

RESEARCH ARTICLE

# Levels of Daily Light Doses Under Changed Day-Night Cycles Regulate Temporal Segregation of Photosynthesis and N<sub>2</sub> Fixation in the Cyanobacterium *Trichodesmium erythraeum* IMS101

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## Abstract

While the diazotrophic cyanobacterium *Trichodesmium* is known to display inverse diurnal performances of photosynthesis and N<sub>2</sub> fixation, such a phenomenon has not been well documented under different day-night (L-D) cycles and different levels of light dose exposed to the cells. Here, we show differences in growth, N<sub>2</sub> fixation and photosynthetic carbon fixation as well as photochemical performances of *Trichodesmium* IMS101 grown under 12L:12D, 8L:16D and 16L:8D L-D cycles at 70 μmol photons m<sup>-2</sup> s<sup>-1</sup> PAR (LL) and 350 μmol photons m<sup>-2</sup> s<sup>-1</sup> PAR (HL). The specific growth rate was the highest under LL and the lowest under HL under 16L:8D, and it increased under LL and decreased under HL with increased levels of daytime light doses exposed under the different light regimes, respectively. N<sub>2</sub> fixation and photosynthetic carbon fixation were affected differentially by changes in the day-night regimes, with the former increasing directly under LL with increased daytime light doses and decreased under HL over growth-saturating light levels. Temporal segregation of N<sub>2</sub> fixation from photosynthetic carbon fixation was evidenced under all day-night regimes, showing a time lag between the peak in N<sub>2</sub> fixation and dip in carbon fixation. Elongation of light period led to higher N<sub>2</sub> fixation rate under LL than under HL, while shortening the light exposure to 8 h delayed the N<sub>2</sub> fixation peaking time (at the end of light period) and extended it to night period. Photosynthetic carbon fixation rates and transfer of light photons were always higher under HL than LL, regardless of the day-night cycles. Conclusively, diel performance of N<sub>2</sub> fixation possesses functional plasticity, which was regulated by levels of light energy supplies either via changing light levels or length of light exposure.

## Introduction

*Trichodesmium*. spp are the most abundant diazotrophic cyanobacteria [1] in oligotrophic tropical and subtropical oceans [2]. They provide biologically available nitrogen sources and

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often form large extensive blooms. Current estimates of marine nitrogen fixation ranging from 100 to 200 Tg ( $1-2 \times 10^{14}$  g)  $\text{yr}^{-1}$ , with *Trichodesmium* contributing about half of the total [3].

Diazotrophs have developed specific molecular and physiological strategies to protect nitrogenase from  $\text{O}_2$  evolved during photosynthesis [4–9] since nitrogenase is extremely sensitive to  $\text{O}_2$ . While some unicellular diazotrophic cyanobacteria fix  $\text{N}_2$  at night to avoid photosynthetic oxygen inhibition of the nitrogenase complex (temporal separation), many filamentous diazotrophic cyanobacteria develop specialized  $\text{N}_2$ -fixing cells, heterocysts, with thickened cell walls, which do not evolve  $\text{O}_2$  [10, 11]. However, the non-heterocystous *Trichodesmium*. spp allow photosynthesis and  $\text{N}_2$  fixation to proceed simultaneously during the daytime in the same trichome, and the mechanisms involved are intriguing and controversial [12].  $\text{N}_2$  fixation and photosynthesis in *Trichodesmium* may be controlled by a circadian rhythm since it possesses the “clock” genes (kaiABC) [13, 14]. *Trichodesmium thiebautii* shows circadian patterns of  $\text{N}_2$  fixation in parallel with the transcription of *nifH* [15]. The changes in nitrogenase activity in *Trichodesmium* can reflect light-dependent activation and deactivation of the Fe protein [16]. Moreover, respiration, photosynthetic  $\text{O}_2$  evolution and nitrogen fixation all show some correlated diurnal variations with a peak in nitrogen fixation at midday corresponding to a dip in photosynthetic  $\text{O}_2$  evolution [17]. However, little is known about the regulation of photosynthetic carbon fixation and nitrogen fixation activity upon exposing *Trichodesmium* to different light-dark regimes.

Since  $\text{N}_2$  fixation requires energy derived via photosynthesis but evolved  $\text{O}_2$  inhibits it in *Trichodesmium*, we hypothesize that both light intensity and length of the light period (light dose) would affect its diurnal patterns and levels of  $\text{N}_2$  fixation. We therefore investigated how  $\text{N}_2$  fixation and photosynthetic carbon fixation in the marine non-heterocystous cyanobacterium *Trichodesmium erythraeum* IMS101 respond to changes in light-dark cycles under different light levels. We report here that the activity of  $\text{N}_2$  fixation oscillated diurnally with differential peaking time under different light regimes, which was the latest for the shortest light period and contrary to that of photosynthetic carbon fixation.

## Materials and Methods

### Culture condition and experimental set-up

*Trichodesmium erythraeum* IMS101, originally isolated from the North Atlantic Ocean, were grown in 1 L glass flasks (500–800 ml cultures) in YBC-II medium without combined nitrogen source [18]. The culture was maintained in a plant growth chamber (GXZ, Ningbo, China) at 25°C under  $70 \pm 5$  (LL) and  $350 \pm 19$  (HL)  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  PAR (photosynthetically active radiation, 400–700 nm), representing sub-saturating and super-saturating values for *Trichodesmium* according to [19]. The light was supplied by white fluorescent tubes (Philips) within the chamber. These two different light levels were achieved by using neutral density screen, and were measured using a LI-COR  $2\pi$  PAR sensor (PMA2100, Solar light, USA). Three independent cultures under each light level and each light regime were run and used to measure the growth rate and all subsequent physiological parameters.

The cultures were initially run with a 12L:12D (Light: Dark) cycle under the two light levels for 60 generations (more than 180 days) before being shifted to 8L:16D or 16L:8D regime. Under each light regime, subsequently, the cultures were run in triplicates and semi-continuously diluted every 4–5 days to achieve steady-state exponential growth for 50–55 days (10–40 generations) prior to the measurements of growth,  $\text{N}_2$  fixation and photosynthetic parameters. The measurements of  $\text{N}_2$  and carbon fixation were carried out in 2 h intervals during the light period and at the onset of the dark period.

Biomass of the cultures was estimated by measuring chlorophyll *a* concentration. Even though the chl *a* content per cell would be different under different regimes or different light levels, the content was shown to be constant after the cells had been acclimated to different light levels in semi-continuously diluted cultures of the same strain [19]. Therefore, the growth rate under each light regime was estimated as  $\mu = (\ln C_2 - \ln C_1) / (t_2 - t_1)$ , where  $C_1$  and  $C_2$  represent the chl *a* concentrations per ml at time  $t_1$  (after dilution) and  $t_2$  (before the next dilution), respectively. Chl *a* contents were determined by filtering samples onto glass-fiber filters (GF/F, Whatman) and then extracted in 4 ml 100% methanol at 4°C overnight and quantified from the absorption spectra (400–700 nm) of the supernatants with a spectrophotometer (DU800, Beckman, USA). The chl *a* concentration in the supernatant was estimated according to the equation:  $[\text{Chl } a] (\mu\text{g mL}^{-1}) = 12.94 \times (A_{665} - A_{750})$  [20].

## N<sub>2</sub> fixation

Rate of N<sub>2</sub> fixation were measured in sequence with 2 h intervals using the acetylene reduction assay (ARA) [21]. Samples of 5 ml subculture were placed in 13 ml glass vials. Gas-tight syringes were used to inject 1 ml acetylene into the headspace. The vials were incubated for 1 h under the growth conditions with continual shaking, a 500  $\mu\text{L}$  headspace sample was then analyzed in a gas chromatograph equipped with a flame-ionization detector and quantified relative to an ethylene standard. The ethylene produced was calculated using the Bunsen gas solubility coefficients according to [22], and the ethylene production to N<sub>2</sub> fixation conversion factor of 4 was used to derive N<sub>2</sub> fixation rates, which were normalized to chl *a*.

