

Response to Referee #1

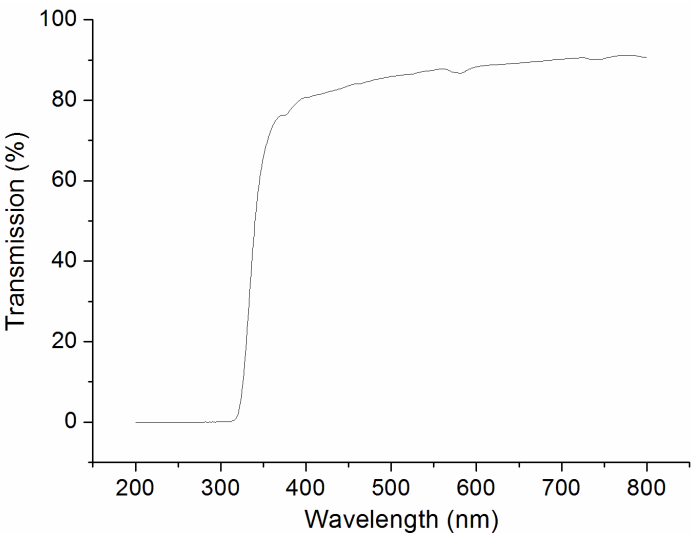
Reviewer: General Comments: This paper focuses on the effects of changing light and CO₂ on diel gene expression of key metabolic genes in the model diatom *Phaeodactylum tricornutum*. The work is motivated by a desire to better understand the effects of climate change drivers (higher CO₂, higher mixed layer irradiances) on diatoms in the ocean. In addition to dramatic diel changes in the expression of many genes, this work documents significant effects of both CO₂ and light on the expression of certain genes. Given the amount of work that has been done on climate change effects on diatoms there has been surprisingly little work on gene expression and so this work does fill a gap there.

However, gene expression is only relevant if interpreted in the context of the overall physiological response of the diatom to changing environmental variables, and in particular if it helps explain biogeochemically relevant responses such as changes in growth rate, nutrient uptake, etc. Here the gene expression data is interpreted in a piecemeal fashion, and while there is an attempt made to connect the response of individual genes to particular physiological responses (e.g. changes in Lhcf3 to NPQ responses) there is **no holistic interpretation** of the data. Finally the experimental design, which includes **rapid, large changes in CO₂ and light intensity** applied for **short duration**, is problematic since the research aims to address climate change variables that will **develop gradually and persist**. The treatments are only applied for several days and so the cells are not likely to have acclimated to the new conditions. **Lack of acclimation** is clear in the gene expression data.

Response: We agree with the reviewer in that the climate change variables will develop gradually and persist, and evolutionary responses to OA (changed carbonate chemistry with elevated CO₂) treatment should be considered, as reported from our group and other labs (Jin et al., 2013, Evolution; Collins et al., 2014, Evol Appl; Mackey et al., 2015, Oceanography; Torstensson et al., 2015, Proc Biol Sci). However, we followed the OA research guide (Riebesell U., Fabry V. J., Hansson L. & Gattuso J.-P. (Eds.), 2010) as we reported previously (Wu et al., BG, 2010; Gao et al., 2012, Nat Clim Change; Jin et al., 2015, Nat Comm) and the acclimation to OA conditions for more than 10 generations in the present work can hardly be considered as lack of acclimation, though adaptational changes would not be apparent in that time frame. In terms of light condition changes, naturally, cells in deeper layers receiving low light could be readily transported to the surface layer by mixing and thus be exposed to high levels of sunlight. In this work, the cells had been acclimated to OA conditions for over 12 generations and to OA+fluctuating light conditions for over 6 generations. Therefore, we believe that our experiments do not suffer from any lack of acclimation. 6-10 generations have been considered enough to examine molecular responses in microalgae to environmental changes, as reflected in other papers (Diner et al., 2015, Mar. Biol.; Hoppe et al., 2015, New Phytologist; Li et al., 2015, BG; Shi et al., 2015, LO). Gradual elevation of pCO₂ has been shown to result in similar physiological changes as one step increase to 800-1000 μ atm in some algae (Zheng Y Q, Ph.D. Thesis, 2009; Low -D écarie et al., 2011, GCB).

