The Influence of pCO2 and Temperature on Gene Expression of Carbon and Nitrogen Pathways in Trichodesmium IMS101

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Abstract

Growth, protein amount, and activity levels of metabolic pathways in Trichodesmium are influenced by environmental changes such as elevated pCO2 and temperature. This study examines changes in the expression of essential metabolic genes in Trichodesmium grown under a matrix of pCO2 (400 and 900 μatm) and temperature (25 and 31°C). Using RT-qPCR, we studied 21 genes related to four metabolic functional groups: CO2 concentrating mechanism (bicA1, bicA2, ccmM, ccmK2, ccmF3, ndhF4, ndhD4, ndhL, chpX), energy metabolism (atpB, sod, prx, glcD), nitrogen metabolism (glnA, hetR, nifH), and inorganic carbon fixation and photosynthesis (rbcL, rca, psaB, psaC, psbA). nifH and most photosynthetic genes exhibited relatively high abundance and their expression was influenced by both environmental parameters. A two to three orders of magnitude increase was observed for glnA and hetR only when both pCO2 and temperature were elevated. CO2 concentrating mechanism genes were not affected by pCO2 and temperature and their expression levels were markedly lower than that of the nitrogen metabolism and photosynthetic genes. Many of the CO2 concentrating mechanism genes were co-expressed throughout the day. Our results demonstrate that in Trichodesmium, CO2 concentrating mechanism genes are constitutively expressed. Co-expression of genes from different functional groups were frequently observed during the first half of the photoperiod when oxygenic photosynthesis and N2 fixation take place, pointing at the tight and complex regulation of gene expression in Trichodesmium. Here we provide new data linking environmental changes of pCO2 and temperature to gene expression in Trichodesmium. Although gene expression indicates an active metabolic pathway, there is often an uncoupling between transcription and enzyme activity, such that transcript level cannot usually be directly extrapolated to metabolic activity.

Introduction

The marine filamentous N2 fixing (diazotroph) cyanobacteria Trichodesmium spp. form extensive blooms contributing 25 to 50% of the estimated rates of N2 fixation in the oligotrophic subtropical and tropical oceans [1]. Trichodesmium’s dominant role in carbon and nitrogen cycling has prompted investigations examining the effects of rising sea surface temperatures and elevated atmospheric pCO2 (leading to ocean acidification) on the growth and abundance of this organism.

Elevated pCO2 supports enhanced N2 fixation and growth rates in Trichodesmium [2–7]. These trends are further accentuated when elevated pCO2 and higher temperatures are combined [3,5]. The higher N2 fixation and growth rates are enabled via flexible phosphorus stoichiometry, changes in the activity of the CO2 concentrating mechanism (CCM), and modified protein activity [4–8].

In Trichodesmium, as in other cyanobacteria, metabolic pathways (e.g. respiration, photosynthesis, C1 fixation, N2 fixation, and combined nitrogen assimilation) share cellular complexes such as plastoquinone (PQ) pool, succinate dehydrogenase and ferredoxin [9–11]. Trichodesmium’s unique metabolism allows oxygenic photosynthesis and oxygen-sensitive N2 fixation to occur concurrently during the photoperiod via a complex spatial-temporal separation of these processes [11–13]. Photosynthetic activity in Trichodesmium is coupled with CCM activity. PSI driven electron transport is responsible for generating energy needed to pump HCO3− into the cell. This HCO3− is subsequently converted to CO2 by the ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO) within the carboxysomes [6]. Regulation of the photosynthetic and N2 fixation processes occurs at the transcription, translation, and post-translational (activity) levels [5,8,10,12,13,16,17]. While elevated pCO2 and temperature resulted in higher growth rates, higher N2 fixation rates, and higher C:P ratios, photosynthesis, protein pools, and total cellular allocation of carbon and nitrogen were not significantly affected [5]. Importantly, the abundance of nitrogenase and glutamine synthetase (mediating combined nitrogen assimilation) did not
increase in parallel to the increased N₂ fixation rates, implying that environmental factors can allow higher reaction turnover rates through the same protein amounts [5,8]. In addition, our previous study showed that pCO₂ changed the mRNA diel expression patterns, but not the abundance, of five genes (psaH, glcb, hefK, pshA, and pshB), resulting in a more synchronized expression pattern under elevated pCO₂ [5]. We therefore decided to check the combined effect of pCO₂ and temperature on the expression levels of 21 genes of interest (GOI) representing key metabolic aspects in *Trichodesmium*, as part of *Trichodesmium*’s acclimation response.

Genomic analyses demonstrate that *Trichodesmium* (IMS101) has a partial suite of CCM components ([18,19]; http://genome.jgi-psf.org/finished_microbes/trier/trier.home.html). Accordingly, *Trichodesmium* possesses β-carboxysomes, a cellular compartment containing RubisCO, and a low-affinity, high-flux HCO₃⁻ uptake system called BicA [18–20]. *Trichodesmium* also has a specialized NADPH dehydrogenase, NDH-I₄, which acts as a low-affinity CO₂ uptake system, converting CO₂ to HCO₃⁻ using the ChpX protein [18]. The presence of a true internal carbonic anhydrase (CA) was not found in the genome and direct measurements by means of ¹³O₂ exchange method [21] revealed only a low activity, close to the detection limit of the method [6]. Yet, there is a distinct possibility that the N-terminal domain of the essential β-carboxysomal *ccmM* gene found in *Trichodesmium* can act as a γ-CA in an oxidized β-carboxysome interior as was observed in *Thermosynechococcus elongatus* [19,22,23].

