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REEVALUATION OF THE NITROGEN FIXATION BEHAVIOR IN THE MARINE NON-HETEROCYSTOUS CYANOBACTERIUM *LYNGBYA MAJUSCULA*[1]

Pernilla Lundgren, Karolina Bauer,[2] *Charles Lugomela,*[3] *Erik Söderbäck, and Birgitta Bergman*

Department of Botany, Stockholm University, S-10691 Stockholm, Sweden

***Lyngbya majuscula* Harvey ex Gomont is a common marine cyanobacterium in tropical and subtropical near-shore waters. A few reports have indicated that *L. majuscula* fixes nitrogen only in the light. Because this feature is uncommon among non-heterocystous cyanobacteria, we attempted a reevaluation. Nitrogenase activity, regulation, and localization were examined over diel cycles on natural populations of *L. majuscula* growing in subtidal zones off Zanzibar in the western Indian Ocean. The data show that *L. majuscula* fixed nitrogen and synthesized nitrogenase in all cells during the dark phase of a diel cycle. During the light phase, nitrogenase was degraded to undetectable levels.**

***Key index words:* cyanobacteria; Indian Ocean; *Lyngbya majuscula*; *nifH*; nitrogenase; Zanzibar**

Lyngbya majuscula Harvey ex Gomont is a common cyanobacterium in marine tropical and subtropical subtidal zones (Dennison et al. 1999, Hoffmann 1999, Lugomela et al. 2001). It often grows "attached" to substrates, which can be organic or inorganic. It forms fronds on sea grasses and macroalgae and goes under the nickname "mermaid hair." *Lyngbya majuscula* is non-heterocystous, and the cells are typically 30–60 μm wide and 2–4 μm long (Gomont 1892). The trichomes have a brownish pigmentation, and the fronds can be up to 40 cm long (Dennison et al. 1999). *Lyngbya majuscula* may cause "swimmers itch," possibly by its highly inflammatory and vesicatory toxin, lyngbyatoxin A (Cardellina et al. 1979).

Lyngbya majuscula has been reported to fix nitrogen in the light (Jones 1990, Dennison et al. 1999), whereas another study suggested that nitrogen fixation took place during both day and night (Jones 1992). *Lyngbya aestuarii* has also been reported to fix nitrogen in the light (Paerl et al. 1991). The enzyme responsible for nitrogen fixation, nitrogenase, is highly oxygen labile. Nitrogen fixation in the light is apparently a rare feature of non-heterocystous cyanobacteria and has only been convincingly characterized for two genera, *Trichodesmium* and *Katagnymene* (Berman-Frank et al. 2001, Lundgren et al. 2001). The gene encoding dinitrogenase reductase, *nifH*, has a 97.8% sequence similarity in these two cyanobacteria (Lundgren et al. 2001). Other cyanobacteria that do not differentiate heterocysts primarily fix nitrogen at night, for example, the unicellular genera *Gloeothece* (Gallon et al. 1988) and *Cyanothece* (Reddy et al. 1993), or fix nitrogen under microaerobic conditions in combination with a temporal separation of oxygenic photosynthesis and nitrogen fixation, for example, *Plectonema boryanum* (Rai et al. 1992).

This study was aimed at a reevaluation of the nitrogen fixation behavior of *L. majuscula*, because nitrogen fixation in the light is an uncommon behavior of non-heterocystous cyanobacteria. Nitrogenase activities, regulation, and localization were examined during diel cycles in natural populations. We also sequenced *nifH* to assess the phylogenetic relation between *L. majuscula* and other diazotrophs.

MATERIALS AND METHODS

Collection and identification of field samples. Samples of *L. majuscula* were collected from the subtidal zone off Changuu Island, Zanzibar. The method of Gomont (1892) was used for the morphological identification.

