

Uncoupling the Effects of pH and $p\text{CO}_2$: How *Phaeocystis globosa* Adapts its Nitrogen Assimilation to an Acidifying Ocean

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Abstract

Ocean acidification (OA) fundamentally reshapes the chemical landscape of coastal waters, yet the mechanistic basis for the resilience and metabolic adaptation of the bloom-forming alga *Phaeocystis globosa* remains poorly understood. By disentangling the individual and interactive effects of elevated $p\text{CO}_2$ and reduced pH, this study shows that *P. globosa* exhibits distinct physiological and metabolic responses to these stressors. Reduced pH emerged as the stronger physiological constraint, significantly inhibiting growth, whereas the reduction in relative maximum electron transport rate ($r\text{ETR}_{\text{max}}$) was not statistically significant; these responses override the potential benefits of CO_2 fertilization. Transcriptomic profiling revealed diver-specific reprogramming of carbon and nitrogen metabolism: reduced pH induced coordinated down-regulation, elevated $p\text{CO}_2$ coordinated up-regulation, and combined stress opposing trends. Key nitrogen genes (NR, NiR, GS) shifted phase-dependently. The absence of detectable CPS and OTC transcripts, together with multiple pathways potentially contributing to endogenous ammonium supply, points to a potentially truncated urea cycle and flexible nitrogen utilization. As an exploratory transcriptomic analysis, these results suggest that *P. globosa* may possess ecological resilience under future acidification.

1 Introduction

Atmospheric CO_2 has risen rapidly from approximately 280 ppm pre-industrially (Zhang and Fischer 2014) to the current 427.35 ppm (Lan et al. 2025), and is projected to reach 900–1000 ppm by 2100 under high-emission scenarios (IPCC 2023). Ocean uptake of approximately one-third of anthropogenic CO_2 drives ocean acidification (OA), with surface pH projected to decline by 0.3–0.4 units to 7.7–7.8 by 2100 (Huang and Drange 1996; Sabine et al. 2004; Orr et al. 2005). These concurrent changes in seawater carbonate chemistry can significantly alter phytoplankton physiological and community composition (Dutkiewicz et al. 2015), with particular concern for harmful algal bloom (HAB) species that may gain selective advantages under elevated CO_2 , and are capable of forming blooms at CO_2 concentrations projected for the end of this century (Riebesell et al. 2018). However, disentangling the biological responses to $p\text{CO}_2$ enrichment from those driven by pH reduction is essential for resolving the conflicting signals of carbon fertilization and proton toxicity—two bioenergetically opposing forces that together shape phytoplankton resilience in an acidifying ocean. To date, uncoupling experiments have demonstrated that the responses of marine microalgae to OA are multifaceted. For instance, in coccolithophores *Emiliania huxleyi*, calcification is primarily regulated by HCO_3^- concentration rather than by pH or carbonate saturation, whereas photosynthesis is limited under low CO_2 but is also directly affected by low pH (Bach et al. 2013). In diatoms, the suppressive effect of acidification is often attributed to proton stress rather than the direct influence of CO_2 (Shi et al. 2010). Nevertheless, most studies have focused on carbon acquisition or calcification, leaving nitrogen metabolic networks—a critical determinant of bloom dynamics (Anderson et al. 2002)—largely unexplored, particularly in haptophyte species such as *Phaeocystis globosa*. Understanding species-specific responses to OA is therefore crucial for predicting future HAB dynamics and their ecological consequences.