Currently, there is no genetic system for *Trichodesmium* transformations, limiting the physiological study of CCM activity to the examinations of fluxes of inorganic carbon (Ci) and O₂ [6]. Here we present the expression and abundance of genes related to CCM (bicA₁, bicA₂, *ccmM*, *ccmK₂*, *ccmK₃*, ndhF₄, ndhD₄, ndhL and *cbpX*), energy metabolism (*atpB*, *sod*, *pxr*, *gD*), nitrogen metabolism (*glnA*, *hetR*, *nifH*), and light harvesting (*psaC*, *psbA*) in *Trichodesmium* acclimated to a matrix of pCO₂ (400 and 900 µatm) and temperature (25 and 31°C). Since diurnal regulation is essential for metabolic functions in *Trichodesmium*, we performed our measurements over the day and sampled 1, 5, 9 and 13 h after the onset of light. The sampling times were chosen for time periods that represent different metabolic preferences in *Trichodesmium* [13]: time of maximal photosynthesis (1 h), maximal N₂ fixation rates (5 h), late afternoon (9 h) and 1 h after dark induction (13 h). We compare the expression levels and patterns of these genes and look at the correlation of their coordinated expression.

**Materials and Methods**

**Culturing and growth**

*Trichodesmium* IMS101 stock cultures were grown in YBCII medium [24] at 25°C, 12:12 light/dark cycle at ~80 µmol photons m⁻² s⁻¹ white light and 400 µatm pCO₂. Diluted batch cultures were grown in sterile square 1 L Nalgene bottles as single filaments with gentle bubbling, sufficient for preventing aggregates formation without harming the integrity of the filaments. Stock cultures were unialgal and under exponential growth the bacterial biomass was negligible and was not observed under light microscopy or by DAPI staining. Experimental cultures were enriched with CO₂ and air mixes of 400 µatm (current) and 900 µatm (expected 2100) and were gradually acclimated to 31°C (1°C increase per week). Cultures were acclimated for at least 1.5–2 months before sampling. Biomass was kept under 0.2 µg chl a ml⁻¹, thereby maintaining a low enough biomass that did not additionally influence the chemical experiment of the experimental setup. For more information about carbonate chemistry in similar experimental setups, see Kranz et al. [6].

**Sample collection for RNA, RNA-Extraction and reverse transcription RT-qPCR**

Samples of *Trichodesmium* IMS101 were collected at 4 time points during the diurnal cycle; 1, 5, 9 and 13 h after the onset of light (the last point is 1 h after dark induction). Acclimated cultures were filtered on polycarbonate filters of 1 µm pore size; 25 mm diameter filters (Osmonics). Filters were placed in sterile DNase and RNase free centrifuge tubes and put directly into liquid nitrogen until transfer to -80°C for storage.

mRNA was extracted with the RNEasy Plant Mini Kit (Qiagen Cat.74904) according to the producers instructions. Additionally a DNase treatment was accomplished with RNase-Free DNase Set (Qiagen Cat.79254) on column during the extraction as well as with TURBO DNA free (Ambion Cat.AM1907) after the extraction, following the manufacturer’s specifications for rigorous DNase treatment to remove any gDNA contamination. RNA concentration was measured with a NanoDrop ND-1000 Spectrophotometer (peqLab Biotechnologie) and quality was tested with 1% agarose gels. Reverse transcription was conducted with the Invitrogen or Applied Biosystems) and are presented, by name and function, in Table 1.

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thresholds for the GOI and the endogenous reference gene [26]. In time course experiments, the gene expression is often compared internally by normalization to a calibrator, which can be the time zero point. Here we have chosen the average ΔCt values of the nifH from the 400 μM/25°C treatments (our control treatment, already normalized to 16S rRNA) as a calibrator, since we wanted to compare the relative abundance of the different genes, as well as their time dependence. The expression of this gene was relatively constant over the day.

The rnpB gene, encoding RNase P, was examined as a potential endogenous reference gene and revealed unexpected large variations in its expression. Therefore we decided to use 16S rRNA for further calculations, as its expression was stable under all the different conditions. Following Bustin et al. [28], standard deviations were chosen to present statistical differences between independent replicates of mRNA transcript enrichments.

### Statistical analysis and presentation

mRNA abundances of all 21 GOIs are presented in Figure 1 as the average mRNA abundance from all acclimations at each time point, n = 12–13.

To examine the influence of sampling time and the applied environmental factors, pCO₂ and temperature, we performed a 3-Way ANOVA (time, pCO₂ and temperature, p<0.05) for the enrichment values of each GOI over the time course measured. Number of independent replicates was n = 24–25 for each pCO₂ concentrations, and n = 12–13 for each measuring time point (Figure 2 and Table 2).

Pearson correlations for the enrichment values of our 21 GOIs were done for each sampling point, n = 11–13. Correlations in which the Pearson correlation coefficient r>0.75 are presented by color coding in Figure 3 (CCM-pink, energy metabolism-yellow, nitrogen metabolism-blue, Ci fixation and photosynthesis-green). All correlation coefficients and significances according to the Pearson correlation are supplied in the supplemental data (Table S1 i-iv).