## Carbon fixation

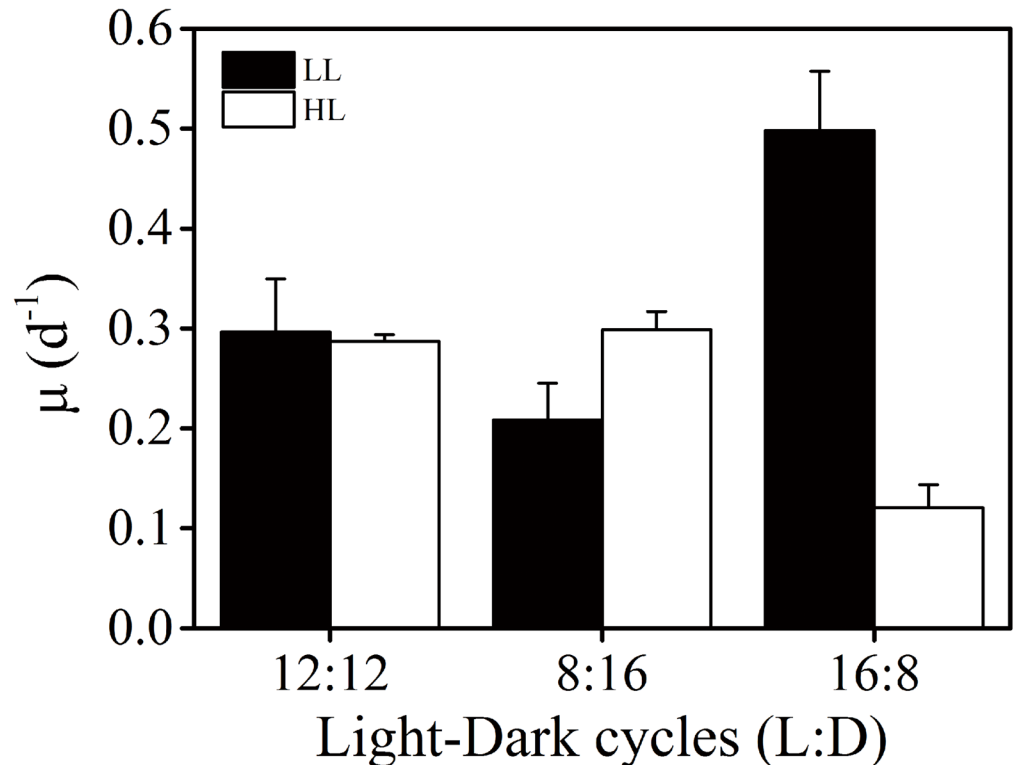
Subsamples of 20 ml were taken every 2 h to measure the carbon fixation rate in parallel with the N<sub>2</sub> fixation measurements. The incubation (1 h) was initiated by inoculating 100  $\mu\text{L}$  (5  $\mu\text{Ci}$ ) of  $\text{NaH}^{14}\text{CO}_3$  (ICN Radiochemicals, Irvine, California, USA) and maintained under the growth conditions. The cells were collected onto Whatman GF/F glass fiber filters ( $\Phi$  25 mm) and stored at -20°C until analysis. To determine the radioactivity, the filters were exposed to HCl fumes overnight and dried at 60°C to get rid of non-assimilated C<sup>14</sup> before being digested in scintillation cocktail (Hisafe 3, Perkin-Elmer, Shelton, CT, USA), and measured with a scintillation counter (Tri-Carb 2800TR, Perkin-Elmer, Shelton, CT, USA), as previously described [23].

## Fluorescence

Fluorescence parameters was measured every 2 h using a Fluorescence Induction and Relaxation Fluorometer (FIRe, Satlantic, Halifax, Canada) with a green (530 nm with 30 nm bandwidth) excitation light. A saturating ( $5 \times 10^4 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ), single turnover flash (120  $\mu\text{s}$ ) was applied and the actinic light was set at the growth light level. The quantum yield of PSII was calculated as  $\Phi\text{PSII} = (F_M' - F_S) / F_M'$ , where the  $F_S$  and  $F_M'$  were the steady state and maximum chlorophyll fluorescence measured under the light, respectively. In the dark period, the quantum yield was measured without actinic light. The quantum yield was estimated as  $F_V / F_M = (F_M - F_O) / F_M$ , where the  $F_O$  and  $F_M$  were the minimal and maximum chlorophyll fluorescence measured in the darkness. The relative ETR (rETR) was calculated as  $r\text{ETR} = \Phi\text{PSII} \times \text{Growth PAR}$ .

## Statistics

Triplicate cultures were operated under each light level and/or each day-night cycles, and all of the data were obtained with 3 replication from the triplicate cultures. The data were expressed



**Fig 1. Specific growth rate of *Trichodesmium* IMS101.** Growth rates of *Trichodesmium* IMS101 of LL (70  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , dark column) and HL (350  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , open column) cultures grown under different Light Dark (LD) regimes. Values represent mean  $\pm$  SD of triplicate cultures under each regime.

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as mean value  $\pm$  SD for three independent cultures. The daily fixed amount of carbon was derived by integrating the carbon fixation rate during the light period under the different L-D regimes and light levels, while the daily fixed amount of  $\text{N}_2$  was derived by integrating the  $\text{N}_2$  fixation rate during the light period and at the onset of the dark period. Daily light dose was calculated as  $I_{\text{eq}} \times \text{daytime length (s)}$ . One-way ANOVA and Tukey test were used to establish differences among the treatments at a significance level of  $p < 0.05$ .

## Results

### Growth

Under the light regime of 12L:12D, growth rate of *Trichodesmium* IMS101 did not show significant difference between 70 (LL) and 350 (HL)  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  ( $p = 0.83$ ) (Fig 1). After the LL and HL-grown cells had been transferred to 8L:16D regime for 50 days, the growth rate of LL-grown cells was significantly reduced by 30% ( $P = 0.02$ ) compared to that of 12L:12D regime, while that of the HL was not affected ( $P = 0.94$ ). When the light period was extended to 16 h (dark period to 8 h), the growth rate significantly increased in the LL-grown cells by 68% ( $P = 0.006$ ), but was significantly reduced by 57% in the HL-grown ones ( $P = 0.001$ ), compared to that in the 12L:12D regime (Fig 1).

### $\text{N}_2$ fixation

After the cells had been grown under the regimes of 8L:16D and 16L:8D, respectively, for 50 days, the  $\text{N}_2$  fixation rates showed distinct variations during the light period under both LL and

HL conditions (Fig 2). The LL- and HL- grown cells showed similar diurnal patterns of N<sub>2</sub> fixation (Fig 2). Under the 12L:12D regime, cells began to fix N<sub>2</sub> shortly after the onset of the light with the rate peaked around 4.5 h into the light period, and then decreased gradually to zero at the beginning of the dark period (Fig 2A). Shortening the light period to 8 h, the peak of N<sub>2</sub> fixation was reached later during the day (Fig 2B), with the N<sub>2</sub> fixation peaked at the end of light period and extended to dark period (1 h after darkness onset). In addition, the maximal N<sub>2</sub> fixation rates were substantially higher by 50% ( $p = 0.006$ ) under LL and by 28% ( $p = 0.08$ ) under HL compared to that of 12L:12D cultures, respectively (Table 1). Nevertheless, the total daily N<sub>2</sub> fixation was lower than that in 12L:12D cultures. In contrast, when the light period was extended to 16 h (16L:8D), the N<sub>2</sub> fixation peaked at the same time (4.5 h into the light period) as that in 12L:12D (Fig 2C). However, elongation of the light period led to 3.8 times higher maximal N<sub>2</sub> fixation rates in the LL grown cells than that in the HL grown ones (Fig 2C), the amount of N<sub>2</sub> fixed in 24 h of LL grown cells were 5 times higher than HL cells (Table 1).

