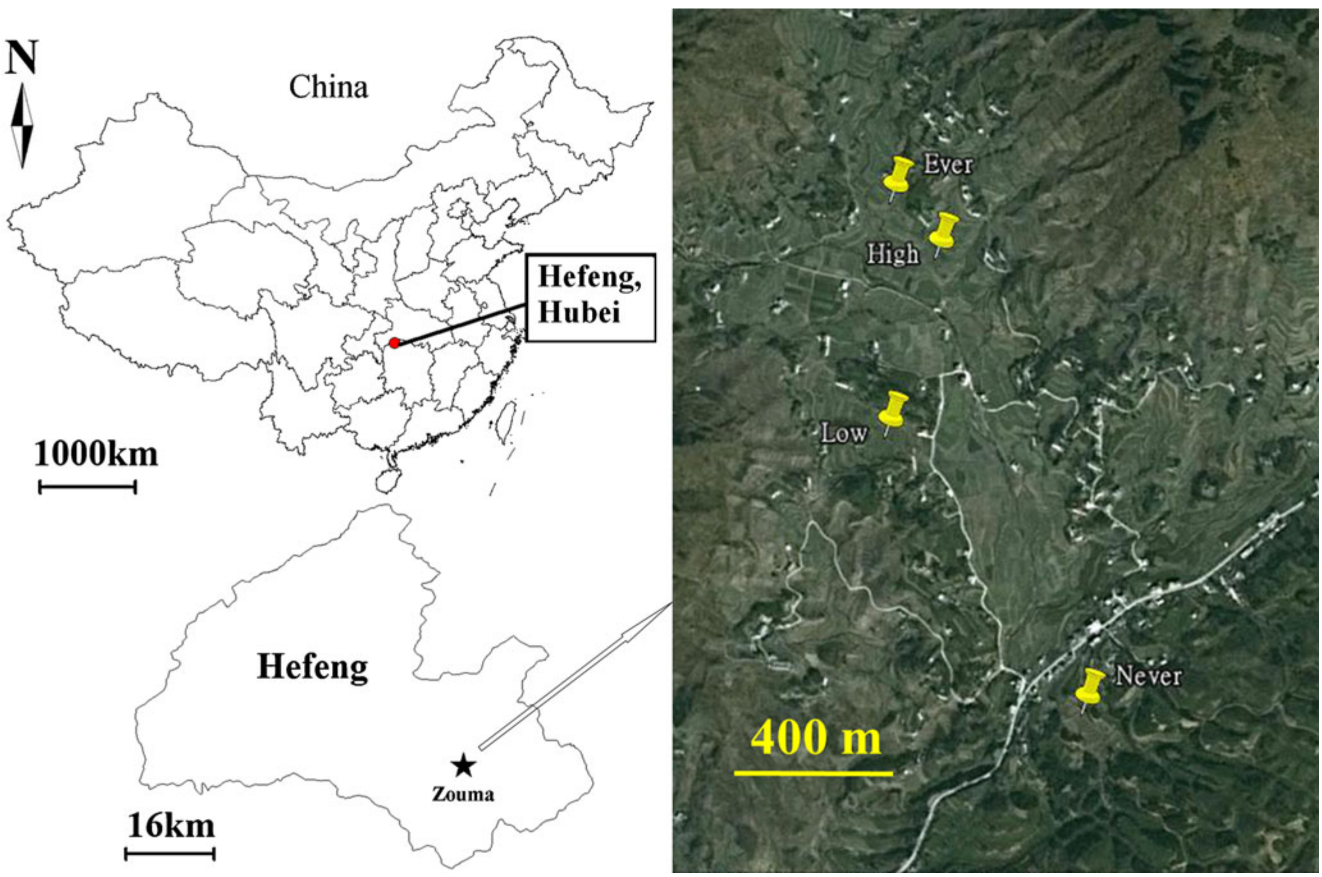

Fig. 2 The distribution of four sampling sites in the town of Zouma. *High* high-yield field, *Low* low-yield field, *Ever* once grown but not present, *Never* did not suit for growth historically

Table 1 Physicochemical parameters of both water in paddy field and water for irrigation harvested from four different paddy fields: high, low, ever, and never, respectively