Haptophyceae *Phaeocystis globosa* is a species of global concern, forming recurrent blooms in temperate and subtropical coastal waters worldwide (Schoemann et al. 2005; Wang et al. 2021) that cause oxygen depletion, foam formation, disruption of marine food webs (Riegman et al. 1992), and production of hemolytic compounds that threaten aquaculture (Song et al. 2023). A defining feature of *P. globosa* ecology is its complex life cycle, alternating between free-living flagellated cells and mucilaginous colonies (Rousseau et al. 1994), which confer resistance to grazing (Verity et al. 2007). However, intraspecific variation exists within the genus, with some species remaining exclusively solitary throughout their life cycle (Rousseau et al. 2007; Verity et al. 2007). Under favorable environmental conditions, *P. globosa* tends to form dense blooms predominantly in the colonial form (Lancelot et al. 1994). The solitary cell stage serves as a precursor to colony formation, and colony development is associated with dramatic changes in ecosystem structure and biogeochemical cycling (Lancelot et al. 1994; Verity et al. 2007). This species exhibits high sensitivity to environmental variability, with growth, colony formation, and metabolic activity strongly regulated by temperature, irradiance, salinity, and nutrient availability (Xu et al. 2017; Wang et al. 2021). In particular, the ongoing trend of ocean acidification has emerged as a decisive factor modulating its ecological fitness. For instance, elevated $p\text{CO}_2$ has been shown to profoundly alter the photophysiology of *P. globosa* and shift its morphotype partitioning, which significantly increases colony growth rate (Wang et al. 2010). These CO_2 -driven effects are further mediated by light intensity; for example, high $p\text{CO}_2$ increases non-photochemical quenching (NPQ) under strong sunlight but reduces it in low light (Chen and Gao 2011). Chen et al. (2014) demonstrated that *P. globosa* acclimates to reduced pH by increasing energy capture and decreasing non-photochemical energy loss, indicating a reallocation of cellular energy resources (Chen et al. 2014). However, the extent to which acidification-driven adjustments in photosynthetic energy use are accompanied by transcriptional reprogramming of nitrogen assimilation remains unclear.

OA fundamentally alters seawater carbonate chemistry and modifies phytoplankton C:N and C:P stoichiometry (Riebesell et al. 2007; van de Waal et al. 2010). These shifts necessitate metabolic adjustments of phytoplankton to maintain balanced growth under altered resource availability. Critically, OA effects on phytoplankton are strongly modulated by nitrogen availability: in oligotrophic waters, elevated CO_2 ($\sim 700 \mu\text{atm}$, simulating the 2100 moderate-emission RCP 6.0 scenario) suppressed eukaryotic phytoplankton growth by up to 30%, effects fully reversed by nitrate enrichment (Dai et al. 2025). Similarly, Proteomic analyses of the model marine diatom *Thalassiosira pseudonana* further demonstrated coordinated downregulation of key nitrogen assimilation enzymes, including glutamine synthetase and aspartate aminotransferase, under CO_2 limitation, consistent with active carbon-nitrogen homeostasis during inorganic carbon acclimation (Clement et al. 2017). Nitrogen metabolism of phytoplankton is therefore central to OA acclimation. For *P. globosa*, despite proteomic evidence that nitrate metabolism proteins are upregulated during blooms (Zhang et al. 2022c), transcriptional regulation of nitrogen pathways under OA remains unexplored. This knowledge gap limits understanding of how *P. globosa* will adjust nitrogen metabolism to cope with future ocean acidification and how these adjustments may influence its competitive fitness and bloom potential.

Transcriptomic profiling provides a powerful approach for dissecting metabolic regulation in phytoplankton, enabling identification of genes whose expression is remodeled under environmental stress (Beszteri et al. 2012; Rastogi et al. 2018). In this study, solitary cells of *P. globosa* were cultured under conditions simulating projected end-of-century OA (1,000 ppm CO₂, pH 7.8) alongside present-day controls (400 ppm CO₂, pH 8.1), with additional treatments isolating the effects of elevated CO₂ (1,000 ppm CO₂, pH 8.1) and reduced pH (400 ppm CO₂, pH 7.8). Physiological responses were assessed through measurements of cell abundance and photosynthetic performance. At the same time, transcriptomic profiling via RNA-sequencing systematically characterized expression changes in nitrogen metabolic pathways, including genes involved in nitrate and ammonium uptake, assimilation, and allocation. Expression patterns of key nitrogen metabolism genes were further validated by quantitative real-time PCR (RT-qPCR). Accordingly, this study aimed to disentangle the effects of reduced pH and elevated *p*CO₂ on the physiological and nitrogen metabolic responses of *P. globosa* under OA. It was hypothesized that (1) reduced pH would exert a stronger constraint on growth and photosynthetic performance than elevated *p*CO₂, (2) elevated *p*CO₂ would promote carbon metabolism and modify nitrogen metabolic responses, and (3) combined reduced pH and elevated *p*CO₂ would induce a distinct transcriptional response in nitrogen metabolism compared with either stressor alone. These hypotheses were examined by integrating physiological measurements, transcriptomic profiling, and time-resolved qPCR validation of key nitrogen assimilation genes.