### Carbon fixation

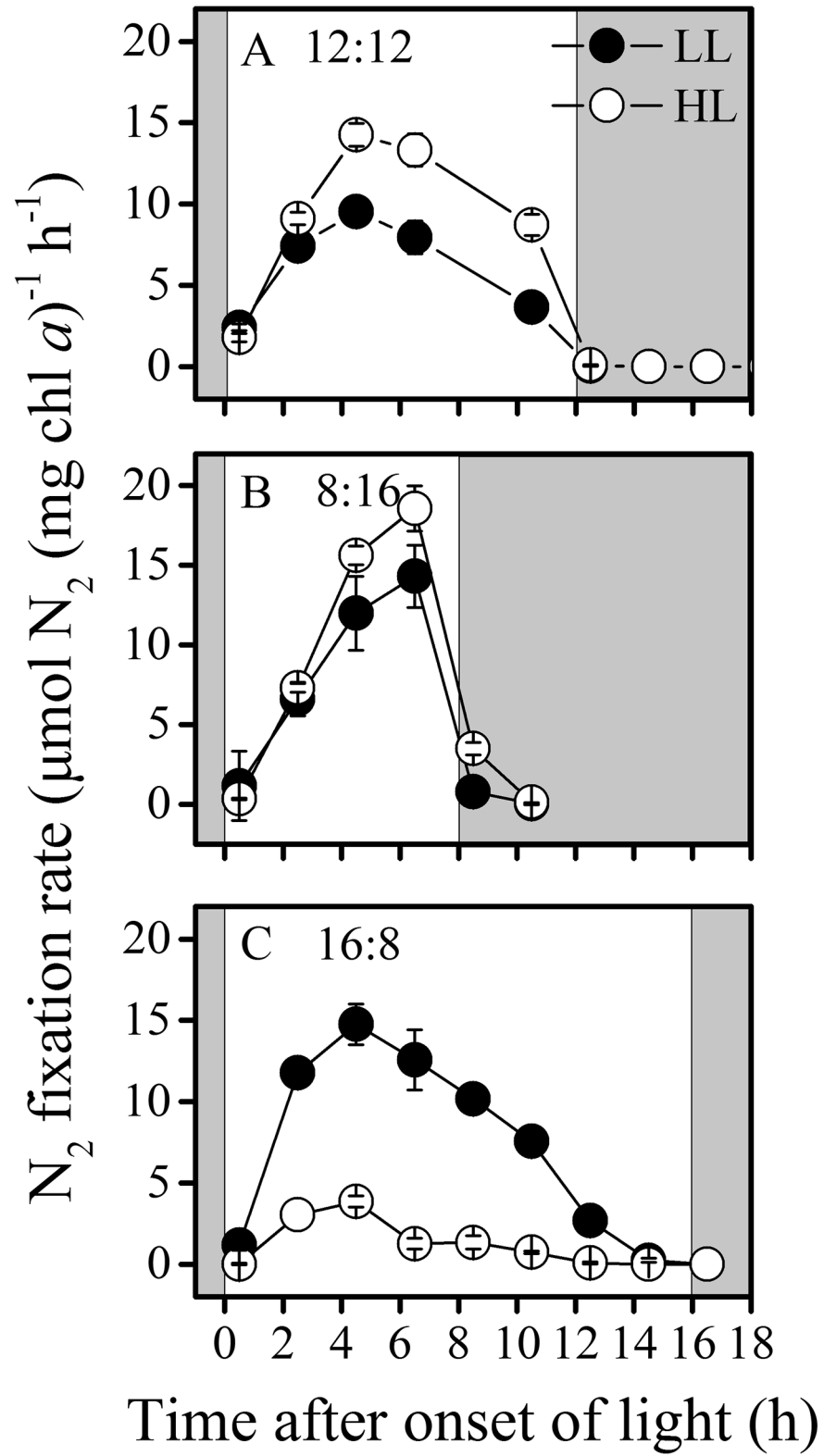
The carbon fixation rates were higher in HL-grown cells compared to LL-grown cells in all light regimes (Fig 3). In the HL-grown cells, carbon fixation decreased first, dipped around 2.5 h into the light period and then increased, peaked at the 2–4 h before the light was off. However, in the LL grown cells, the carbon fixation rate first decreased at the onset of light period and then gradually increased toward the end of light period regardless of the light regimes (Fig 3). In terms of the total amount of carbon fixed in light, extending the light period to 16 h significantly increased the amount of carbon fixed by 50% ( $p < 0.001$ ) in HL-grown cells compare to that under 12L:12D regime, and increased by 4% ( $p = 0.26$ ) in LL-grown cells compared to that under 12L:12D regime. The amplitude and periodicity of carbon fixation of LL-exposed cultures varied much less than those of HL cultures. The rates and amounts of total carbon fixed in light as well as C:N assimilation ratio were the lowest in LL-grown cells at 8L:16D regime, while the highest values were found in the HL-grown cells at 16L:8D regime (Table 1).

### Fluorescence

The effective quantum yield ( $\Phi_{PSII}$ ) (Fig 4) and relative electron transport rate (rETR) (Fig 5) showed similar patterns among the three light-dark regimes. The effective quantum yield ( $\Phi_{PSII}$ ) of LL cells was about 5 times higher than HL cells (Fig 4), while the maximal rETR were about 1.5 to 2 times higher in HL than in LL-grown cells. In terms of the diel variations, in 12L:12D and 8L:16D regimes,  $\Phi_{PSII}$  of both LL and HL-grown cells gradually increased toward the end of light period, once in the dark period, the quantum yield dramatically decreased. In the 16L:8D cultures,  $\Phi_{PSII}$  of LL-grown cells first decreased in the early stage of light period, then gradually increased to a maximum value before the dark period, while for the HL-grown cells, the  $\Phi_{PSII}$  peaked in the late light period and thereafter decreased towards the end of light period.

### Relationship with daily light doses

The daily fixed amount of N<sub>2</sub> and carbon were affected differentially by increased daytime light doses, with the former increasing under LL and decreased under HL with the light doses over 16 mol photons m<sup>-2</sup> d<sup>-1</sup> and the latter showing linear increase with increased daytime light doses (Fig 6A and 6B). The specific growth rate increased under LL and decreased under HL with increased levels of daytime light doses exposed under the different light regimes, respectively (Fig 6C).



**Fig 2. Diel variations of N<sub>2</sub> fixation.** Diel variations of N<sub>2</sub> fixation of LL (closed circles) and HL (open circles) cultures acclimated to different LD regimes. (A) 12L:12D, (B) 8L:16D and (C) 16L:8D regimes. Shaded areas represent dark periods. Values represent mean ± SD of triplicate cultures under each regime.

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**Table 1. Means or daily N<sub>2</sub> and carbon fixation and ratios of C/N.**

LD regime	Light level	Maximum N <sub>2</sub> fixation rate	Total N <sub>2</sub> fixed in the light	Total N <sub>2</sub> fixed in 24 hours	Percentage of N <sub>2</sub> fixed in the dark	Maximum C fixation rate	Total C fixed in the light	C:N <sub>2</sub> fixation ratio in the light
(h:h)		( $\mu\text{mol N}_2$ (mg chl a) <sup>-1</sup> h <sup>-1</sup> )	( $\mu\text{mol N}_2$ (mg chl a) <sup>-1</sup> )	( $\mu\text{mol N}_2$ (mg chl a) <sup>-1</sup> )	(%)	( $\mu\text{mol C}$ (mg chl a) <sup>-1</sup> h <sup>-1</sup> )	( $\mu\text{mol C}$ (mg chl a) <sup>-1</sup> )	(mol:mol)
12:12	LL	9.5±0.8	71.0±9.0	71.0± 9.0	0	282.3±51.9	2457.3±22	34.6
	HL	14.3±0.7	114.8±4.2	114.8± 4.2	0	397.4±9.1	3274.3±93	28.5
8:16	LL	14.3±2.0	66.5±12.9	68.6± 16.0	2.99	110.6±5.0	616.4±22	9.3
	HL	18.6±1.4	84.2± 11.7	90.5± 9.4	6.96	302.0±84.5	1658.5±310	19.7
16:8	LL	14.8±1.3	120.8± 10.0	120.8± 10.0	0	273.6±34.1	2574.4±316	21.3
	HL	3.9±0.4	20.7 ±1.9	20.7± 1.9	0	594.2±44.1	4966.8±290	239.9

Means of daytime or daily N<sub>2</sub> fixation and carbon fixation (± SD) under different light: dark (LD) regimes and ratios of C/N assimilation during daytime. The C:N<sub>2</sub> fixation ratio were based on the amount of carbon and N<sub>2</sub> fixed in the light period.