Reviewer: Specific Comments: 1. P 15813 line 10. Were the outdoor incubators screened or exposed to full sunlight? A shift from 130 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ to full sunlight is quite severe and certainly more extreme than would be expected under climate change scenarios.

Response: In terms of spectrum of solar radiation, the cells received nearly full sunlight, with most of UV-B irradiances screened out (please note the following transmission figure), because the UV-B could not penetrate the glass bottle. This information was added in M&M (Page 6 line 119).



In terms of mean daily light (PAR) dose, the outdoor cultures received $\sim 220 \mu\text{mol m}^{-2} \text{s}^{-1}$ (mean daytime PAR). Since the cells were transferred to outdoor condition after sunset, they were exposed to gradually increasing sunlight during the next morning. In the natural environment, low-light grown cells can be subject to mixing of the water column and thus periodically exposed to high light. Since we saw differential responses of diatoms growth to OA under different levels of sunlight (Gao et al., 2012 Nat Clim Change), in this work, we designed the experiment to see molecular changes along with physiological performances at different light levels or light regimes.

Reviewer: 2. P 15818 line 18. Upregulation of nitrite reductase at high CO₂ is in contrast to a down regulation of nitrate reductase (gene and activity) at high CO₂ in *T. pseudonana* as found by Shi et al. LO 60 1805-1822. While these are different genes, nitrite reductase is part of the nitrate acquisition pathway and so presumably reflects activity of this pathway. It is interesting to see such different responses between diatom species.

Response: We are grateful for the reviewer to recognize our Xiamen Univ. colleague's work in L&O. Yes, there should be species-specific responses. In addition, since the culture conditions are quite different, we have provided a Table making the comparisons between the two papers.

	Shi et al., 2015 LO	Li et al., BGD
Species	<i>T. pseudonana</i> (CCMP 1335)	<i>P. tricornutum</i> (CCMA 106)
Light	continuous light:	Indoor: 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (12:12=L: D)
conditions	30(low) / 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (high)	Outdoor: Fluctuated light condition

		(supplementary data)																
N source	Nitrate	Aquil medium (Nitrate)																
Treatments		Indoor cultures	Outdoor cultures															
	<table><tr><th>Treatment name</th><th>Light $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$</th><th>$p\text{CO}_2$ (μatm)</th></tr><tr><td>LL400NO₃⁻</td><td>30</td><td>400</td></tr><tr><td>LL800NO₃⁻</td><td>30</td><td>800</td></tr><tr><td>HL400NO₃⁻</td><td>250</td><td>400</td></tr><tr><td>HL800NO₃⁻</td><td>250</td><td>800</td></tr></table>	Treatment name	Light $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	$p\text{CO}_2$ (μatm)	LL400NO ₃ ⁻	30	400	LL800NO ₃ ⁻	30	800	HL400NO ₃ ⁻	250	400	HL800NO ₃ ⁻	250	800	<div>400 μatm 1000 μatm</div> <div>After 12 generations, both LC and HC-grown cells were transferred to outdoor condition.</div>	<div>400 μatm 1000 μatm</div>
Treatment name	Light $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	$p\text{CO}_2$ (μatm)																
LL400NO ₃ ⁻	30	400																
LL800NO ₃ ⁻	30	800																
HL400NO ₃ ⁻	250	400																
HL800NO ₃ ⁻	250	800																
	Cells were harvested after growth in LC and HC for 25(small volume)+6 (larger volume culturing) generations	Cells were harvested after growth in LC and HC for 18 generations under indoor and 6 generations under outdoor conditions, respectively																
Results	The activity, protein and gene expression of NR were lower in high CO ₂ treated cells with the latter being insignificant.	The relative expression of <i>nir</i> was significantly up-regulated by elevated CO ₂ .																