### Results and Discussion

We examined the expression levels of 21 GOIs over the day and under different pCO₂ concentrations (400 and 900 μM) and temperatures (25 and 31°C). The samples for the RT-qPCR analysis were taken from the exact experimental set-up described in Levitan et al. [8]. The physiological characteristics of *Trichodesmium* IMS101 cultures used in these experiments are summarized in Table 3: growth rates (chl d⁻¹), elemental stoichiometry [C:N, C:P, N:P (mol:mol)], nitrogen fixation rates (nmol N₂ chl⁻¹ h⁻¹), and NifH amounts (pmol μg protein⁻¹). These data were presented and discussed in Levitan et al. [8].

The GOIs can be divided into 4 functional groups: CCM (bic1, bic2, ccmM, ccmk2, ndhF4, ndhD4, ndhL), CCM-pink), energy metabolism (atpB, sod, prx, glcD), nitrogen metabolism (glnA, hetR, nifH), and photosynthesis and Ci fixation (rbcL, rca, psaB, psaC, psbA). The gene description and the primers sequences are presented in Table 1.

### Expression levels of the selected GOIs

We examined the mean enrichment levels of the different GOIs over the day, arranged by their metabolic function, using the

| Gene | Description | Forward Primer (5’ to 3’) | Reverse Primer (5’ to 3’) |
|------|-------------|--------------------------|--------------------------|
| 16s  | 16s rRNA    | GCCAACCCCTCGTTATTAGTT    | TTGTCACCGGACGTCCTTC      |
| rnpB | RNaseP      | TTGGTAAAGACCATCCAGATGATA | CCGGTGTTGCTCTTCACTCCAAAA |
| bicA1| Low affinity HCO₃⁻ transporter | GTCCGGCTGCTGCGGTATTAG | AACAGTGGCTGCAAAACACCC |
| bicA2| Low affinity HCO₃⁻ transporter | TTGGATACGGTGGGCAAGTTA  | TCCACGAAATATGCGGATCC    |
| ccmM | β-Carboxysome shell/gamma CA | TCGGCTTCTGTTACGTTTTTAA | AACTATACATCCTTCACGGAAG |
| ccmK2| β-Carboxysome shell | CGAGGAGATGTTTCAGGATG | CCAACCATATCTTTATGCTGGC |
| ccmK3| β-Carboxysome shell | TCGGCAATGGACGAGCTGA | GCCACACGATTTGCGGAGG |
| ndhF4| NADPH dehydrogenase - NDH-1 complex | TTGGCGAGTGCTATTAACCCAGAA | CTTAAATCATGAGACTCGCAAC |
| ndhD4| NADPH dehydrogenase - NDH-1 complex | TTGGCGAGTGCTATTAACCCAGAA | CTTAAATCATGAGACTCGCAAC |
| ndhL | NADPH dehydrogenase - NDH-1 complex | TTGGCGAGTGCTATTAACCCAGAA | CTTAAATCATGAGACTCGCAAC |
| chpX | NADPH dehydrogenase - NDH-1 complex | TTGGCGAGTGCTATTAACCCAGAA | CTTAAATCATGAGACTCGCAAC |
| atpB | ATP synthase – β subunit | CAAGATGTATCCGTGACCTGTGAA | TTGGCGAGTGCTATTAACCCAGAA |
| sod  | Superoxide-Dismutase | TTGGCGAGTGCTATTAACCCAGAA | CTTAAATCATGAGACTCGCAAC |
| glcD | Glycolate oxidase | CCCAGCCTCCCTGCTGAA | CTTAAATCATGAGACTCGCAAC |
| prx  | 1-Cys peroxiredoxin | TGCAAGACGGTGAGTACCAAAGTC | CTTAAATCATGAGACTCGCAAC |
| glnA | Glutamine synthetase | AAATTGAGAAAGACGGAGGACC | AAATTGAGAAAGACGGAGGACC |
| hetR | Key regulatory gene in heterocyst differentiation | TTATATATGATACGCTGG | CCGATCCTCTAATACGCCAG |
| nifH | Fe-protein of nitrogenase | TGCGGCGGTATATTACGCTGAC | GCAAATACGGAGAGGAGG |
| rbcL | Rubisco Large subunit | ACTGCCGGCTCCTGAAAGAGA | CTTCTATGAGGAAATACGCCAG |
| rca  | Rubisco activase Small subunit | GCTTTATCTAATACGCTGAC | ACGAATACGGAGAGGAGG |
| psaB | Subunit of PSI | TGGAGATTTGGAATATTGAG | CTTCTATGAGGAAATACGCCAG |
| psaC | Subunit of PSI | TGGAGATTTGGAATATTGAG | CTTCTATGAGGAAATACGCCAG |
| psbA | Subunit of PSI (D1) | TGGAGATTTGGAATATTGAG | CTTCTATGAGGAAATACGCCAG |

Table 1. Description and sequences of forward and reverse primers for our target genes.
average enrichment values of all acclimations (Figure 1). The expression levels of each of our GOIs were on the same order of magnitude for all acclimations (excluding the high pCO2/high temperature acclimation for hetR and glnA; Figure 1).

The transcript abundance of the CCM and energy metabolism GOIs were low compared to the nitrogen metabolism and Ci fixation and photosynthetic genes. The CCM and energy metabolism genes spanned over 2 orders of magnitude, with the exception of ccmK2 and pxr (Figure 1). The pxr gene, encoding for the cyanobacterial peroxiredoxin 1-Cys revealed a unique diurnal trend and had higher transcript abundance relative to the other genes examined for energy metabolism (Figure 1). The ccmK2 gene is encoding for the main β-Carboxysome shell protein. The pores in the CcmK2 protein hexamers may enable diffusion of small essential metabolites into the carboxysome lumen [23]. The ccmK2 gene revealed a trend similar to the other CCM genes, yet its expression was slightly higher (Figure 1). Most GOIs related to nitrogen metabolism (glnA, hetR and nifH) and photosynthesis and Ci fixation (rbcL, rca, psbA, psaC, psaB) exhibited 1–4 orders of magnitude higher average enrichment levels than the CCM and energy metabolism genes with varying expression patterns (Figure 1). Our findings correspond with results published from a community gene expression of a Trichodesmium spp. bloom in the Southwest Pacific Ocean showing that during the day, the highest abundance of Trichodesmium related genes was that of the photosynthetic and nitrogen metabolism pathways [29].