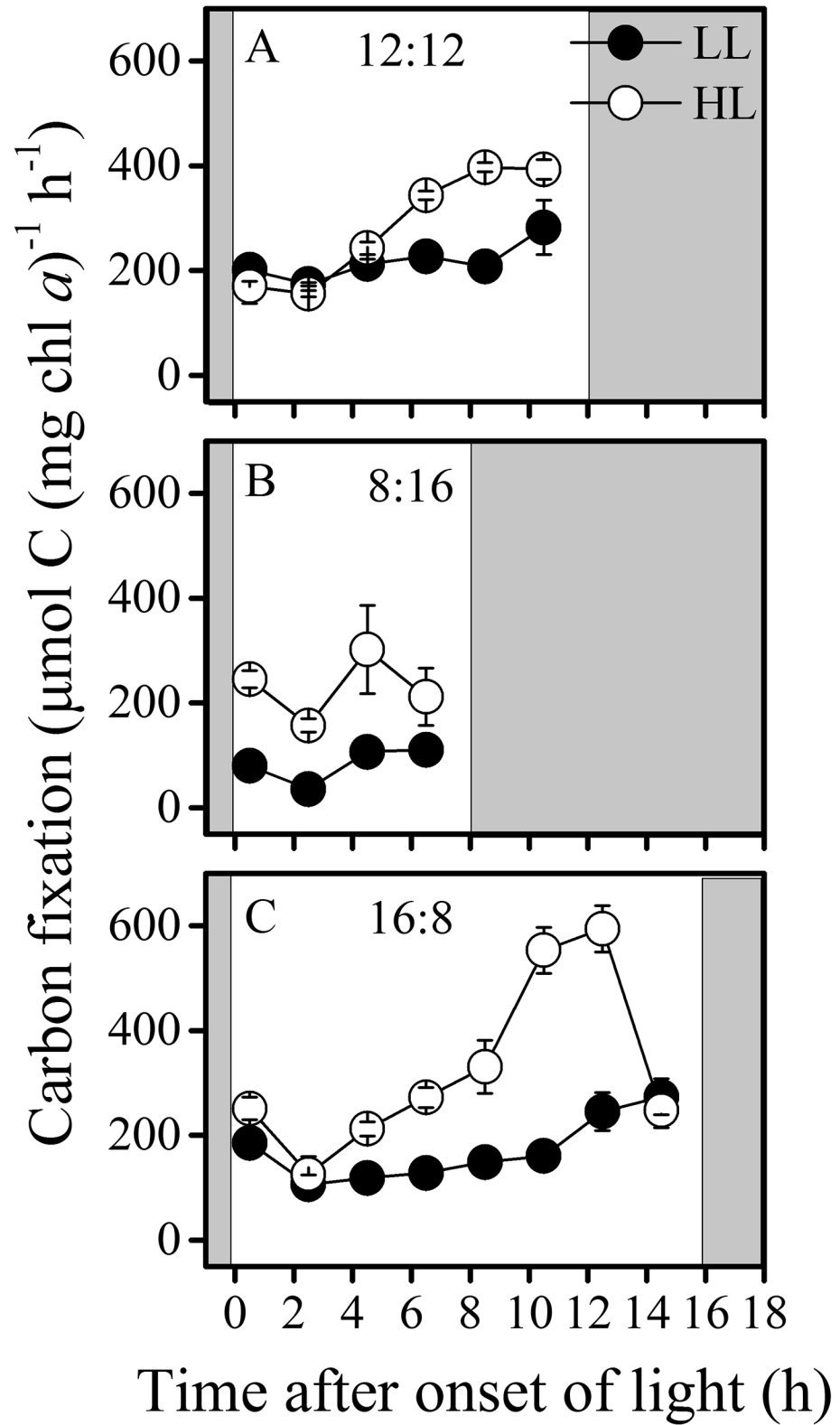
doi:10.1371/journal.pone.0135401.t001

## Discussion

Our results demonstrated that the different day-night cycles as well as different levels of light doses per day affected the diurnal performance of N<sub>2</sub> fixation and carbon fixation in *Trichodesmium*. Temporal segregation of N<sub>2</sub> fixation from photosynthetic carbon fixation was evidenced under all day-night regimes, with different magnitudes under different levels or regimes of light. Elongation and shortening of light period affected not only the maximal rates and daily fixed amount of N<sub>2</sub> and carbon as well as the timing of maximal N<sub>2</sub> fixation, which occurred at the end of the shortest but at early phase of the longest light period. The imbalance between daily carbon and nitrogen fixation was suggested to cause the changes in the growth rate under different light regimes.

N<sub>2</sub> fixation in *Trichodesmium* spp. is known to follow a circadian rhythm that developed after illumination, peaked at the middle and decreased near the end of the light period [13, 16]. In this study, N<sub>2</sub> fixation rates peaked during the light periods at different timings: relatively early in 16L:8D, at midday in 12L:12D, and late in 8L:16D day-night cycles (Fig 2), reflecting a functional plasticity of the N<sub>2</sub> fixation. Daily *de novo* synthesis, transcriptional and post-translational levels of nitrogenase [13, 24–26] could be expected to display similar plasticity. Buildup of cellular glutamine and glutamate pools [27] must have led to a feedback inhibition to N<sub>2</sub> fixation through glutamine /glutamate (GS/GOGAT) pathway [28, 29]. On the other hand, the fact that N<sub>2</sub> fixation peaked at the end of light period and extend to dark period under the 8L:16D regime (Fig 2B) implies an additional N requirement with extra energetic drive at the late light period. Although a rapid decrease in nitrogenase activity after a short dark incubation was observed [16], energy reserve from photosynthesis in the light period was supposed to be capable of supporting N<sub>2</sub> fixation during early night period [30]. A unicellular N<sub>2</sub>-fixing cyanobacterium *Cyanothece* sp. showed maximal N<sub>2</sub> fixation at different timings during dark period when transferred to different light-dark regimes [31]. Obviously, both daytime and nighttime N<sub>2</sub>-fixing diazotrophs alter their diel N<sub>2</sub> fixation performance when grown under different day-night cycles.