Nitrogenase activity. Acetylene reduction assays were carried out on *L. majuscula* either incubated *in situ* during the entire diel period or *in situ* during day and in the laboratory at night. Further, *L. majuscula* was collected in the dark (at 04:00) and light (at 14:00) and subjected to the following treatments: 1) oxic, light; 2) oxic, dark; 3) anoxic, light; 4) anoxic, dark; and 5) anoxic, light, with additions of DCMU and fructose. Fructose was added at 1 mM, DCMU at 10 μM, and anoxic conditions were provided by flushing the incubation vials with nitrogen for 1 min. Artificial light treatments were carried out in a water bath (26–29° C) with a photon flux density of 300 μmol photons·m^{-2}·s^{-1}. For acetylene reduction measurements, triplicate samples of *L. majuscula* were transferred to 10-mL serum vials containing 4 mL sterile filtered seawater. These were sealed, and 10% acetylene, generated from calcium carbide, was added to the gas phase. After 2 h, 4 mL of the gas phase was withdrawn and injected into a vacutainer (Becton Dickinson, Meylan, France). Subsequently, the ethylene content was analyzed by gas chromatography (Shimadzu GC-8A (Shimadzu, Kyoto, Japan), equipped with a Porapac N column), and the rates of ethylene produced were normalized to chl *a* content (Tandeau de Marsac and Houmard 1988). Background ethylene values in the acetylene gas were subtracted, that is, from controls with no cyanobacteria. Samples were taken every 2 or 3 h during a 24-h period for both the acetylene reduction assay. Four complete diel experiments were carried out.

Diurnal variation of nitrogenase. During the diel studies, pieces of *L. majuscula* were suspended in 150 μL of sample buffer (2% SDS, 2.5% 2-mercaptoethanol, 10% glycerol, 25 mM Tris-HCl pH 6.8, bromophenol blue and a protease inhibitor cocktail; Complete, Boehringer Mannheim, Mannheim, Germany) every 2 h. The samples were frozen and later thawed and heated at 95° C for 5 min before centrifugation at 15,800*g* for 5 min. Whole-cell extracts were applied to the wells of a 10% NuPAGE Bis-Tris gel

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[2]Author for correspondence: e-mail bauer@botan.su.se.

[3]Present address: Department of Zoology and Marine Biology, University of Dar es Salaam, PO Box 35064, Dar es Salaam, Tanzania.

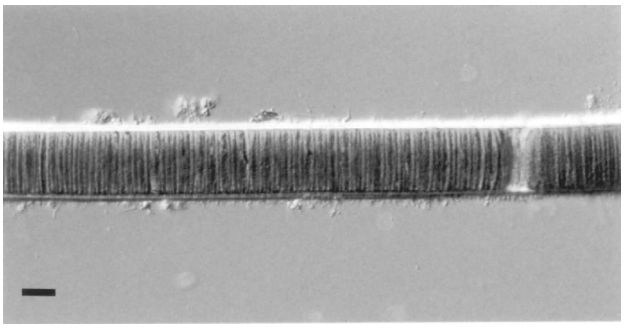

FIG. 1. Differential interference contrast micrograph of *Lyngbya majuscula* Harvey ex Gomont collected at Changuu Island, Tanzania. Bar, 20 μm.

(Novex, San Diego, CA, USA) and electrophoresed, using MOPS running buffer, for 50 min at a constant voltage of 200 V. Proteins were transferred by wet western blotting onto polyvinylidene difluoride membranes (Hybond-P, Amersham Pharmacia Biotech, Uppsala, Sweden) for 60 min at 30 V, using the Novex system with NuPAGE transfer buffer. Blotted membranes were blocked for 30 min in 5% skim milk in TBS (20 mM Tris-base, 0.14 M NaCl, pH adjusted to 7.6). Primary antibody (against dinitrogenase reductase from *Rhodospirillum rubrum*, raised in rabbit) was incubated for 1 h at a 1:10,000 dilution in TBS. After washing for 3 × 10 min in TBS, the membranes were incubated in the secondary antibody (goat anti-rabbit/horseradish peroxidase, Amersham Pharmacia Biotech) at a 1:20,000 dilution. After washing for 3 × 10 min in TBS, the immunodetected proteins were visualized using the ECL Plus detection system (Amersham Pharmacia Biotech). The occurrence of the dinitrogenase reductase protein was examined during three complete diel studies.