We previously showed the pCO2 influences gene expression patterns over the diurnal cycle for five of the above GOIs, nifH, hetR, glnA, psaB and psbA, in Trichodesmium IMS101 (data from another set of experiments, [5]). Three of these genes, nifH, psaB and psbA, had similar enrichment levels in both studies (Figure 1, [5]). The combination of elevated temperature and high pCO2, (not examined in [5]) significantly increased the transcript abundance of glnA and hetR. This acclimation resulted in ~2 orders of magnitude higher transcript levels than previously reported for glnA (Figure 2, [5]). The increase in glnA transcript abundance was not reflected in the GlnA protein pool size, and there was no significant difference for the GlnA amount between treatments and over the diurnal cycle (as measured by a quantitative western blot, one way ANOVA, p<0.05; Table 3; [5]). The combined influence of elevated pCO2 and high temperature increased the average hetR enrichment levels to levels similar to those previously reported [5]. In Trichodesmium, the hetR gene was suggested to be constitutively expressed (under a 12:12 Light/Dark cycle), yet its diurnal abundance ranged 3–10 fold and was regulated by combined nitrogen concentration levels [30]. Based on our results, we believe that the sensitivity of hetR to changes in pCO2, temperature and time, further points to an environmental sensitivity of this gene.

The influence of CO2 and temperature on the selected GOIs

To determine whether the diurnal cycle interacted with the applied environmental factors to influence the transcript abundance of the GOIs, we applied a 3-Way ANOVA (pCO2, temperature and time of day) for all the GOIs tested. The results are summarized in Table 2 and the enrichment levels of the influenced genes for all acclimations are presented in Figure 2. Out of 21 GOIs, six genes (bicA1, ccmM, ccmK3, ndhL, atpB and psaC) were influenced by the diel cycle alone and six other genes (bicA2, ndhF4, ndhD4, ccmK2, rca and sod) were influenced by neither time nor the applied environmental factors. Transcripts abundances of GOIs that were not affected by any of the three main factors, or were influenced by time only, are presented in the
supplemental data (Figure S1). Only nine GOIs were significantly affected by pCO2 and/or temperature (nifH, glnA, hetR, rbcL, psbA, psbA prx, glcD, and ccmK2). Seven of the nine GOIs that appeared sensitive to pCO2 and/or temperature (nifH, glnA, hetR, rbcL, psbA, and prx; Figure 2) correspond with the nine genes expressed at the highest abundance (Figure 1). These genes are representative of the photosynthetic and Ci fixation, nitrogen metabolism and energy generation pathways.

While photosynthesis in Trichodesmium is relatively insensitive to changes in pCO2 [4,6], N2 fixation rates vary significantly with changes in ambient pCO2 [3–5,8]. N2 fixation, and possibly the sequential assimilation of ammonium, were affected by pCO2 at the mRNA and activity level, while protein pools remained relatively constant (Table 3; [5]). Abundance of the nitrogenase Fe-protein gene, nifH, was affected by pCO2, time of day, and the combined influence of pCO2 and temperature (Figure 2; [5]). This corroborates findings showing that nifH expression is pCO2 sensitive [5] and controlled by a circadian rhythm [31]. Relative stability of nifH expression to temperature changes was also reported for Trichodesmium IMS101 grown at 24, 28.5 and 31°C [31]. Similarly, temperature did not appreciably affect the abundance of the NifH protein and the nitrogenase N2 fixation rates in the temperature range applied here (Table 3; [5]). Trichodesmium cultures tested under a broader temperature range revealed changes in growth and N2 fixation rates [32]. As natural populations of Trichodesmium spp. range from 20 to 34°C (reviewed in [33]), it would be advisable to further examine the acclimation responses and levels of regulation under a wider temperature range.

glnA and hetR transcripts were statistically influenced by all three variables: pCO2, temperature and time of day (Table 2), in line with the reported influence of pCO2 and time on both genes [5,30,34]. To our knowledge, scant data on the effect of environmental conditions on glnA and hetR expression in
Table 2. The influence of changing environmental conditions on the enrichment of all our GOIs.

| Significant interactions between factors (additional to main factors) | Significant main factors | Time is the only influencing main factor | No influencing factor |
|---|---|---|---|
| Time + pCO₂ + temperature | pCO₂ + time | pCO₂ + temperature | Time, temperature | Time, pCO₂ |
| nifH | glnA | prx | nifH | hetR | prx | nifH | bicA1 | bicA2 |
| glnA | glnA | ccmK2 | glnA | psaB | ccmM | ndhF4 |
| rbcL | ccmK3 | ndhD4 |
| glcD | ndhL | chpX |
| glnA | atpB | rca |
| hetR | psaC | sod |
| psbA | psbA |

The environmental conditions are pCO₂, temperature, and time. Gene expression was determined using 2^-ΔΔCt method. Statistical analysis was made using 3-Way ANOVA (p<0.05). n = 24–25 for both temperatures, n = 23–25 for both pCO₂, and n = 11–13 for each measuring time point.