	Samples	WT (°C)	pH	DO (mg L^{-1})	Conductivity (μS)
Paddy water	High	26.7	8.72	13.66	433
	Low	28.6	8.92	10.10	513
	Ever	28.2	8.57	11.86	437
	Never	26.3	6.30	4.74	126
Irrigation water	High	26.8	8.37	9.84	582
	Low	28.6	8.40	9.21	543
	Ever	28.2	8.35	9.86	563
	Never	26.2	7.63	7.39	258

Data are means±standard deviation (SD) with n=3. The levels of significant differences between high and the other three groups were indicated by *P<0.05 (LSD)

Total soluble protein measurement

N. sphaeroides colonies ranging from 3 to 4 mm in diameter were suspended in 0.05 mol L^{-1} PBS (pH 7.8) and then were broken via sonication on ice. The protein extract was centrifuged for 10 min at 10,000 rpm at 4 °C to collect the supernatant and was measured according to Bradford (1976).

Statistical analysis

Significant differences between high-yield field and other three types of fields were analyzed by performing one-way ANOVA followed by the least significant difference (LSD) post hoc test (SPSS, USA), at the 95 % confidence level.

Results

Physical and chemical properties of water samples

The physical and chemical properties of water samples taken from four different paddy fields and their sources for irrigation are shown in Table 1. The WT ranged from 26.2 to 28.6 °C. Obvious differences, such as lower pH, lower DO, as well as lower conductivity, were observed in the never group compared with the other three groups. No obvious differences were observed among high, low, and ever groups, but the high group had a higher DO value than the other two groups.

Group ever and never had higher DTN concentration in the paddy water than groups high and low. The DTN and NO_3^-–N concentrations in water for irrigation of groups ever and never were obviously higher than those of groups high and low, especially the concentrations of DTN (Fig. 3).

Concentrations of DTP and PO_4^{3-} in water collected from the paddy of groups low and ever were higher than those of the other two types of soil samples, and there were no obvious differences between the high and never groups. Both DTP and PO_4^{3-} concentrations of group never were the lowest among the four types of paddy-soil samples (Fig. 4).

Measurement of N and P of soil samples

As shown in Fig. 5, the DTN and NH_4^+–N contents of high-yield paddy field were significantly higher than other three types (P<0.05), especially the DTN content (720.9 μg g^{-1}dry

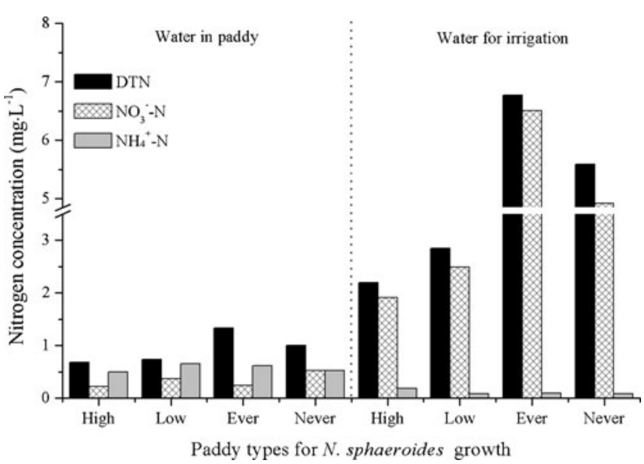

Fig. 3 The concentrations of DTN, NO_3^-–N, and NH_4^+–N of water in paddy field and water for irrigation collected from four different types of fields: high, low, ever, and never, respectively

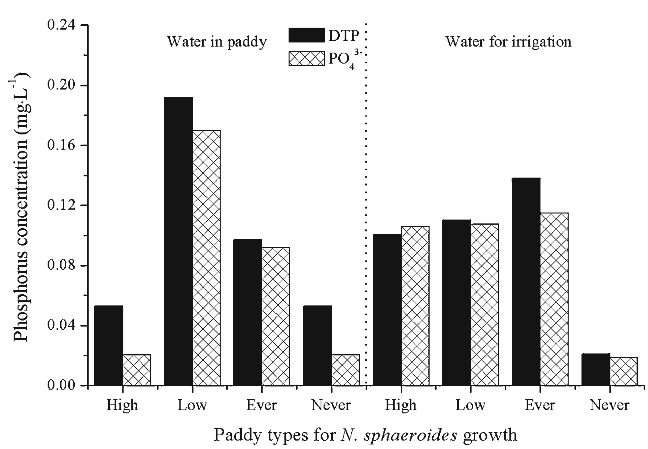

Fig. 4 The concentrations of DTP and PO_4^{3-} of both water in paddy field and water for irrigation collected from four different types of paddy fields: high, low, ever, and never, respectively