Indeed, Shi et al. (2015) showed down-regulated protein expression (but without significant changes in gene expression) and activity of nitrate reductase for *T. pseudonana* under high CO₂ conditions regardless of light levels. Although both nitrate reductase and nitrite reductase are part of the N assimilation pathway, they are encoded by different genes and may show different responses to changes in pH. Additionally, there are a number of genomic differences between *T. pseudonana* and *P. tricornutum* (Armbrust et al., 2004; Bowler et al., 2008). The different roles of the C4 pathway (Reinfelder et al., 2000; Tachibana et al., 2011; Haimovich-Dayana et al., 2013; Samukawa et al., 2014) and differential responses to environmental changes have also been reported (Yang et al., 2011; Gao et al., 2012; Nymark et al., 2013). Additionally, Hofmann et al. (2013) reported that the activity of nitrate reductase was stimulated by elevated CO₂ in calcifying rhodophyte *Corallina officinalis*. To respond to this inspiring comment, we added discussion at page 15 line 327-340.

Reviewer: 3. P 15818 line 23 – P 15819 line 2. It would be worth exploring the contrast between the previous study, which indicated the combination of high CO₂ and high light was detrimental to growth, and the present study further. In particular what were the light levels in the current and previous study and how might that explain the differences in the finds.

Response: The effects of CO₂ on growth were mediated by light levels (Gao et al., 2012; Hoppe et al., 2015) in cells that had been acclimated to elevated CO₂ over 20 generations. In the present study and previous work, this diatom showed enhanced growth rate under a PAR of 50-150 $\mu\text{mol m}^{-2} \text{ s}^{-1}$, and inhibited growth rates under daytime mean PAR >200 $\mu\text{mol m}^{-2} \text{ s}^{-1}$, after the cells had acclimated to OA over 10 generations. In the present work, shifting from indoor low light to outdoor fluctuating sunlight did not change the growth response, probably due to the lacking of acclimation. The related discussion has been revised at Page13 Line 282-290.

Reviewer: 4. P 15819 lines 3-12. The authors attribute previous inconsistencies in the connection between CCM downregulation and enhanced growth at high CO₂ to differences in growth irradiance among experiments. However, the current data would argue against that as

similar effects of CO₂ on both the CCM and growth are observed at dramatically different light levels.

Response: The reasons for difference in light-mediated effects of CO₂ between the current and previous studies are explained above. The expression of β -ca was significantly decreased by the elevated CO₂ regardless of light levels.

Reviewer: Technical corrections: 1. p 15814 line 4. Should read “relative electron transfer rate”.

Response: Corrected.

Response to Referee #2

Reviewer: General comments: In this manuscript, unialgal cultures of a diatom, *Phaeodactylum tricornutum*, were used to study the effect of ocean acidification and light conditions on physiological parameters and mRNA levels of genes involved in carbon concentrating, nitrogen metabolism, photosynthesis, and respiration. Although similar experiments have been performed in the past, the authors indicated that a factorial treatment of light and CO₂ levels would reveal new insights about responsive mechanisms in this diatom. In such a study, it is important to differentiate whether the cultures are in a transient or an acclimated state. According to the not-so-clear description provided in the manuscript, I am assuming that the cultures were maintained under **Low CO₂ condition before hour 0** (p. 15813, line 6). **As a result, immediately after the onset of the incubation, cells in the Low CO₂ condition were fully acclimated while cells in the High CO₂ condition were in a transient state. At this time, a fair comparison was unachievable between High and Low CO₂ conditions.** During the 8-day incubation period (indoor, low light), **only 4 days were sampled.** This **low sampling frequency** causes difficulties for a reader to evaluate the results, especially for those measurements that showed large differences between day 1 and day 8 (e.g. Fig. 4B). The situation was getting more serious when incubation reached the second half. A sudden switch from indoor to outdoor illumination would surely put the cultures in a **transient state**. After the switch, the incubation lasted for **another 5 days** (day 9 – 13), yet only **3 days** were sampled. This created two questions. The authors should explain in what **way this operation simulated an ocean acidification situation so that an understanding of the transient changes would be essential.** In addition, with vastly different pretreatment and sampling schedules between the indoor and the outdoor incubations, it would be difficult to find statistical procedures to reveal the influences of light intensity and fluctuation. In conclusion, I think highly of the motivation of this research, but am deeply concerned about the experimental design. The sporadic sampling schedule and undefined transient states caused difficulties in quantitative comparisons, especially between the indoor and the outdoor incubations. I think such obtained results are incapable of demonstrating the true effects of CO₂ and light on diatom gene expression.