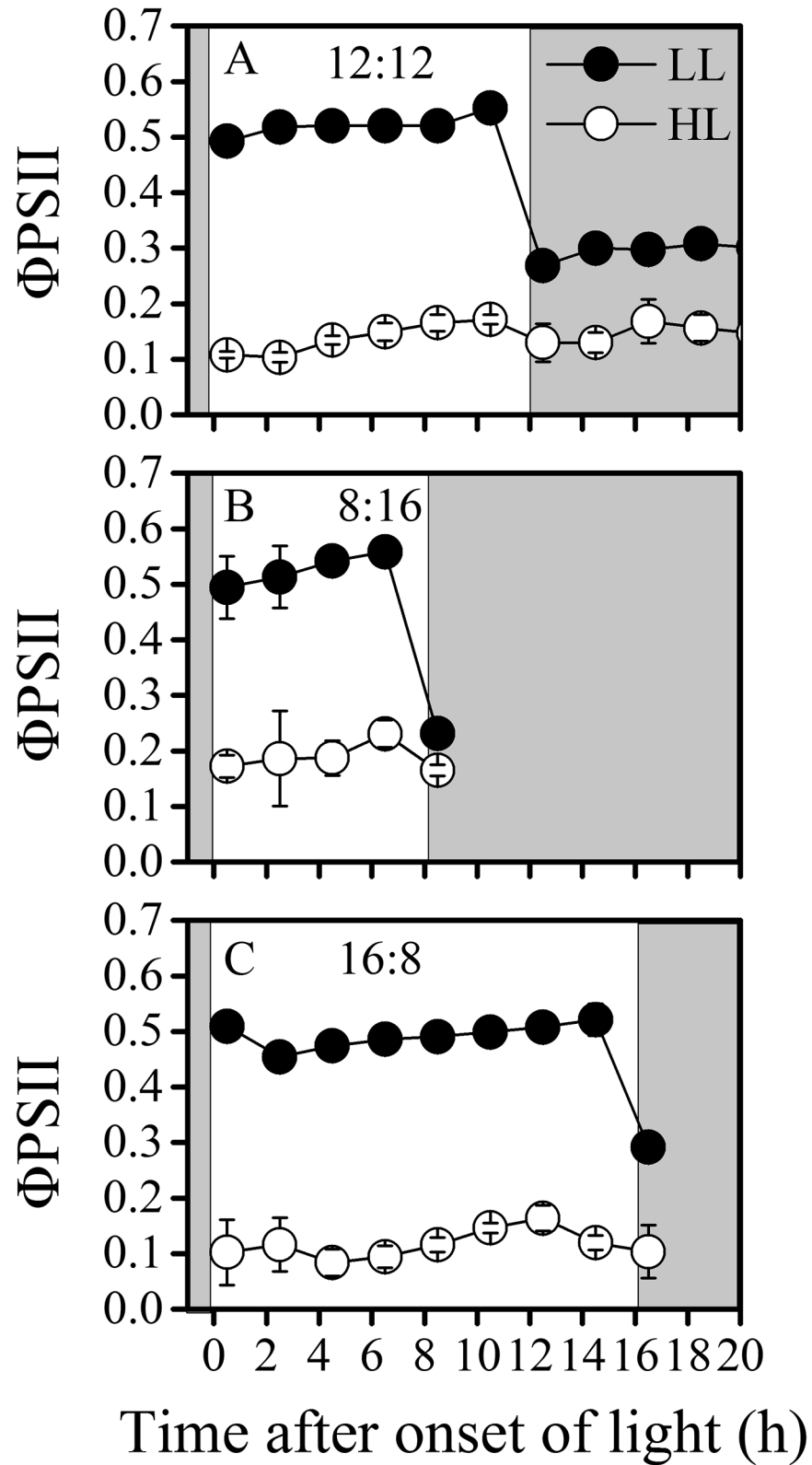
Temporal segregation of photosynthetic O<sub>2</sub> evolution and N<sub>2</sub> fixation has been reported in *Trichodesmium* [17] and in a few other filamentous [32, 33] and unicellular non-heterocystous cyanobacteria [34]. Lower net O<sub>2</sub> evolution coincides with lower CO<sub>2</sub> fixation at mid-day [17]. In this study, such a temporal segregation of N<sub>2</sub> fixation from photosynthetic carbon fixation was observed under all day-night regimes, but the minimal carbon fixation and maximal N<sub>2</sub>



**Fig 3. Diel variations of carbon fixation.** Diel variations of carbon fixation of LL (closed circles) and HL (open circles) cultures acclimated to different LD regimes. (A) 12L:12D, (B) 8L:16D and (C) 16L:8D regimes. Shaded areas represent dark periods. Values represent mean  $\pm$  SD of triplicate cultures under each regime.