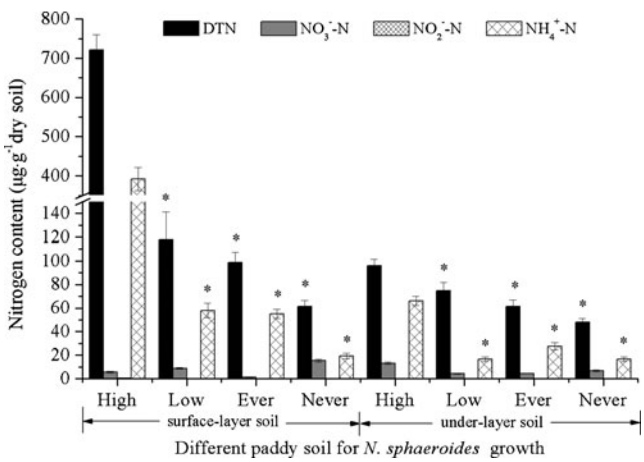

Fig. 5 The contents of DTN, NO_3^-–N, NO_2^-–N, and NH_4^+–N of both surface-layer and under-layer soil collected from four different types of fields: high, low, ever, and never, respectively. Data are means±standard deviation (SD) with n=3 (the same for Figs. 6 and 7). The levels of significant differences between high and the other three groups are indicated by *P<0.05 (LSD; the same for Fig. 6–7)

soil) in surface-layer soil, which was more than 10 times than that of the never group (61.4 μg g^{-1} dry soil).

Compared to the high-yield paddy group, low group and ever group had higher TP and lower AP contents of the surface-layer soil (P<0.05, Figs. 6 and 7), while the TP and AP contents in both surface-layer and under-layer soil of never group was obviously lower than the other soil samples (P<0.05, Figs. 6 and 7).

Changes in biochemical composition of the three sources of *N. sphaeroides*

The contents of PC, APC, and PE were all markedly increased in the short-term and long-term indoor cultivation

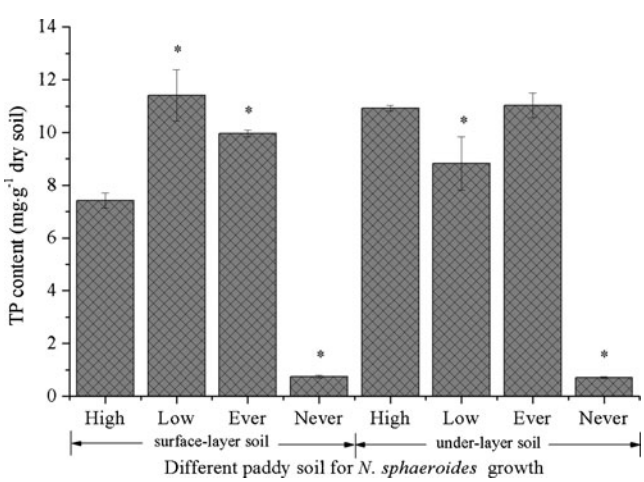

Fig. 6 The contents of TP of both surface-layer and under-layer soil collected from four different types of paddy fields: high, low, ever, and never, respectively

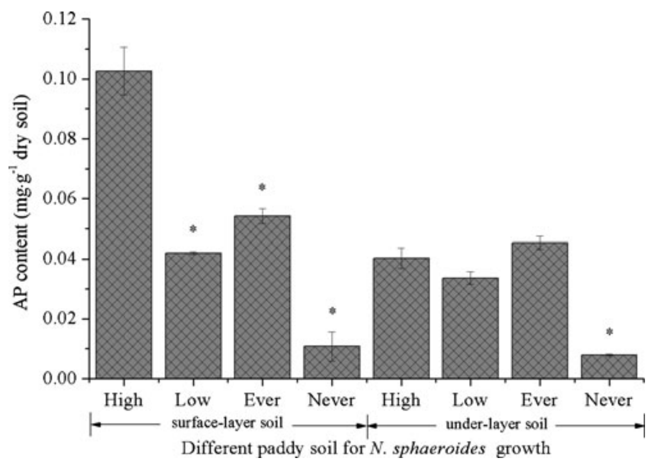

Fig. 7 The contents of AP of both surface-layer and under-layer soil collected from four different types of paddy fields: high, low, ever, and never, respectively