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Figure 3. Correlations between the abundance of the 21 GOIs during the daily cycle. Sampling times were 1, 5, 9, 13 hours after the onset of light, presented from top-left to bottom-right. Colored cells represent a Pearson correlation coefficient r≥0.75. Correlations are presented regardless to the different acclimations. Genes are divided to 4 groups: CCM (pink), energy metabolism (yellow), nitrogen metabolism (blue), and photosynthesis and Ci fixation (green). When correlations were between genes from the same group, the cells were colored in the groups’ color. When correlations were between genes from two different groups, the cells were colored using a gradient from one groups’ color to the other. All the correlation coefficients and significances according to the Pearson correlation are supplied in the supplemental data (Table S1 i-iv). Relative abundance estimated according to the 2^-ΔΔCt method, with 16S rRNA as the endogenous reference gene, and average ΔCt values of the nifH from the 400 μatm/25°C acclimation (control) as a calibrator. n = 11–13 for each gene at a given sampling time.

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Table 3. Physiological characteristics of *Trichodesmium* IMS101 cultures acclimated to a matrix of pCO₂ and temperature.

| Treatment                      | Growth rate (chl d⁻¹) | C:N | CN (moll⁻¹) | C:P | N:P (moll⁻¹) | Maximal N₂ fixation rate (nmol N₂ chl⁻¹ h⁻¹) | GlcA (pmol ug protein⁻¹) |
|--------------------------------|-----------------------|-----|-------------|-----|-------------|----------------------------------------------|-------------------------|
| 400 μatm pCO₂/25 °C            | 0.17±0.04             | 73±0.9 | 139±28 | 19.5±5.4 | 4.8±1.4 | 0.05±0.01                                     | 0.07±0.02               |
| 400 μatm pCO₂/31 °C            | 0.27±0.01             | 66±0.7 | 164±48 | 25.2±9.0 | 7.2±3.1 | 0.05±0.02                                     | 0.07±0.02               |
| 900 μatm pCO₂/25 °C            | 0.33±0.03             | 6.5±0.4 | 118±32 | 18.3±5.5 | 20±5.3 | 0.27±0.03                                     | 0.28±0.09               |
| 900 μatm pCO₂/31 °C            | 0.38±0.08             | 5.97±0.51 | 196±27 | 33.1±4.9 | 15.6±3.2 | 0.28±0.09                                     | 0.07±0.02               |

* n = 3–4
| n = 12–13

Acclimation pCO₂ levels were 400 and 900 μatm and temperatures were 25 and 31 °C. The extended data set and discussion of the physiological responses were previously reported (Levitan et al., 2010a) and are summarized here to emphasize physiological changes associated with the gene abundance presented from the same cultures.

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*Trichodesmium* shows that fixed-nitrogen sources and diurnal rhythmicity regulated *glnA* expression in natural populations of marine Synechococcus spp [35] and *hetR* expression in *Trichodesmium* [30]. The *hetR* gene, previously suggested to be involved only in heterocyst differentiation, was also found in the non-heterocystous filamentous diazotroph *Symploca* PCC8002 [36] and *Lyngbya* PCC8106 [37]. The existence of *hetR* in *Trichodesmium*, *Symploca* PCC8002a and *Lyngbya* PCC8106, its regulation by combined nitrogen status and time [36,37], and the apparent sensitivity of *Trichodesmium*'s *hetR* to pCO₂ (also affecting N₂ fixation; Figure 2, Table 2), further suggest that *hetR* must play a critical role in diazotrophic nitrogen metabolism and is not limited to heterocyst differentiation [37].

The regulation of photosynthetic genes is essential in *Trichodesmium* where photosynthetic O₂ evolution is separated from N₂ fixation by a complex spatial-temporal strategy. To enable N₂ fixation, down regulation of photosystem II (PSII), possibly fixation, down regulation of photosystem II (PSII), possibly the expression pattern of *psa* genes in *Trichodesmium*’s *psa* operon is located in the 5'-flanking region on the *rbcL-rbcS* operon [39,40]. However, our genomic analysis reveals that *rbcL* and *cmmK2* are not closely oriented in the *Trichodesmium* genome. In *Synechocystis* PCC6803, *rbcL* expression was insensitive to changes in pCO₂ (0 to 3% CO₂ in air; [41]). Our findings show that *rbcL* expression was modified only when combining high pCO₂ with high temperature, yet its abundance was still at the same order of magnitude for all acclimations. In *Synechocystis* PCC6803 the Ci derived transcriptional changes in *rbcL* transcript amount were uncoupled from changes in RbcL protein level, possibly resulting from low protein turnover rate due to the protective effect of the carboxysome, slowing down the protein degradation [42]. This was also indicated for *Trichodesmium* in our study. While pCO₂ alone and the combination of pCO₂ and temperature influenced the RbcL protein amount, the highest protein level was at 900 μatm/25 °C while the highest transcript level appeared at 900 μatm/31 °C (unpublished data; Figure 2).