Response: We believe the reviewer has made an important and inspiring comment here. In natural environments, LC-acclimated or low-light-acclimated cells could be

periodically exposed to high CO₂ waters (such as upwelling areas or other mixed waters) or to high-light exposures when mixed or advected by strong mixing from deeper layers to surface layers. Therefore, laboratory LC-maintained species can be used for OA-related studies, as reported for many other species (Wu et al., 2010; Jin et al., 2013; Pierangelini et al., 2014; Hoppe et al., 2015; Jin et al., 2015; Shi et al., 2015). The important scientific efforts concerning OA-effect studies are to explore mechanistic responses to changed carbonate chemistry. In this work, *Phaeodactylum tricornutum* (strain CCMA 106) was maintained in lab conditions under 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of Photosynthetic Active Radiation (PAR, L: D = 12: 12) and 20 °C without aeration. During the experiment, the cells were cultured in an illuminated CO₂ plant incubator under the same light and temperature conditions but with aeration of ambient (LC) or with air enriched with CO₂. The outdoor culture and sampling were only a check to see if there is difference under the two light regimes, therefore, the outdoor growth can be considered as extended cultures under the same LC and HC with an extra light forcing. Following the internationally recognized OA research protocols (Riebesell U., Fabry V. J., Hansson L. & Gattuso J.-P. (Eds.), 2010), 6-10 generations have been considered enough for phytoplankton to acclimate due to their high growth rates. Our response to the reviewer's comment can be supported by many other studies (Wu et al., 2014, LO; Spijkerman et al., 2014, Photosynth Res; Diner et al., 2015, Mar. Biol.; Hoppe et al., 2015, New Phytologist; Li et al., 2015, BG). We have provided more details about the experimental design at 2.1 Species and culture conditions (Page 5, line 101-108) and 2.2 Experimental set up (Page 5, line 115-126)

Specific comments:

Reviewer: Page 15812 Line 18. Please provide the following information: the dilution rate (% per day) and the time of day when dilution was performed.

Response: At the end of the light period, the dilution was performed with the dilution rate about 300% (one part of the culture was added to two parts autoclaved seawater enriched with Aquil medium and pre-equilibrated with the targeted CO₂ levels). This information was added in 2.3 Growth rates (Page 6 line135-137).

Reviewer: Page 15813 Line 12. The outdoor incubations apparently had a different sampling schedule. Please describe in details.

Response: Based on the light conditions (Fig.S1), the light: dark cycle for outdoor incubations was about 14:10, which was different from the indoor cultures. But both the HC and LC were transferred to outdoor cultures at 20:00 after dilution on the eighth day and we sampled at 12:00, 16:00 and 24:00, respectively, which was the same as the indoor cultures (Fig.2). The outdoor growth can be considered as extended cultures under the same LC and HC with an extra light forcing, so we defined the samples time as: (1) indoor culture: 4 h-l, 8 h-l, 16 h-d, 28 h-l, 32 h-l, 40 h-d, 76 h-l, 80 h-l, 88 h-d, 172 h-l, 176 h-l, 184 h-d; (2) outdoor culture: 196 h-l, 200 h-l, 208 h-d, 220 h-l, 224 h-l, 232 h-d, 268 h-l, 272 h-l and 280 h-d. However, the RNA

extraction from samples at 220 h-l, 224 h-l failed , so, the RT-PCR was skipped for these two points. This information was added in the M&M at 2.2 Experimental set up (Page 6 line 123-126).

Reviewer: Page 15813 Line 22. Here, the growth rate was measured on a daily basis. However, growth rates in Fig. 1 were presented as the mean value of triplicate containers (n=3). Please explain the growth rates on which day (or days) were used to generate the mean.

Response: For the indoor culture, the specific growth rates were obtained from day 3 to day 8; for the outdoor culture, because the cell concentration was determined only on the first day, the growth rate in Fig.1 was presented as growth on the first day. This information was added in 3.1 Growth and photosynthetic performance (Page 9 line 209-210).