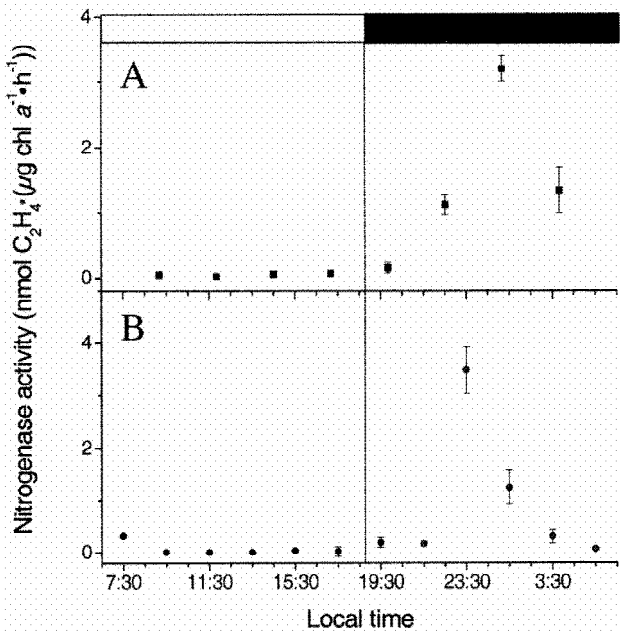

FIG. 2. Nitrogenase activity in *Lyngbya majuscula*. (A) Assay carried out under *in situ* conditions during the day and under controlled conditions in the laboratory at night. (B) Assays carried out *in situ* during the whole time period. Black and white bars represent dark and light periods, respectively. Error bars indicate SD from the mean (triplicate samples).

TABLE 1. Nitrogenase activities in *Lyngbya majuscula* when subjected to various treatments.

Incubation	Additions[a]	Activity	
		Day	Night
Control	−	0.0	**2.7 ± 0.8**
Oxic	−	0.0	0.03 ± 0.0
Oxic	−	**0.0**	**2.7 ± 0.8**
Anoxic	−	0.0	0.08 ± 0.04
Anoxic	−	**0.0**	**0.18 ± 0.1**
Anoxic	+	0.0	5.3 ± 0.8

Triplicate samples were incubated for 2 h at 14:00 and 04:00 during a natural 24-h light:dark cycle. Activities are expressed as nmol C_2H_4•μg chl a^{-1}•h^{-1} (± SE). Bold type indicates samples incubated in darkness; otherwise samples were incubated in the light.

[a]Additions of 10 μM (w/v) DCMU and 1 mM (w/v) fructose.

Intracellular immunolocalization of nitrogenase. Trichomes of *L. majuscula* were identified under a dissecting microscope, immobilized in 2% agar, fixed in 3% (w/v) paraformaldehyde for 30 min, and dehydrated through an ethanol series up to 70%. Subsequently, trichomes were further dehydrated to 100% ethanol, embedded in LR White, sectioned on an ultramicrotome (MT-7000 ULTRA, RMC, Tucson, AZ, USA), and collected on gold grids. The sections were blocked for 1 h with 10% BSA in PBS followed by incubation for 1 h in the primary anti-dinitrogenase reductase antibody (raised in rabbit, as above) diluted 1:100. The grids were washed in PBS (3 × 15 min) and then incubated for 1 h with the secondary antibody, goat anti-rabbit IgG (Amersham Pharmacia Biotech) conjugated to 10-nm gold particles, diluted 1:20 in PBS. After 2 × 15 min washes in PBS and 2 × 15 min in distilled water, the grids were air dried and examined with a microscope (model EM 906, Zeiss, Oberkochen, Germany) operating at 80 kV.

nifH *sequencing.* A single filament of *L. majuscula* was identified morphologically by LM, rinsed in sterile filtered seawater and distilled water, and placed in a PCR tube containing 10 μL of double-distilled H_2O. The sample was frozen, thawed, and used directly as template in the PCR reaction also containing 200 nM dNTP, 0.5 mM $MgCl_2$, Dynazyme reaction buffer, 0.5 U Dynazyme polymerase (Finnzymes Oy, Espoo, Finland), and 0.5 μM of each primer in a total reaction volume of 25 μL. The primers used (PN1: 5′-CGTCACGGTCAAAGAATCAT-3′ and PN2: 5′-ACACCACCAGCATGAGCATA-3′) amplified a 446-

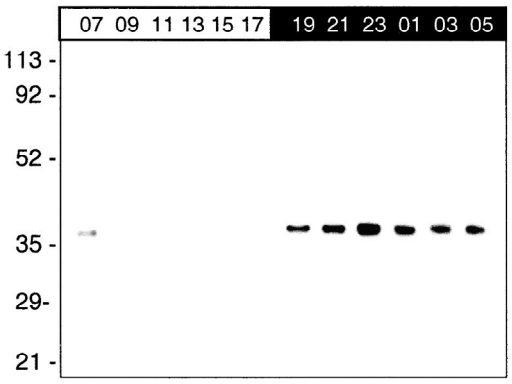

FIG. 3. Western blot analysis of dinitrogenase reductase in *Lyngbya majuscula*. The same amount of proteins was loaded to each lane. The estimated mass of the immunodetected band was about 40 kDa. Black and white bars represent dark and light periods, respectively. Results are typical of the three separate diel studies.