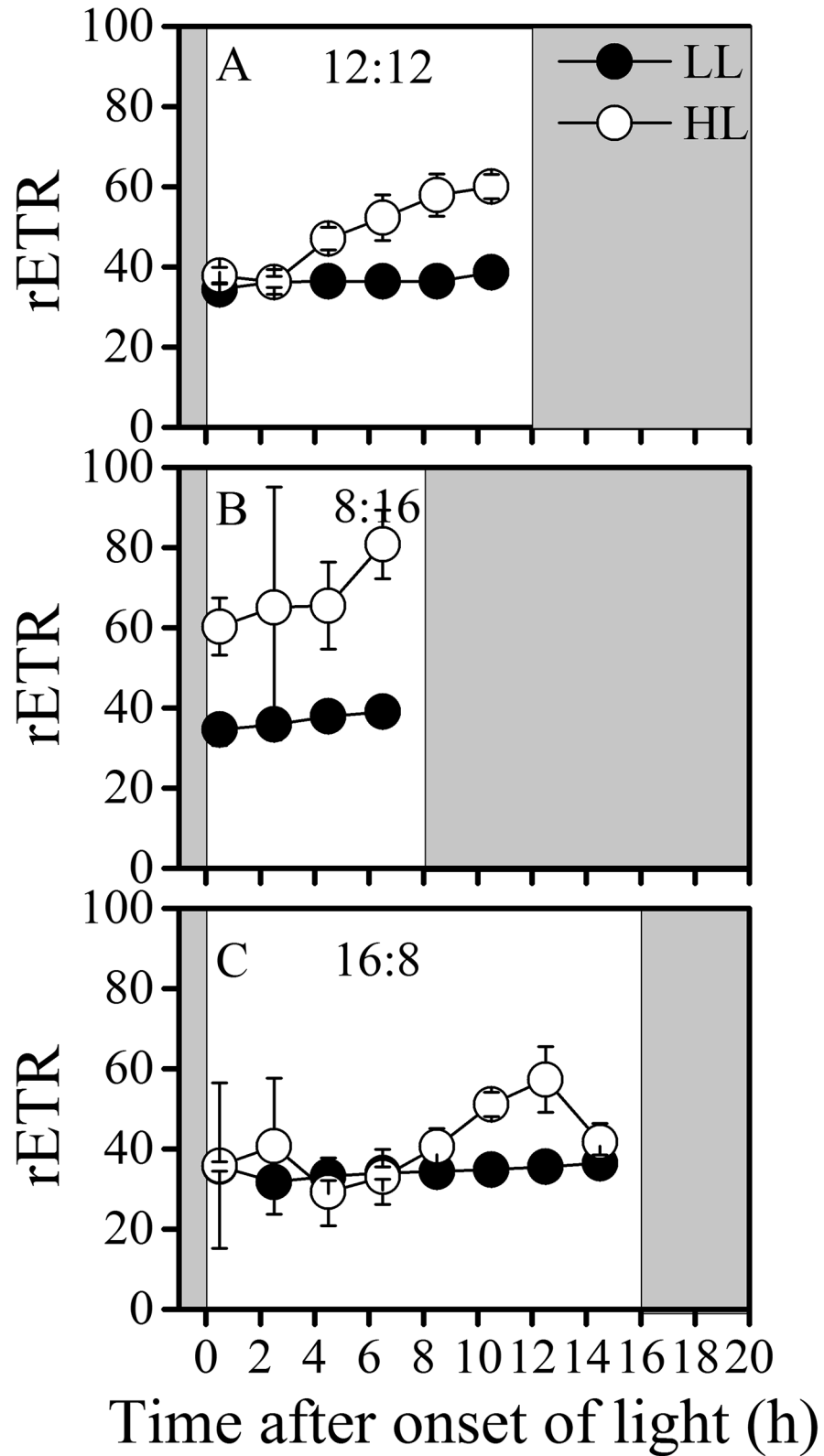
doi:10.1371/journal.pone.0135401.g003





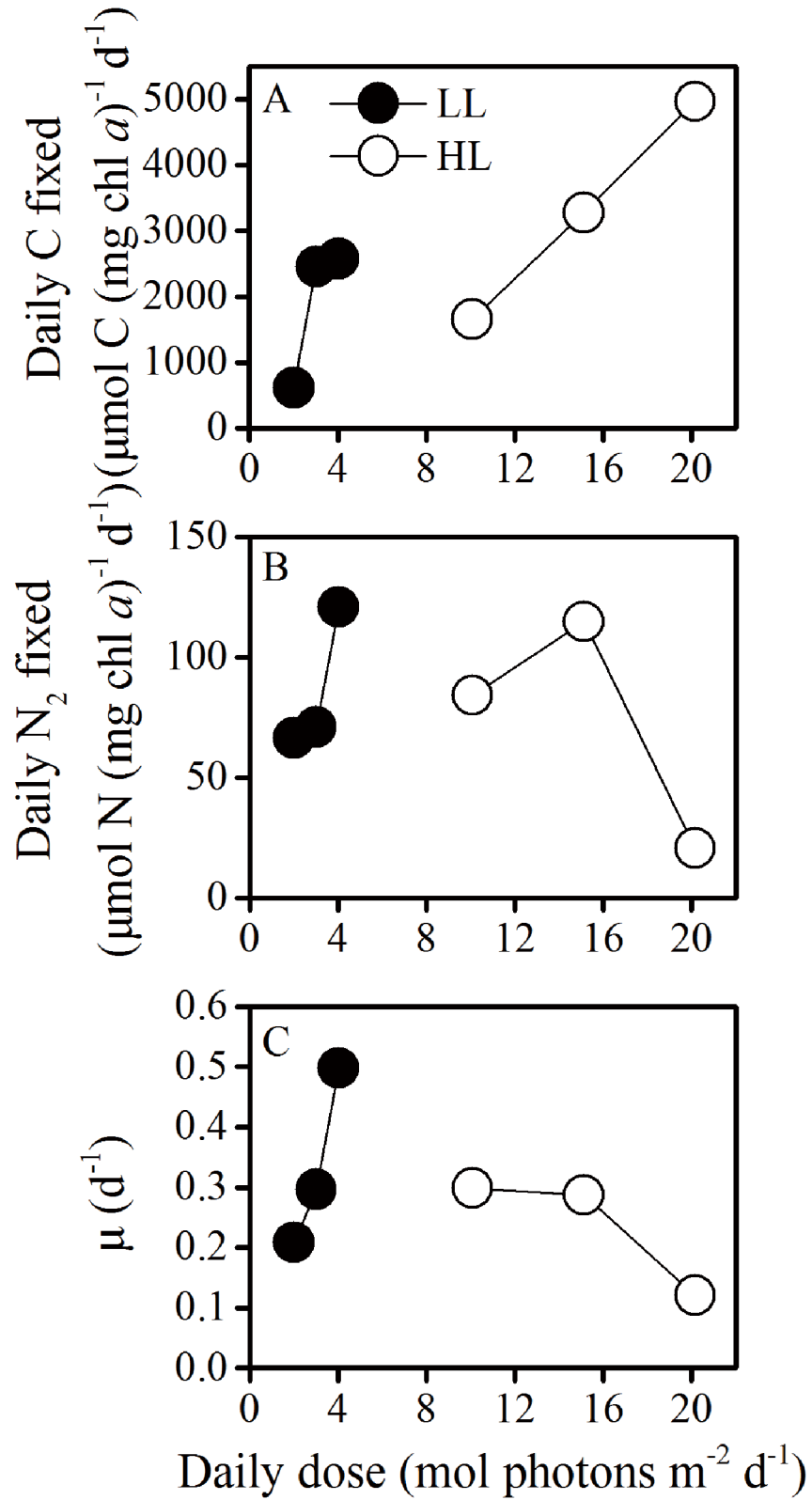
**Fig 4. Diel variations of  $\Phi_{PSII}$ .** Diel variations of quantum yield of PSII ( $\Phi_{PSII}$ ) of low light (LL, closed circles) and high light (HL, open circles) cultures acclimated to different LD regimes, (A) 12L:12D, (B) 8L:16D and (C) 16L:8D. Shaded areas represent dark periods. Values represent mean  $\pm$  SD of triplicate cultures under each regime.

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**Fig 5. Diel variations of rETR.** Diel variations of relative ETR of low light (LL, closed circles) and high light (HL, open circles) cultures acclimated to different LD regimes, (A) 12L:12D, (B) 8L:16D and (C) 16L:8D. The values of rETR was determined from the instant  $\Phi$ PSII multiplied by growth light irradiances. Shaded areas represent dark periods. Values represent mean  $\pm$  SD of triplicate cultures under each regime.

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**Fig 6. Relationship with daily light doses.** Correlations of light doses with (A) daily amount of carbon fixed, (B) daily amount of N<sub>2</sub> fixed and (C) growth rate in the cells grown under of low (LL, closed circles) and high (HL, open circles) light levels.

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fixation did not occur at the same time, with the peaks of  $N_2$  fixation occurring prior to that of carbon fixation (Figs 2,3). Such a time lag between minimal carbon fixation and maximal  $N_2$  fixation indicates an initial photosynthesis-dependent  $N_2$  fixation following with a subsequent photosynthetic suppression with peaked  $N_2$  fixation, which implies N dependency of continuous operation of photosynthetic machinery. This seems to reflect, a time-delay in *nif* gene expression due to photosynthetic metabolic feedback. Since regulation of nitrogenase synthesis by  $O_2$  occurs at the level of *nif* gene transcription [35], suppression of photosynthesis may open a window for  $N_2$  fixation in the following hours, while the metabolic feedback to gene expression might be on a slower time scale (hours) [36, 37].

For the photosynthetic performance, a correlation between the carbon fixation and the rETR was observed (Figs 3 and 5), since photosynthetic electron transport or energy transfer is the direct driver for the carbon fixation [38]. Nevertheless, in cyanobacteria, the photosynthetic and dark respiratory electron transport chain share the same plastoquinone pool [39], thus the respiration electron transport during the light period affects the actual redox state of the plastoquinone pool, which in turn affect the quantum yield of PSII ( $\Phi$ PSII) [19]. Therefore, the fluctuation amplitude of rETR was lower than that of carbon fixation (Figs 3 and 5). In addition, carbon fixation is not the only sink of electrons generated from light reaction.  $N_2$  fixation, Mehler reaction [9], photorespiration [40] and inorganic carbon uptake can also compete for energy [41]. The  $\Phi$ PSII as well as rETR showed higher values in short-day regime (8L:16D) of HL, coinciding with higher  $N_2$  fixation rate, which appeared to support the proposition that the  $N_2$  fixation may be a major sink of photosynthetic electrons [42]. The phenomenon that rETR values in the 8L:16D regime were higher compared to other L:D regimes indicates a photosynthetic strategy by which relatively more photons could be captured within shorter period, and the mechanisms involved should be studied in future works.

The daily fixed amount of carbon and  $N_2$  of LL-grown cells both increased with increased daily light doses (Fig 6A and 6B), leading to increased growth rates (Fig 6C). Such a phenomenon indicates light energy limitation in the LL-grown cells. In the HL cultures, the daily amount of carbon fixed increased with increasing light doses, while the daily amount of  $N_2$  fixed decreased with light dose over  $16 \text{ mol photons m}^{-2} \text{ d}^{-1}$ , reflecting a severe inhibition of  $N_2$  fixation of HL-grown cells under prolonged light regime (Fig 2C), which must have resulted in reduced growth rates (Figs 1 and 6C). Previous studies on *Trichodesmium* IMS101 showed that nitrogenase activity saturates at light levels of  $200\text{--}400 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  in the cells grown at 50 or  $500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  [42], while other strains of this genus saturates at lower light levels [43, 44], with saturation of nitrogenase activity occurring prior to that of photosynthetic electron transport [42]. Since the HL level is highly over the saturating light levels for the growth and nitrogen fixation according to previous studies [19, 42], photosynthetic  $O_2$  evolution was nearly saturated under this light level [45], therefore, accumulation of photosynthetically evolved  $O_2$  might have harmed nitrogenase and decreased nitrogen fixation. Additionally, long-day condition can also cause photoinhibition due to increased reaction oxygen species [46]. In the present study, longer exposure to high light induced severe inhibition to  $N_2$  fixation but not to the photosynthetic carbon fixation (Figs 2 and 3), indicating differential energy demand between C and  $N_2$  fixations in *Trichodesmium*. Nitrogenase gene expression and  $N_2$  fixation were shown to severely wither under continuous light [13], and the inhibition of  $N_2$  fixation under continuous light depends on light intensities [47]. In the present work, both light intensity and doses affected diurnal performance of photosynthesis, growth rates and  $N_2$  fixation. In view of the relationship of these parameters with light doses, carbon fixation increased with increasing growth light doses, while  $N_2$  fixation increased under the low to moderate levels but decreased under the high levels of light doses (above  $16 \text{ mol photons m}^{-2} \text{ d}^{-1}$ ) (Fig 6).