The oxygenase activity of RubisCO forms 2-phosphoglycolate (2PG), considered toxic for CI fixation in the Calvin cycle. The GlcD protein helps protect the Calvin cycle by converting two molecules of 2PG into one 3-phosphoglycerate (3PGA) molecule (the product of RubisCO’s carboxylase activity), and thus enables Ci fixation to proceed. GlcD metabolism was found essential for the viability of the cells and oxygenic photosynthesis in the cyanobacterium *Synechocystis* PCC6803 at ambient CO₂ conditions [43]. Statistical analysis (3-Way ANOVA, p<0.05) revealed that the *gld* mRNA abundance was sensitive to the combined influence of pCO₂ and temperature, yet its abundance was the same for all our acclimations (Figure 2, Table 2). Although *gld* is also found in *Arabidopsis* and *Anabaena* [43], there is generally scarce information regarding the expression and regulation of this gene.

Temperature, time of day and their interaction affected the 1-cys peroxiredoxin gene, *prx* (Table 2), increasing its abundance by 3 orders of magnitude from 1 to 9 h after the onset of light (Figure 2). 1-cys *prx* mRNA increased in response to different metabolic imbalances in *Synechocystis* PCC6803, including irradiation, salinity, and iron deficiency [44]. No data are currently available on changes of *prx* at different pCO₂ and/or temperatures in *Trichodesmium* and other cyanobacteria. O₂ generated in PSII is reduced to H₂O₂ by PSI related components [45]. In cyanobacteria peroxiredoxin reduces H₂O₂ to H₂O using electrons donated from a variety of substrates [46]. Increased expression of iron and oxidative stress genes at the end of the high N₂ fixation period was detected for cultures of the unicellular diazotroph *Crocophila watanuki* [47]. Biological fixation of one N₂ molecule requires at least 16 ATP molecules that can be generated via cyclic electron flow around PSI [48]. Thus, in *Trichodesmium*, the higher expression of *prx* in the second half of the photoperiod may be required to recover from the high energetic demand for N₂.
fixation, leaving the cell susceptible to oxidative stress. In addition, peroxidases function as regulators of redox-mediated signal transduction in some eukaryotes [49,50], and are therefore important components for the cellular antioxidant defense system [46]. Redox state of shared components between photosynthesis and respiration regulates gene expression in *Trichodesmium* [13,51]. Hence, changes in *prx* expression reported here (Figure 2, Table 2) indicate that oxidative defense, photosynthesis and/or respiratory redox state in *Trichodesmium* are temperature and time dependent.

Our results indicate that these nine genes (*ndhH*, *glcA*, *hetR*, *rbcL*, *psbA*, *psbB* *prx*, *gkd* and *ccmK2*), are non-constitutively expressed and are regulated both by a diurnal cycle and by environmental factors such as pCO2 and temperatures.

**Expression of CCM genes**

The nine CCM-related GOIs that were tested are representative of all known CCM complexes in *Trichodesmium*: the carboxysome (*ccmM*, *ccmK2*, *ccmK3*) that contains the cellular *Ci* fixation enzyme RubisCO (*rbcL* [19]), HCO3− transporter named BicA (*bicA1*, *bicA2*), and the specialized NADPH dehydrogenase NDH-I4 (*ndhF4*, *ndhD4*, *ndhL*, and *chpX*). *Trichodesmium* lacks any genes of inducible-high affinity uptake system for both CO2 and HCO3−, such as NDH-I4 (CO2), BCT1 or SbtA (HCO3−) [18,19], and has no recognizable carbonic anhydrase (CA) genes [18].

CCM operation in algae is regulated by environmental factors with elevated CO2 levels expected to reduce the cellular requirements for concentrating *Ci* and enabling enhanced growth [52]. All of our nine examined CCM-related GOIs (Table 1) exhibited similar expression patterns and low expression levels when compared to *Ci* fixation, photosynthesis and nitrogen metabolism genes (Figure 1). Only one gene, the *ccmK2*, was affected by changes in environmental conditions (Figure 2, Table 2). For all time points measured, the expression of the CCM related genes had the highest correlations of all the GOIs metabolic groups. This applies within the CCM group and also with the other functional groups (Figure 3).

CCM genes of high *Ci* affinity are known to be regulated at the transcript level [53]. Our experimental setup is different in two aspects from “classical” cyanobacterial CCM induction experiments: 1. we report on a steady state expression of CCM genes under long term constant CO2 conditions, whereas in most publications cells are rapidly transferred (usually less than 1 day) from one CO2 concentration to another; 2. Trying to work on publications cells are rapidly transferred (usually less then 1 day). Our experimental setup is different in two components: 1. we report on a steady state expression of CCM genes [53]. Our experimental setup is different in two aspects from “classical” cyanobacterial CCM induction experiments: 1. we report on a steady state expression of CCM genes under long term constant CO2 conditions, whereas in most publications cells are rapidly transferred (usually less than 1 day) from one CO2 concentration to another; 2. Trying to work on publications cells are rapidly transferred (usually less then 1 day).

In *Synechocystis* PCC6803, genes of low affinity CCM components such as *ndhD4*, *ndhF4*, *chpX* and *ccmK-N* [41,42] and *bicA* [42,53] were *Ci* insensitive, constitutively expressed, and revealed relatively low transcript abundance as we found for *Trichodesmium* (Figures 1 and 3, Table 2). In *Synechococcus* PCC7002 *bicA* is regulated by a *ccmR* gene [53], which is absent in the *Trichodesmium* genome [19].

In *Trichodesmium’s* genome, most of the CCM-related genes are not arranged in operons or clusters (http://genome.jgi-psf.org/finished_microbes/trier/trier.home.html), as was previously shown for *Synechocystis* PCC6803 [54]. This also applies for the three CCM-related-gene-pairs that were co-expressed over the day, *ccmM-ccmK3*, *ccmK3-ccmK2* (carboxysome shell) and *ndhF4-ndhD4* (NDH-I4; Table 4). We conclude that in *Trichodesmium*, CCM genes are constitutively expressed and are mostly unaffected by the applied changes in pCO2 and temperature.