Reviewer: Page 15814 Line 22. Please provide the following information: during the incubation period, on which few days that the measurement of P-C curves were performed.

Response: The measurement of P-C curves was performed on the eighth day for indoor cultures and on the first day for the outdoor cultures. This information was added in 2.5 sections (Page 7 line 161).

Reviewer: Page 15815 Line 24. Please provide the following information: expression level at which sampling point in the Low CO₂ group was used as the calibrator.

Response: The first sampling point in the Low CO₂ group, that is LC-4 h-l, was used as the calibrator to calculate the relative mRNA expression. This information was added in 2.6 sections (Page 9 line 196-197).

Reviewer: Page 15816 Line 5. In addition to CO₂ concentrations and indoor/outdoor incubations, light:dark cycle and the progress of acclimation also affect gene expressions in this study. More descriptions are needed to explain how to set up statistical procedures that can properly identify the sources of variations.

Response: Although more factors potentially affect gene expression in this study, we compared the effects of LC and HC at each sampling time point, with all factors kept the same except for the CO₂ level. We added this information in the M&M (Page 9 line 204). Moreover, the influence of the light:dark cycle was obvious in Figs 2.4.5.

Reviewer: Page 15816 Line 18. Please identify the interval that represented the acclimated status with increased F_v/F_m.

Response: We recalculated the increase of F_v/F_m by elevated CO₂ during all the acclimation period, the value was 1.4% (p = 0.36). This information was added in M&M (Page 10 line 219).

Reviewer: Page 15816 Line 21. I am not sure about the result of similar rETR between indoor illumination and fluctuating sunlight. In Fig. 2B, the rETR obviously decreased on the last day under fluctuating sunlight.

Response: That is right. Here, the similar rETR just means that diurnal oscillations were observed in both indoor illumination and fluctuating sunlight. And “the rETR obviously decreased on the last day under fluctuating sunlight” was added in the M & M (page 10 line 227-228).

Reviewer: Page 15817 Line 13. By comparing data points in Figs. 2, 4, and 5, two data points for mRNA expression were missing for all genes measured. Why?

Response: The outdoor growth can be considered as extended cultures under the same LC and HC with extra light forcing, so we defined the samples time as: (1) indoor culture: 4 h-l, 8 h-l, 16 h-d, 28 h-l, 32 h-l, 40 h-d, 76 h-l, 80 h-l, 88 h-d, 172 h-l, 176 h-l, 184 h-d; (2) outdoor culture: 196 h-l, 200 h-l, 208 h-d, 220 h-l, 224 h-l, 232 h-d, 268 h-l, 272 h-l and 280 h-d. However, the RNA extraction for the samples at 220 h-l, 224 h-l failed, so, the RT-PCR was skipped for the two points. This information was added in the M&M at 2.2 Experimental set up (page 6 line 123-126) and 3.3 gene expression (page 11 line 241-242).

Reviewer: Page 15817 Line 17. I did not see higher expression for *Lhcf 3* under high/fluctuating sunlight in Fig. 4B.

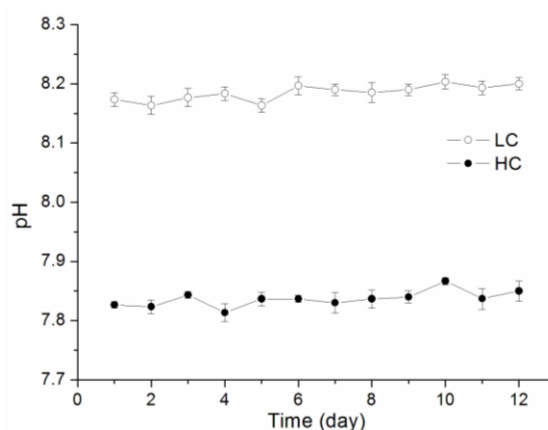
Response: The gene expression for *lhcf 3*, encoding the antennae in the light harvesting complex (LHC), was significantly down-regulated ($p = 0.04$) after the cells had acclimated to HC for a period of 4–48 h, with the highest values obtained 8 h after the onset of light and the lowest values 4 h after the onset of darkness. Significant decreased expression of *lhcf 3* was observed with the elapse of the culture time, but after cells were transferred from indoor to outdoor cultures for 1 day, a slight increase in expression was observed, especially for LC-grown cells (Fig. 4b). This information was added in 3.3 gene expression (Page 11 line 248-354).