Because of the imbalance between carbon and N fixation, the C:N<sub>2</sub> fixation ratio ranged from 9 to 239 (mol: mol) (Table 1). The lowest C:N<sub>2</sub> ratio was found in the LL-grown cells under short-day condition, while the highest C:N<sub>2</sub> fixation ratio was found in the HL-grown cells under long-day condition, which could be due to higher rates of photosynthetic carbon fixation and lower levels of N<sub>2</sub> fixation over the longer light period (Fig 3C). While C: N<sub>2</sub> fixation ratios are often higher than the Redfield ratio [48, 49] and can be up to 400–700 in *Trichodesmium* [50, 51], the observed uncoupling of carbon and N<sub>2</sub> fixation in our study might be due to exudation of newly fixed C especially in long-day treatment of HL cells [51]. Since growth limitation by internal carbon and nitrogen status follows Liebig's law (growth is not controlled by the total amount of resources available, but by the scarcest resources), the unbalanced C:N<sub>2</sub> fixation appeared to be responsible for the reduction of growth rates under the elongated light period to 16 h of the high light cultures (Figs 1 and 6).

While *Trichodesmium* has a maximal biomass density at the depth of 20 to 40 m, where the light intensities would be 600 to 900  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  during noon period [2], it usually forms blooms in surface waters of tropical and sub-tropical oceans with the latitudes between 30°N and 30°S, where the shortest and longest day-time are 10 and 14 h, respectively [52]. However, this ecologically important organism may be drifted to higher latitudes via currents and encounter shorter or longer day-time [52]. 2.9–6.9% of daily N<sub>2</sub> fixed in the night under short-day regimes should be taken into account when estimating N<sub>2</sub> fixation by *Trichodesmium* spp. in the field. On the contrary, less N<sub>2</sub> fixation by 82% under the extended daytime to 16 h under HL should also be considered. Furthermore, different weather conditions also determines daily light doses, so that ecological and biogeochemical roles of this cyanobacterium could be influenced differentially under changing environments in different waters.

## Conclusions

The carbon and N<sub>2</sub> assimilation as well as growth rate in *Trichodesmium* were light dose dependent, with carbon fixation showing positive and N<sub>2</sub> fixation having negative relationships with increasing light doses over growth-saturating light levels. The shortest light period led to latest peaking timing of maximal N<sub>2</sub> fixation, while the longest light period led to N<sub>2</sub> fixation maximum at early light phase, with photosynthetic carbon fixation showing inverse patterns. The N<sub>2</sub> fixation extended to dark period when the cells were grown under 8L:16D regime. These results indicate that N<sub>2</sub> fixation was regulated by light energy supplies in addition to circadian rhythms. The imbalance in C:N<sub>2</sub> assimilation ratio under different levels of light energy supplies correlated with growth rates, with the maximal growth rate at about 20–30 and the minimal growth at over 200 C:N<sub>2</sub> ratios.

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## Author Contributions

Conceived and designed the experiments: XC KG. Performed the experiments: XC. Analyzed the data: XC. Wrote the paper: XC KG.

## References

1. Zehr JP. Nitrogen fixation by marine cyanobacteria. *Trends Microbiol.* 2011; 19: 162–173. doi: [10.1016/j.tim.2010.12.004](https://doi.org/10.1016/j.tim.2010.12.004) PMID: [21227699](https://pubmed.ncbi.nlm.nih.gov/21227699/)
2. Capone DG, Zehr JP, Paerl HW, Bergman B. *Trichodesmium*, a globally significant marine cyanobacterium. *Science.* 1997; 276: 1221–1229.
3. Mahaffey C, Michaels AF, Capone DG. The conundrum of marine N<sub>2</sub> fixation. *AM J SCI.* 2005; 305: 546–595.
4. Stal LJ, Krumbein WE. Oxygen protection of nitrogenase in the aerobically nitrogen fixing, non-heterocystous cyanobacterium *Oscillatoria* sp. *Arch Microbiol.* 1985; 143: 72–76.
5. Fay P. Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiol Rev.* 1992; 56: 340–373. PMID: [1620069](https://pubmed.ncbi.nlm.nih.gov/1620069/)
6. Kana TM. Oxygen Cycling in Cyanobacteria, with Specific Reference to Oxygen Protection in *Trichodesmium* spp. In: Carpenter EJ, D. G. Capone DG, Rueter JG, editors. *Marine Pelagic Cyanobacteria: Trichodesmium and other Diazotrophs.* Springer Netherlands; 1992. pp. 29–41.
7. Kana TM. Rapid oxygen cycling in *Trichodesmium thiebautii*. *Limnol Oceanogr.* 1993; 38: 18–24.
8. Gallon JR. N<sub>2</sub> fixation in phototrophs: adaptation to a specialized way of life. *Plant Soil.* 2001; 230: 39–48.
9. Milligan AJ, Berman-Frank I, Gerchman Y, Dismukes GC, Falkowski PG. Light-dependent oxygen consumption in nitrogen-fixing cyanobacteria plays a key role in nitrogenase protection. *J Phycol.* 2007; 43: 845–852.
10. Wolk CP, Ernst A, Elhai J. Heterocyst Metabolism and Development. In: Bryant D., editors. *The Molecular Biology of Cyanobacteria.* Springer Netherlands; 2004. pp. 769–823.
11. Kumar K, Mella-Herrera RA, Golden JW. Cyanobacterial heterocysts. *Cold Spring Harb Perspect Biol.* 2010; 2: a000315. doi: [10.1101/cshperspect.a000315](https://doi.org/10.1101/cshperspect.a000315) PMID: [20452939](https://pubmed.ncbi.nlm.nih.gov/20452939/)
12. Bergman B, Sandh G, Lin S, Larsson J, Carpenter EJ. *Trichodesmium*—a widespread marine cyanobacterium with unusual nitrogen fixation properties. *FEMS Microbiol Rev.* 2013; 37: 286–302. doi: [10.1111/j.1574-6976.2012.00352.x](https://doi.org/10.1111/j.1574-6976.2012.00352.x) PMID: [22928644](https://pubmed.ncbi.nlm.nih.gov/22928644/)
13. Chen YB, Dominic B, Mellon MT, Zehr JP. Circadian rhythm of nitrogenase gene expression in the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. strain IMS 101. *J Bacteriol.* 1998; 180: 3598–3605. PMID: [9658003](https://pubmed.ncbi.nlm.nih.gov/9658003/)
14. Axmann IM, Hertel S, Wiegard A, Dörrich AK and Wilde A. Diversity of KaiC-based timing systems in marine Cyanobacteria. *Mar Genom.* 2014; 14: 3–16.
15. Wyman M, Zehr JP. Temporal Variability in Nitrogenase Gene Expression in Natural Populations of the Marine Cyanobacterium *Trichodesmium thiebautii*. *Microbiology.* 1996; 62: 1073–1075.
16. Ohki K, Zehr JP, Fujita Y. Regulation of nitrogenase activity in relation to the light-dark regime in the filamentous non-heterocystous cyanobacterium *Trichodesmium* sp. NIBB 1067. *J Gen Microbiol.* 1992; 138: 2679–2685.
17. Berman-Frank I, Lundgren P, Chen YB, Küpper H, Kolber Z, Bergman B, et al. Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. *Science.* 2001; 294: 1534–1537. PMID: [11711677](https://pubmed.ncbi.nlm.nih.gov/11711677/)
18. Chen YB, Zehr JP, Mellon M. Growth and nitrogen fixation of the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. IMS101 in defined media: evidence for a circadian rhythm. *J Phycol.* 1996; 32: 916–923.
19. Cai X, Gao K, Fu F, Campbell DA, Beardall J, Hutchins DA. Electron transport kinetics in the diazotrophic cyanobacterium *Trichodesmium* spp. grown across a range of light levels. *Photosynth Res.* 2015; 124: 45–56. doi: [10.1007/s11120-015-0081-5](https://doi.org/10.1007/s11120-015-0081-5) PMID: [25616859](https://pubmed.ncbi.nlm.nih.gov/25616859/)
20. Ritchie RJ. Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. *Photosynth Res.* 2006; 89: 27–41. PMID: [16763878](https://pubmed.ncbi.nlm.nih.gov/16763878/)
21. Capone DG. Determination of nitrogenase activity in aquatic samples using the acetylene reduction procedure. In Kemp PF, Sherr BF, Sherr EB, Cole JJ, editors. *Handbook of methods in aquatic microbial ecology.* Lewis Publishers, Boca Raton, Fla. 1993; pp: 621–631.
22. Breitbarth E, Mills MM, Friedrichs G, LaRoche J. The Bunsen gas solubility coefficient of ethylene as a function of temperature and salinity and its importance for nitrogen fixation assays. *Limnol Oceanogr: Meth.* 2004; 2: 282–288.
23. Gao K, Wu Y, Li G, Wu H, Villafane VE, Helbling EW. Solar UV radiation drives CO<sub>2</sub> fixation in marine phytoplankton: a double-edged sword. *Plant Physiol.* 2007; 144: 54–59. PMID: [17494919](https://pubmed.ncbi.nlm.nih.gov/17494919/)