Genomic analyses indicate that *Trichodesmium* lacks inducible inorganic carbon (*Ci*) uptake systems [18,19]. Yet, physiological measurements of *Ci* uptake showed that *Trichodesmium* changes its *Ci* uptake characteristics when acclimated to high CO2 (900 μatm; [6,7]). While the cell’s affinity to total DIC decreased with elevated pCO2 [6], the cell’s CO2 uptake increased [7]. Under a range of pCO2 (150-900 μatm pCO2), *Trichodesmium* uses HCO3− for over 90% of its *Ci* source [6]; Kranz and Levitan, unpublished data).

Based on the genetic analysis (Figure 1; [19]), the Km of *Ci* uptake [6] and BicA being a low-affinity but high flux HCO3− uptake system [20], it is likely that a major part of *Trichodesmium’s* *Ci* uptake is via the Na+ dependent HCO3− transporter BicA.

The operation of a *Ci* uptake system that maintains constant transcription levels while its affinity is modified indicates that CCM operation in *Trichodesmium* is controlled at the translational or post-translational levels. Changes in CCM operation without altering gene expression or the cell’s capacity to transport *Ci* was proposed by Beardall and Giordano [52], i.e. via fluctuations in the redox state of the PQ pool. A rapid increase in HCO3− transport activity appears to involve phosphorylation events, possibly by activating two or three component regulatory systems, is of considerable importance when looking at CCM regulation [55,56]. The thioredoxin regulatory system and internal *Ci* pools can also act in controlling CCM operation, away from the transcript level [55,57]. Moreover, it is possible that large transcript changes were not detected in *Trichodesmium’s* CCM genes due to the long acclimations (>2 months) of the cultures, whereas a rapid transfer of *Trichodesmium* from low to high CO2 may result in changes in transcript abundance. Finally, although there are physiological changes in cells grown at different pCO2s [2-8], it could be that the acclimation to 900 μatm pCO2 does not simulate a large enough increase to detect significant differences in CCM gene abundance.

Our analyses show low transcript abundance of co-expressed CCM genes in *Trichodesmium* (Figures 1 and 3) that are insensitive to changes in pCO2 and temperature (Figure 2; Table 2). This, together with genomic analysis [19] and physiological data, suggest that CCM genes in *Trichodesmium* are constitutively expressed under our applied conditions.

### Co-expression of GOIs

We explored co-expression of GOIs by examining the correlations between their enrichment at all measured time points. Figure 3 presents GOIs with Pearson correlation coefficients.

| Gene pair                  | Pearson correlation coefficient | Significance |
|----------------------------|---------------------------------|-------------|
| ccmM-ccmK3                 | 0.767 (0.004)                   | 0.802 (0.000) | 0.828 (0.002) |
| ccmK3-ccmK2                | 0.792 (0.002)                   | 0.911 (0.000) | 0.778 (0.005) |
| ndhF4-ndhD4                | 0.913 (0.000)                   | 0.963 (0.000) | 0.832 (0.001) |
| hetR-glnA                  | 0.912 (0.000)                   | 0.969 (0.000) | 0.978 (0.000) |

Presented values are the Pearson correlation coefficient and the significance is in the parentheses. Sampling points were 1, 5, 9, 13 hours after the onset of light.

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higher than 0.75 (r>0.75, p<0.01, all correlations are given in Table S1 i-iv in the supplemental data). The highest number of significant correlations between GOIs of different metabolic functions appeared 5 h after the onset of light, when high N₂ fixation rates and assimilation are detected [5,13,30]. A large number of correlations were also observed between genes 1 h after the onset of light. Later in the day, at 9 and 13 h, a significantly lower number of correlations were detected, especially between GOIs related to different metabolic groups, indicating only limited co-expression.

Expression levels of nitrogen metabolism GOIs (glnA, hetR, nifH) were correlated with GOIs of other metabolic functional groups predominantly at 5 and 9 hours after the onset of light, yet the highest number of correlations was found at 5 h (Figure 3). The limited literature on gene expression patterns in Trichodesmium demonstrated diurnal regulation of genes correlated with photosynthesis and nitrogen metabolism [12,30,31,33,59]. At 9 h, only nifH was co-expressed with GOIs of other metabolic functional groups, especially with the carboxysomal, NDH-I4 and photosynthesis related genes. Chen et al. [12] demonstrated a time-dependent cycling and coupling between nifH and photosynthetic transcripts in Trichodesmium. Our results showed that nifH was co-expressed with the photosynthesis-related genes (psaA, psbD and rbcL), with all CCM components, and with two of the energy metabolism genes (atpB and sod).

Out of the three nitrogen metabolism genes tested, only the glc-hetR gene pair was co-expressed for all time points measured (Figure 3, Table 4), although they are not closely localized in the Trichodesmium genome (http://genome.jgi-psf.org/finished_microbes/trier/trier.home.html). hetR was also highly correlated to the NDH-I4 genes, to atpB, and to sod. The co-expression of glc and hetR did not correspond with nifH expression (Figure 3), in agreement with other studies showing that hetR expression was inversely correlated with nifH expression in Trichodesmium [30,34]. In Trichodesmium, both glnA and hetR are likely under ntcA regulation [34], yet we couldn’t verify this in our experiment.