2 Materials and methods

2.1 Algal strain, medium preparation, and culture conditions

P. globosa used in this study was maintained in the College of Environmental Science and Engineering, Ocean University of China. This strain represents the solitary morphotype that remains in the free-living state throughout its life cycle, allowing focused investigation of cellular-level responses without the confounding effects of colony formation and extracellular matrix production. Use of the solitary morphotype facilitates physiological measurements and molecular analyses while still capturing fundamental metabolic responses relevant to bloom initiation in natural populations. Seawater (salinity ~ 35) was collected from coastal waters off Qingdao, filtered through a 0.45 µm membrane, and sterilized in an autoclave (MLS-3750, SANYO) at 121°C and 100 kPa for 20 min. After cooling to room temperature, nutrient components were added following the f/2 medium recipe (Smith and Chanley 1975).

Exponentially growing *P. globosa* cells pre-acclimated to each treatment were inoculated into acid-washed, autoclaved Erlenmeyer flasks and gently mixed. Cultures were maintained in an illuminated incubator (GXZ-280C, JiangNan) under a 12 h:12 h light-dark cycle at 20 ± 1°C, with a light intensity of 80–100 µmol m⁻² s⁻¹. Cell density was regulated by aeration rate and cellular biomass, and pH fluctuations were kept within 0.05 units to ensure system stability. The initial cell density was adjusted to

1.0×10^5 cells mL⁻¹. Medium was renewed every 24 h by removing a calculated volume of culture based on cell density and adding fresh medium to maintain sufficient nutrient supply and sustain exponential growth (Hutchins et al. 2003). Cultures were gently shaken three times daily to prevent cell sedimentation.

2.2 Seawater acidification treatments

To distinguish the individual and combined effects of pH and $p\text{CO}_2$ on *P. globosa*, two pH levels and two CO_2 partial pressures were applied. The selected $p\text{CO}_2$ levels represented present-day seawater conditions (400 ppm) and the projected end-of-century concentration (1,000 ppm) under high-emission IPCC scenarios. Corresponding pH values were set to 8.1 (current) and 7.8 (expected at ~ 1000 ppm CO_2). Four $p\text{CO}_2$ -pH treatment combinations were therefore established: treatment A (400 ppm, pH 8.1; PG48), treatment B (400 ppm, pH 7.8; PG47), treatment C (1000 ppm, pH 8.1; PG18), and treatment D (1000 ppm, pH 7.8; PG17). Because pH tends to increase as biomass accumulates, maintaining stable pH conditions throughout cultivation can be difficult. To limit pH drift, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (HEPPS) was added as a buffering agent (Shi et al. 2009). A concentrated HEPPS stock solution was prepared, filtered through a 0.22 μm syringe filter (Millipore), and stored at 4°C. The final concentration of HEPPS in cultures was maintained at 5 mM.

Acidified conditions were generated using a CO_2 enrichment system (Qingdao Haixing Instrument Co., Ltd.), which mixed high-concentration CO_2 with air to achieve the target $p\text{CO}_2$ levels. To avoid microbial contamination during aeration, the mixed gas was passed through a 0.22 μm syringe filter before entering the culture vessel. Gas exchange occurred across the air-liquid interface rather than by direct bubbling, preventing mechanical damage to cells while maintaining carbonate system equilibrium.