24. Zehr JP, Wyman M, Miller V, Duguay L, Capone DG. Modification of the Fe Protein of Nitrogenase in Natural Populations of *Trichodesmium thiebautii*. *Appl Environ Microbiol*. 1993; 59: 669–676. PMID: [16348883](#)
25. Zehr JP, Capone DG. Problems and Promises of Assaying the Genetic Potential for Nitrogen Fixation in the Marine Environment. *Microb Ecol*. 1996; 32: 263–281. PMID: [8849422](#)
26. Capone DG, O'Neil JM, Zehr JP, Carpenter EJ. Basis for Diel Variation in Nitrogenase Activity in the Marine Planktonic Cyanobacterium *Trichodesmium thiebautii*. *Appl Environ Microb*. 1990; 56: 3532–3536.
27. Capone DG, Ferrier MD, Carpenter EJ. Amino Acid Cycling in Colonies of the Planktonic Marine Cyanobacterium *Trichodesmium thiebautii*. *Appl Environ Microbiol*. 1994; 60: 3989–3995. PMID: [16349431](#)
28. Carpenter EJ, Bergman B, Dawson R, Siddiqui PJ, Söderbäck E, Capone DG. Glutamine synthetase and nitrogen cycling in colonies of the marine diazotrophic cyanobacteria *Trichodesmium* spp. *Appl Environ Microbiol*. 1992; 58: 3122–3129. PMID: [1359837](#)
29. Mulholland MR, Capone DG. The nitrogen physiology of the marine N<sub>2</sub>-fixing cyanobacteria *Trichodesmium* spp. *Trends Plant Sci*. 2000; 5: 148–153. PMID: [10740295](#)
30. Rabouille S, Staal M, Stal LJ, Soetaert K. Modeling the dynamic regulation of nitrogen fixation in the cyanobacterium *Trichodesmium* sp. *Appl Environ Microb*. 2006; 72: 3217–3227.
31. Rabouille S, Van de Waal DB, Matthijs HCP, Huisman J. Nitrogen fixation and respiratory electron transport in the cyanobacterium *Cyanothece* under different light:dark cycles. *FEMS Microbiol Ecol*. 2014; 87: 630–638. doi: [10.1111/1574-6941.12251](#) PMID: [24236731](#)
32. Rai A, Borthakur M, Bergman B. Nitrogenase derepression, its regulation and metabolic changes associated with diazotrophy in the non-heterocystous cyanobacterium *Plectonema boryanum* PCC 73110. *J Gen Microbiol*. 1992; 138: 481–491.
33. Misra H, Tuli R. Nitrogen fixation by *Plectonema boryanum* has a photosystem II independent component. *Microbiology*. 1994; 140: 971–976.
34. Bergman B, Gallon J, Rai A, Stal L. N<sub>2</sub> Fixation by non-heterocystous cyanobacteria. *FEMS Microbiol Rev*. 1997; 19: 139–185.
35. Gallon JR. Tansley Review No. 44. Reconciling the Incompatible: N<sub>2</sub> fixation and O<sub>2</sub>. *New Phytol*. 1992; 122: 571–609.
36. Misra HS, Tuli R. Differential expression of photosynthesis and nitrogen fixation genes in the cyanobacterium *Plectonema boryanum*. *Plant Physiol*. 2000; 122: 731–736. PMID: [10712536](#)
37. Masuda T, Tanaka A, Melis A. Chlorophyll antenna size adjustments by irradiance in *Dunaliella salina* involve coordinate regulation of chlorophyll a oxygenase (CAO) and Lhcb gene expression. *Plant Mol Biol*. 2003; 51: 757–771. PMID: [12678561](#)
38. Falkowski PG, Raven JA. *Aquatic photosynthesis*, 2nd edn. Princeton University Press, New Jersey. 2007; pp: 167–170.
39. Campbell D, Hurry V, Clarke AK, Gustafsson P, Oquist G. Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. *Microbiol Mol Biol Rev*. 1998; 62: 667–683. PMID: [9729605](#)
40. Xu J, Gao K. Future CO<sub>2</sub>-induced ocean acidification mediates the physiological performance of a green tide alga. *Plant Physiol*. 2012; 160: 1762–1769. doi: [10.1104/pp.112.206961](#) PMID: [23129205](#)
41. Kranz SA, Eichner M, Rost B. Interactions between CCM and N<sub>2</sub> fixation in *Trichodesmium*. *Photosynth Res*. 2011; 109: 73–84. doi: [10.1007/s11120-010-9611-3](#) PMID: [21190135](#)
42. Wilson ST, Kolber ZS, Tozzi S, Zehr JP, Karl DM. Nitrogen Fixation, Hydrogen Cycling, and Electron Transport Kinetics in *Trichodesmium erythraeum* (Cyanobacteria) Strain IMS101. *J Phycol*. 2012; 48: 595–606.
43. Ohki K, Fujita Y. Aerobic nitrogenase activity measured as acetylene reduction in the marine non-heterocystous cyanobacterium *Trichodesmium* spp. grown under artificial conditions. *Mar Biol*. 1998; 98: 111–114.
44. Bell PRF and Fu F-X. Effect of light on growth, pigmentation and N<sub>2</sub> fixation of cultured *Trichodesmium* sp. from the Great Barrier Reef lagoon. *Hydrobiologia*. 2005; 543: 25–35.
45. Carpenter E, Roenneberg T. The marine planktonic cyanobacteria *Trichodesmium* spp.: photosynthetic rate measurements in the SW Atlantic Ocean. *Mar Ecol Prog Ser*. 1995; 118: 267–273.
46. Dinakar C, Bartels D. Light response, oxidative stress management and nucleic acid stability in closely related Linderniaceae species differing in desiccation tolerance. *Planta*. 2012; 236: 541–555. doi: [10.1007/s00425-012-1628-8](#) PMID: [22437647](#)
47. Stal LJ, Krumbein WE. Temporal separation of nitrogen fixation and photosynthesis in the filamentous, non-heterocystous cyanobacterium *Oscillatoria* sp. *Arch Microbiol*. 1987; 149: 76–80.

48. Orcutt KM, Lipschultz F, Gundersen K, Arimoto R, Michaels AF, Knap AH, Gallon JR. A seasonal study of the significance of N<sub>2</sub> fixation by *Trichodesmium* spp. at the Bermuda Atlantic Time-series Study (BATS) site. *Deep-Sea Res Pt II*. 2001; 48: 1583–1608.
49. Mulholland MR, Bernhardt PW. The Effect of Growth Rate, Phosphorus Concentration, and Temperature on N<sub>2</sub> Fixation, Carbon Fixation, and Nitrogen Release in Continuous Cultures of *Trichodesmium* IMS101. *Limnol Oceanogr*. 2005; 50: 839–849.
50. McCarthy JJ, Carpenter EJ. *Oscillatoria* (*Trichodesmium*) *thiebautii* (Cyanophyta) in the central North Atlantic Ocean. *J Phycol*. 1979; 15: 75–82.
51. Wannicke N, Koch BP, Voss M. Release of fixed N<sub>2</sub> and C as dissolved compounds by *Trichodesmium erythreum* and *Nodularia spumigena* under the influence of high light and high nutrient (P). *Aquat Microb Ecol*. 2009; 57: 175–189.
52. LaRoche J, Breitbarth E. Importance of the diazotrophs as a source of new nitrogen in the ocean. *J Sea Res*. 2005; 53: 67–91.