groups compared with the values of colonies harvested from paddy field (P<0.05, Table 2), especially APC contents of field colonies, which were 360 % and 450 % of the short-term and long-term indoor cultivation groups, respectively.

No significant differences in the level of RWC were observed among three different sources of *N. sphaeroides* (P>0.05). Both contents of chl *a* and carotenoids were significantly higher in the two indoor-cultivated colonies compared with the paddy field-harvested colonies (P<0.05). The content of total soluble protein also showed an increasing trend from paddy field *N. sphaeroides* to short-term cultivations and then to long-term cultivations, while an obviously decreasing trend was observed in both soluble and total carbohydrate (P<0.05, Table 2).

Discussion

It has been widely reported that alkaline conditions are beneficial for the growth of cyanobacteria (Imhoffa et al. 1979; Melack 1981; Li et al. 2006), although it has also been reported that the water suitable for *N. sphaeroides* growth is acidic (pH ranging from 6.2 to 6.3; Qiu et al. 2002). In this study, our local survey results carried out in May 2012 suggested that the acidic water condition (pH 6.30) is not suitable for the growth of *N. sphaeroides*. Liu et al. (2005) also found that alkaline water (pH 8.5) was the optimum condition for *Phormidium corium* growth, while the growth was inhibited if the pH was much lower or higher than pH 8.5. Low pH may be a key limiting factor for the distribution of *N. sphaeroides*. We found that the conductivity of the field in which there had been no *N. sphaeroides* growth historically was the lowest of the four types of paddy fields. This suggests that the water is lacking some nutrients required to support the growth of *N. sphaeroides*. Therefore, in order to restore the

Table 2 The differences of biochemical composition among *N. sphaeroides* harvested from wild paddy field, short-term indoor cultivation, and long-term indoor cultivation, respectively

Parameters	Paddy field	Short-term cultivation	Long-term cultivation
Relative water content (RWC; %)	98.78±0.10	98.35±0.31	98.71±0.39
PC (mg g^{-1} dry weight)	33.34±7.72	44.71±11.41*	60.76±14.93*
APC (mg g^{-1} dry weight)	40.79±10.38	149.69±13.81*	183.53±38.71*
PE (mg g^{-1} dry weight)	14.04±3.63	60.68±3.89*	73.60±15.19*
Chlorophyll *a* (mg g^{-1} dry weight)	3.85±0.19	8.70±0.80*	10.08±0.85*
Carotenoid (μg g^{-1} dry weight)	231.09±3.73	264.56±23.85*	295.40±20.66*
Total soluble protein (mg g^{-1} dry weight)	275.21±21.39	294.34±17.61	376.60±44.92*
Soluble carbohydrate (mg g^{-1} dry weight)	92.55±8.33	64.76±7.15*	52.03±7.24*
Total carbohydrate (mg g^{-1} dry weight)	443.12±55.42	412.74±21.01	336.97±11.64*

Data are means±standard deviation (SD) with n=3. The levels of significant differences between paddy field samples and the other two indoor-cultivated samples are indicated by *P<0.05 (LSD)

habitat and production of *N. sphaeroides*, nutritional supplements and pH adjustment may need to be considered.

Heterocyst development in cyanobacteria such as *Anabaena* and *Nostoc* can be repressed in the presence of high content of NH_4^+–N (Adams and Duggan 1999). High NH_4^+–N is usually caused by microbial decomposition in the surface-layer soil of high-yield field, which contains more microbes including bacteria, actinomyces, and fungi (Qiu et al. 2002). However, NH_4^+–N in paddy soil did not affect the water column condition at low NH_4^+–N content in this study. Our data suggest that all paddy water contained low NH_4^+–N, which should not have harmful effects on *N. sphaeroides*. In addition, higher contents of DTN and NO_3^-–N in the irrigation water of the fields where *N. sphaeroides* does not grow suggest that N is not the limiting factor for *N. sphaeroides* growth in the wild habitat.