TABLE 2. Relative quantities of dinitrogenase reductase (gold particles/μm^2) in *Lyngbya majuscula* as revealed by immunogold TEM localization.

Sample	Label	
	Night	Day
Label ($n = 30$)	5.9	0.7
Background ($n = 30$)	0.2	0.4

Samples were collected at night (24:00) and day (14:30).

base pair (bp) fragment of *nifH* (coding for dinitrogenase reductase), including the 324-bp region described previously (Zehr and McReynolds 1989). PCR was performed with an initial denaturation step at 95° C for 4 min, 30 cycles of 1 min at 93, 50, and 72° C, and a last elongation step at 72° C for 4 min. The PCR product was separated on a 1.5 % agarose gel, cut, purified, and sequenced bidirectionally on an automated sequencer (ABI model 377, PE, Applied Biosystems, Stockholm, Sweden). The *L. majuscula nifH* sequence was aligned using CLUSTAL W 1.7 (Thompson et al. 1994) and deposited in GenBank under the accession number AY115593.

Phylogenetic analysis. The *nifH* nucleotide sequence from *L. majuscula* was subjected to a BLAST search (www.ncbi.nlm.nih.gov/blast; Altschul et al. 1997). Distances between pairs of sequences (324 bp) were calculated using the distance correction of Jukes and Cantor (1969), followed by the construction of a phylogenetic tree using the neighbor-joining method and bootstrap resampling with 500 replicates. This analysis was carried out using the TREECON software package (Van de Peer and de Wachter 1994). Sequences not obtained in this study were retrieved from GenBank under the following accession numbers: *Symploca (Microcoleus chthonoplastes)* PCC 8002 (AF013125), *Anabaena* PCC 7120 (V01482), *Trichodesmium erythraeum* (L00689), *Cyanothece* (AF003336), *Calothrix* (U73130), *Lyngbya lagerheimii* (L15550), *Phormidium* (AF227927), *Scytonema* (U73131), *Gloeothece* (L15554), and *Rhodospirillum rubrum* (M33774).

RESULTS

Collection and identification of field samples. Lyngbya majuscula was encountered off Changuu Island in the Zanzibar Channel of the western Indian Ocean. It grew in fronds, often associated with sea grasses. It was dark brown, and the cells were 35–50 μm wide and 2–4 μm long, and the non-heterocystous filaments were surrounded by a characteristic sheath (Fig. 1).

Nitrogenase activity. When examined during a 12:12-h light:dark cycle, nitrogenase activity was primarily restricted to the dark phase. Results from two separate diel studies are shown in Figure 2. One was incubated under *in situ* conditions during the light phase and in the laboratory during the dark phase (Fig. 2A), and the second was incubated under *in situ* conditions during the entire diel period (Fig. 2B). Nitrogenase activity developed rapidly after the light–dark transition, reached a maximum 4 to 8 h into the dark phase, and then declined. Activity reached zero after the dark–light transition and remained undetected throughout the light phase. This pattern was seen repeatedly in four separate diel studies. The highest activity observed was 3.5 nmol $C_2H_4 \cdot \mu g$ chl $a^{-1} \cdot h^{-1}$ (Fig. 2A).

Nitrogenase activities were also measured in *L. majuscula* subjected to various treatments during the dark and the light phase (Table 1). As opposed to