Energy metabolism related GOIs (atpB, sod, prx, and glcD) were co-expressed and positively correlated with all CCM related GOIs and photosynthetic genes at 1 and 5 h after the onset of light. This could be related to the energetically demands of the CCM, and to the connection between photosynthetic electron transfer and the use of these electrons in sequential processes (Figure 3; [6,7]). prx correlated with many other GOIs only 1 h after the onset of light. This correlation disappeared from 5 h onwards, when prx mRNA abundance rapidly increased (Figures 1 and 2).

Positive correlations between Ci fixation and photosynthesis to CCM GOIs were observed at all measured time points. A high number of correlations were especially noted 1 and 5 h after the onset of light for rbcL and psbA together with the CCM GOIs (i.e. rbcL-ccmK2 at 1, 5 and 9 h). Fewer correlations, predominantly occurring at 5 h, were detected between photosynthetic and nitrogen metabolism GOBs (Figure 3). Co-expression of CCM and photosynthetic genes at the first half of the photoperiod (when photosynthesis and carbon fixation take place) could account for the tight interaction observed between the two mechanisms [6,20].

The co-expression of GOBs we observed fundamentally reflects the diurnal patterns of the predominant metabolic pathways in Trichodesmium (CCM, photosynthesis and carbon fixation, nitrogen metabolism, and energy generation). Transcriptional regulation is the first level of regulation, followed by translational and post-translational regulation. Different levels of metabolic regulations were found in Trichodesmium, for example for nitrogenase [5,16,17,31] and PSII [8,12]. The differing patterns of co-expression between the metabolic gene families during the day (Figure 3) indicate a strategy of a complex and tightly regulated gene expression. In Trichodesmium, such a strategy is required due to the unique spatial-temporal segregation of oxygenic photosynthesis and N₂ fixation [12-14].

Conclusions

Our motivation in this study was to examine changes in expression of essential metabolic genes in Trichodesmium grown under a matrix of pCO₂ and temperature. In Trichodesmium IMS101, nitrogen metabolism, Ci fixation, and photosynthesis related GOBs exhibited the highest abundance of all measured genes (Figure 1). These genes were also mostly affected by changes in pCO₂, temperature and the time within the diurnal period (Figure 2, Table 2), suggesting that these metabolic functions are also controlled at the mRNA transcript level. To our knowledge this is the first report of CCM gene expression in Trichodesmium. We suggest that CCM genes in Trichodesmium are constitutively expressed under our applied conditions, yet, their corresponding protein activity may be altered by changes in pCO₂ [6,7], probably due to translational and/or post-translational regulations [19].

Protein and activity levels of the CCM and fixation pathways in Trichodesmium are influenced by environmental changes [5-8]. Thus, we hypothesized that modifications in the CCM genes expression due to elevated pCO₂ may facilitate the reported physiological changes. Our results negate this hypothesis as the expression of CCM-genes under long term acclimation (steady state conditions) was insensitive to changes in experimental conditions. The comprehensive analysis of abundance and expression patterns of the GOBs presented here, demonstrates that gene expression may be uncoupled from translational and protein activity levels. Thus, although gene expression reflects active metabolic pathways, there is often an uncoupling between transcription and enzyme activity. Therefore we conclude that to examine the effects of environmental parameters on Trichodesmium and its biogeochemical impact, studies of gene transcription levels should be done in parallel with physiological and activity measurements.

Supporting Information

Figure S1 Daily mRNA transcript enrichment of 12 GOBs, not significantly influenced by changing environmental factors. Significant influence of pCO₂ (400 and 900 µatm) temperature (25 and 31 °C) and their interaction, on the GOI expression was done according to a 3-Way ANOVA (p<0.05, Table 3). The left panel represent GOIs that no influencing factor (bicA2, ndhD4, ndhF4, chpX, rca, sod) and the right panel represent genes for which time was the only influencing factor. Circles and triangles represent Trichodesmium acclimated to 400 and 900 µatm pCO₂, respectively. Black and open symbols represent Trichodesmium acclimated to 25 °C and 31 °C, respectively. Black and open symbols represent Trichodesmium acclimated to 400 and 900 µatm pCO₂, respectively. Relative abundance estimated according to the 2⁻ΔΔCt method, with 16S rRNA as the endogenous reference gene, and average ΔCt values of the nifH from the 400 µatm / 25 °C acclimation (control) as a calibrator. White and black bars on top of the graphs represent light and dark hours, respectively. n = 3 for all. Errors are ±1 standard deviation, following Bustin et al. (2009). Note: 1. the different y-axes scales; 2. the results and standard deviations are presented using logarithmic scale y axes.

Table S1 Pearson correlations of the enrichment of the 21 genes of interest over the day, 1-1 h after the onset of
light; ii- 3h after the onset of light; iii- 9 h after the onset of light; iv- 13 h after the onset of light. Relative abundance estimated according to the 2-ΔΔCT method, with 16S rRNA as the endogenous reference gene, and average ΔCT values of the nifH from the 400 μm / 25 μm treatments as a calibrator. n = 11-13 for each gene at a given sampling time. Upper value represents the correlation coefficient (r) and lower values represent the significance.

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Author Contributions

Conceived and designed the experiments: OL, SS, JLR, IBF. Performed the experiments: OL, SS, JLR, IBF. Analyzed the data: OL, SS, JLR, IBF. Contributed reagents/materials/analysis tools: OL, SS, JLR, IBF. Wrote the paper: OL, SS, JLR, IBF. Funding: JLR, IBF.
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