Reviewer: Page 15819 Line 12. I did not see evidence for high CO₂ stimulating RbcL expression in Fig. 4C.

Response: The reviewer is right; the expression of *rbcl* was enhanced by elevated CO₂ at hour 28-40, hour 172-184, but being only significant at hour 32. This was added in M&M (Page 13 line 299-300).

Reviewer: Page 15820 Line 10. If the authors want to conclude that an increase in NiR expression was caused by acidification, the time course of pH values during the incubation period should be shown.

Response: The pH values during the incubation period were shown as follows, and the parameters of the seawater carbonate system calculated from pH and pCO₂ measurements with CO2 SYS software (Lewis and Wallace, 1998), using the values for the equilibrium constants K1 and K2 for carbonic acid dissociation from Roy et al. (1993) and that for KB for boric acid of Dickson (1990) were shown in Supplementary information.



Reviewer: Page 15820 Line 17. The expression levels of both *Lhcf 3* and *mATP* increased at hour 32. I do not think the expression was totally suppressed in the initial phase of high CO₂. In addition, I do not understand how “6 generations” is defined.

Response: (1) For HC and LC grown cells, the expression levels of both *lhcf 3* and *mATP* increased at hour 32, the endogenous rhythm maybe the reason. Additionally, a slight down-regulation was observed in HC-grown cells (Fig.4b and Fig.5a).

(2) The specific growth rate (μ) was about 1.01 and 1.096 d⁻¹ for LC and HC-grown cells, the generations were calculated as: Generations = $\mu / \ln(2)$ * days.

Based on above information, this sentence was rewritten as: In the present study, although the genes encoding FCP and mtATP synthase were slightly suppressed during the initial phase of OA (at the initial 2 days), they increased their expression levels under the OA treatment after the cells had acclimated for 6 generations, indicating a homeostatic response to an acidic perturbation to the cells (Giordano, 2013). This information was added in Page 15 line 345-349.

Additionally, the combined effects of light and CO₂ were complex (Tanaka et al., 2015), light signals may regulate multiple stages of gene expression, which cross-talk with CO₂ signals. All these interactions need to be further studied.

Reviewer: Page 15821 Line4. The induction of beta-CA lasted for only one day. I do not think this result should be viewed as a permanent effect of ocean acidification.

Response: During the light period, the expression of β -ca was down regulated by elevated CO₂, not only for one day (Fig4a). Furthermore, there are many studies showed the similar effects of OA on CA (Johnston and Raven, 1996; Matsuda et al.,

2001; Kucho et al., 2003; Vance and Spalding, 2005; Hopkinson et al., 2011; Matsuda et al., 2011)

Reviewer: Page 15821 Line 9. I do not understand in what ways the gene expressions were linked to photo-protection and energy balance. Another question is why these expression patterns appeared only for one day.

Response: *Lhcf*, encoding photosynthesis light harvesting antenna, and *mATP*, encoding mitochondrial ATP synthase, are responsible for the light absorption and energy transfer processes which were influenced by the light conditions. Other genes including in this study also took part in energy transfer and then were linked to photo-protection. Of course, we should accept that photo-protection and energy balance are complex processes, involving many more genes/proteins. Elevated CO₂ caused different sets of gene regulation in transition and steady state in *T. pseudonana* (Hennon et al., 2015). It is tempting to postulate that the fact that expression patterns of *lhcf* and *mATP* only appeared for one day is due to their roles in energy balance and light harvesting in a transition state during acclimation to elevated CO₂.

Reviewer: Technical corrections: Page 15817, Line 24. Fig. 4C should be cited here.

Response: Corrected as suggested.

Reviewer: Figs 2, 4, 5. Labels on the x-axis should be in multiples of 24.

Response: Corrected as suggested.