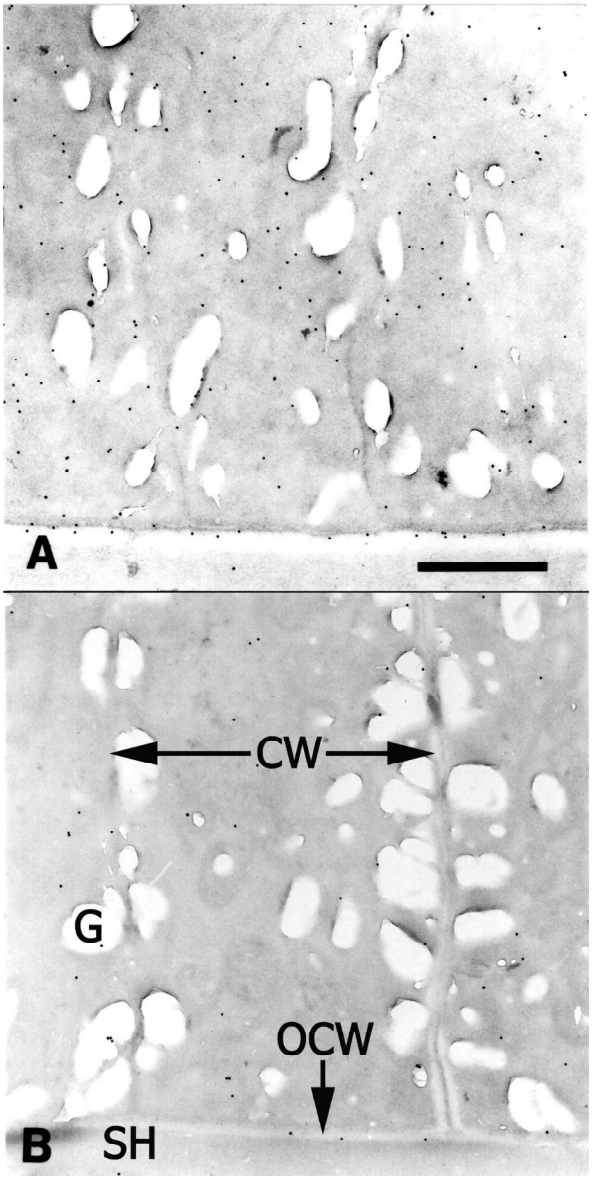

FIG. 4. Immunogold localization TEM of nitrogenase (Fe protein) in trichomes of *Lyngbya majuscula*. Trichomes collected at night (A; 24:00) or at daytime (B; 14:30). Each micrograph depicts a trichome running horizontally with portions of three adjacent cells, separated by vertically running cell walls (CW). The sheath (SH) is just below the outer trichome cell wall (OCW). The electron opaque areas (G) likely represent glycogen granules. The corresponding immunogold label intensity data are given in Table 2. Scale bar, 1 μm.

oxic conditions, samples incubated under anoxic conditions in the dark showed virtually no nitrogenase activity. Samples incubated in the light during the dark phase showed almost no activity. Enhanced nitrogenase activities were only observed during the natural dark phase when incubated anoxically in the light with additions of DCMU and fructose. This treatment resulted in an activity almost twice that of the control. Irrespective of treatments, samples incubated under

varying conditions during the light phase always lacked detectable nitrogenase activity (Table 1).

Diurnal variation of nitrogenase. Whole-cell extracts of *L. majuscula* were prepared every 2 h throughout a 24-h light:dark period. Western blot analysis revealed a single polypeptide of approximately 40 kDa. Nitrogenase (here referring to dinitrogenase reductase) was distinctly present during the dark phase from 18:30 to 06:30, barely detectable at 07:00, and was then absent for the rest of the light phase (Fig. 3). Similar results were obtained in three separate diel studies. No modification of the dinitrogenase reductase polypeptide (Du and Gallon 1993) was observed.

Intracellular immunolocalization of nitrogenase. Actively N_2-fixing *L. majuscula* trichomes collected at night (24:00) labeled positively for nitrogenase, and all cells in the trichomes contained nitrogenase. The label was found dispersed throughout cytoplasmic areas (Table 2, Fig. 4A). Trichomes collected during the light phase showed label intensities similar to background values (Table 2, Fig. 4B).

nifH *sequencing and analysis.* The *nifH* sequence most similar to that of *L. majuscula* was from *Trichodesmium* sp., showing a 93% sequence similarity. In the neighbor-joining tree inferred from *nifH* nucleotide sequences, *L. majuscula* was positioned in the same lineage as *Trichodesmium* sp. with 100% bootstrap support (Fig. 5). So far, only one additional *Lyngbya nifH* sequence is known, the one of *L. lagerheimii*, which had only a 70% similarity to that of *L. majuscula*. Further, the two different *Lyngbya* species were confined to different clades, and *L. lagerheimii* was positioned most ancestral of all the cyanobacterial *nifH* sequences included in the analysis (Fig. 5).