N_2 fixation by cyanobacteria not only helps alleviate N shortages but also increases soil fertility in paddy fields (Schindler 1974; Sinha and Häder 1996). Belnap (2002) suggested that most of the N fixed by crust-forming organisms including *Nostoc* and *Scytonema* was released almost immediately to the surrounding soils. In this work, we found that *N. sphaeroides* significantly increased N contents in both surface (0–5 cm) and sub-surface (5–10 cm) soil samples of the paddy fields. The decomposition of *N. sphaeroides* colonies, especially under the anaerobic environment, is another important pathway for N input in paddy fields. The harmful influences of chlorine contained in many fertilizers on cyanobacteria also have been widely investigated (Whitton and Potts 2000). *N. sphaeroides* may be a pollution-free and environment-friendly substitute for N fertilizers whose reduced use has been proposed by local government.

It has also been suggested that P might play an important role in the growth and distribution of the *N. sphaeroides* in wild habitats (Mandal et al. 1993; Qiu et al. 2002; Chen et al. 2012), and P availability is an important factor controlling phytoplankton productivity and species composition in lakes (Cotner and Wetzel 1992). Available phosphorus (AP) is the soluble PO_4^{3-} in soil absorbed by seasonal plants (Bao 2000). The higher content of AP and lower content of TP in soil of high-yield field suggests that there might be a strong correlation between the distribution of *N. sphaeroides* and the AP content in the local paddy fields.

The supply of AP is considered deficient when the content of TP is lower than 0.44 mg g^{-1} dry soil (Bao 2000). In addition, TP content might be relatively high; however, algae and cyanobacteria can take up only AP in substantial amounts (Cotner and Wetzel 1992). Phosphatase plays a key role in organic matter mineralization (Zhou et al. 2001) and Sayler et al. (1986) found a positive correlation between the activity of alkaline phosphatase and the amount of bacteria in sediment harvested from ponds and streams. The local survey suggested that the soil where *N. sphaeroides* was present contained more bacteria than in rice fields of Hefeng County in China (Qiu et al. 2002). It also has been confirmed that bacteria play an important role in P release from sediments (Gacther and Meyer 1993; Pettersson 1998; Khoshmanesh et al. 2002). Recent reports suggested that the most part of polyphosphates extracted from sediment was bacterial polyphosphate which released under hypoxic conditions (Hupfer et al. 1995; Baldwin 1996). Based on these observations, we speculate that the amount of bacteria in paddy soil might be another important influencing factor on *N. sphaeroides* distribution.

Differences in biochemical composition between indoor cultivations and field products

In recent years, with the reducing resources and driving by economic interests, *N. sphaeroides* is gradually being cultivated (Li 2000; Deng et al. 2008b). The biochemical composition of *N. sphaeroides* changed gradually as the indoor-cultivated period increased. The PBPs all significantly increased under indoor-cultured condition where they were illuminated with continuous light at low PAR, compared with wild habitat. The different light conditions also affected the concentrations of chlorophyll *a* and carotenoids.

Protein synthesis in heterocystous cyanobacteria requires N which is fixed by the heterocysts (Conley et al. 2009; Narayan et al. 2011). Favorable indoor conditions such as constant temperature and adequate N_2 provision are conducive to N_2 fixation and this may be the main reason for *N. sphaeroides* containing more protein when grown indoors.

Carbohydrates, known to play a key role in colony formation (Hoagland et al. 1993), protect cyanobacteria from environmental stressors and these stressors also stimulate their biosynthesis. (Chen et al. 2003; Otero and Vincenzini 2003; Qin et al. 2012). The favorable indoor environment used in our cultures may therefore be the reason for the reduced carbohydrate synthesis indoors.

In conclusion, we suggest that low content of AP in paddy soil together with acidic water environment might be the two important factors limiting the distribution of *N. sphaeroides* in paddy fields. These observations provide theoretical and practical support for the restoration of wild habitats. The observed differences in the biochemical composition of the wild habitat and indoor cultivation of *N. sphaeroides* must also be considered in managing the quality of any later indoor cultures.

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