DISCUSSION

This study provides the first continuous 24-h measurements of nitrogenase activity in *L. majuscula* and demonstrates that nitrogen fixation was restricted to the dark phase under a 12:12-h light:dark cycle. It is apparent from our data that the lack of activity during the light phase is due to the absence of the nitrogenase enzyme during this period. Moreover, even when incubated in the light during the dark phase, nitrogenase activity was close to zero. Therefore, our data do not support those previously published for *L. majuscula*, which either suggested that nitrogenase activity only occurs in the light (Jones 1990, Dennison et al. 1999), or that fixation takes place during both day and night (Jones 1992). As Jones either measured diurnal and nocturnal activities on separate occasions (Jones 1992) or incubated the cells in either continuous 24-h light or darkness (Jones 1990), the experimental procedures may explain such findings. For instance, if the 24-h dark incubation started at the beginning of the subjected light phase, the cyanobacterium would not have been able to build up carbohydrate reserves necessary to sustain nitrogen fixation.

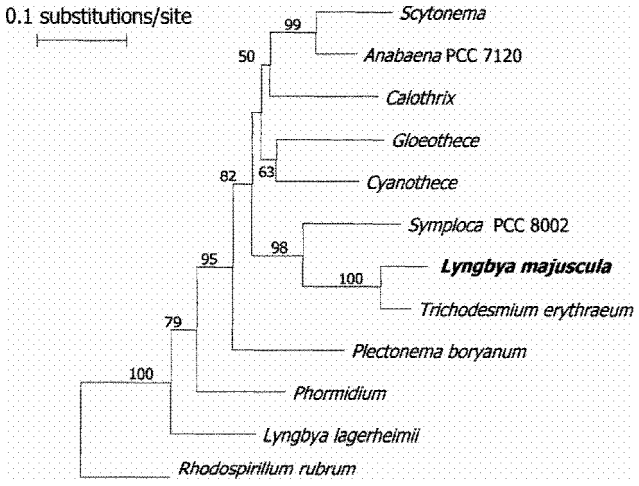

FIG. 5. Neighbor-joining phylogenetic tree based on cyanobacterial *nifH* nucleotide sequences (324 bp). Branch lengths correspond to nucleotide substitutions. Numbers show the percentage of times each branch was found in 500 replicates.

The only filamentous non-heterocystous cyanobacteria that so far consistently have shown aerobic nitrogen fixation during the light phase are members of the genera *Trichodesmium* and *Katagnymene*. Both *Trichodesmium* and *Katagnymene* sequester nitrogenase into subsets of cells (Janson et al. 1994, Lin et al. 1998, Lundgren et al. 2001) and retain nitrogenase throughout the night (Fredriksson and Bergman 1995, Lundgren et al. 2001). These are features clearly separating their adaptations from those described in the present study for *L. majuscula*.

Most other non-heterocystous cyanobacteria, both filamentous and unicellular, primarily fix nitrogen aerobically during the dark phase. These include *Oscillatoria*, *Gloeothece*, *Cyanothece*, and *Synechocystis* (Stal and Krumbein 1985, Huang and Chow 1986, Gallon et al. 1988, Reddy et al. 1993). For these cyanobacteria, like *L. majuscula*, nitrogenase is typically synthesized in all cells (Stal and Bergman 1990, Villbrandt et al. 1992, Bergman et al. 1997, Colón-López et al. 1997) and the nitrogenase enzyme is often degraded during the light phase (Du and Gallon 1993, Colón-López et al. 1997). Another similarity between *L. majuscula* and these non-heterocystous cyanobacteria is that nitrogenase activity is negatively affected by anoxic conditions during the dark phase (Griffiths et al. 1987, Bergman et al. 1997). This may be explained by a need for respiration to supply ATP and reductants to maintain nitrogenase activity in the dark (Gallon et al. 1988, Schneegurt et al. 1994). However, *L. majuscula* also exhibited some unexpected behavior. Unlike most other non-heterocystous cyanobacteria, nitrogenase activity during the dark phase was inhibited by light under both oxic and anoxic conditions. However, when DCMU and fructose were added under anoxic conditions in the light (during the dark phase) this inhibition was circumvented, and the rates were even higher than those of the dark control.

Though more experiments are needed to fully understand the regulation of nitrogenase activity in *L.*

majuscula, it is apparent from our study that nitrogenase is fueled by O_2-dependent respiration and that the rate of N_2 fixation can be stimulated by light in a PSII-independent fashion under certain conditions.

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