2.3 Determination of carbonate system parameters

Accurate characterization of carbonate system conditions is essential for ensuring consistency between the intended and actual acidification treatments. During cultivation, pH and dissolved inorganic carbon (DIC) were quantified as key carbonate system parameters. Throughout the experimental period, the pH meter was calibrated daily using a three-point NBS standard solution, and pH values of the cultures were monitored accordingly. Before and after *P. globosa* cultivation, DIC concentrations in seawater were measured using an inorganic carbon analyzer. The measurement principle was based on establishing a calibration curve using sodium carbonate standards, followed by calculation of DIC concentrations in the samples. Actual $p\text{CO}_2$ levels in the culture medium were calculated using the CO2SYS software package (Pierrot et al. 2006). Temperature and salinity were entered into the “Input conditions” and “Output conditions”, and measured pH and DIC values were subsequently input to compute the corresponding $p\text{CO}_2$ concentrations.

2.4 Measurement of physiological parameters

Each day, 1 mL of culture was collected at a fixed time and preserved using Lugol's solution (Solarbio). A 100 μL aliquot was transferred to a phytoplankton counting chamber (PURITY, Beijing) and enumerated

under a BX51 optical microscope (Olympus, China). The specific growth rate (SGR, μ) was calculated according to Eq. (1):

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1}$$

1

where N_2 and N_1 represent cell densities at times t_2 and t_1 , respectively.

For photophysiological measurements, 2 mL of culture from each treatment was collected daily at a fixed time. After 15 min of dark acclimation, samples were analyzed using a chlorophyll fluorometer (Zequan Technology Co., Ltd.) to determine the maximum quantum yield of photosystem II (F_v/F_m). To further characterize photosynthetic performance, photosynthetically active radiation (PAR) was applied across a series of irradiance levels. Rapid light curves (RLC) were generated and fitted using the exponential-photoinhibition (EP) model, allowing calculation of the photosynthetic efficiency (α) and the relative maximum electron transport rate ($rETR_{max}$), which reflect the light-harvesting capacity of the phytoplankton.

2.5 RNA extraction and transcriptome sequencing

To minimize growth phase-associated transcriptional noise and capture transcriptional reprogramming under sustained acidification rather than acute stress, the samples for transcriptomic analysis were collected on day 6, when cultures had reached a quasi-steady state under semi-continuous conditions. 30 mL of culture from each of the 4 treatments was concentrated by filtration using 10- μ m pore-size membranes (Jinjing), with 3 biological replicates per treatment. Cells retained on the membranes were rinsed with 1 mL of PBS into 1.5-mL microcentrifuge tubes and centrifuged at 8000 g for 2 min at 4°C. The supernatant was discarded, and the resulting cell pellets were transferred to cryovials. The 3 biological replicates from each treatment were subsequently pooled, rapidly frozen in liquid nitrogen for 15 min, and stored at -80°C to prevent RNA degradation. Total RNA was extracted following the manufacturer's instructions of the RNAiso Easy kit (TaKaRa). Library preparation and paired-end sequencing were performed on the Illumina NovaSeq 6000 platform (Nanjing Personal Gene Technology Co., Ltd.).

2.6 Transcriptomic data processing

Raw sequencing data (FASTQ files) were filtered to remove adapter-containing and low-quality reads, yielding clean reads for subsequent analyses. To assess the feasibility to a reference-based assembly strategy, the clean reads were mapped against the chromosome-scale genome of *P. globosa* (strain CNS00066) recently published by Chen et al. (2024). However, the overall mapping rate was extremely low (only 0.92%), which is consistent with the substantial intraspecific genomic variations documented among different *P. globosa* strains (Chen et al. 2024). Given this exceptionally low mapping rate, a reference-guided approach was deemed unsuitable. Therefore, transcript assembly was performed using

Trinity. For functional annotation, the longest transcript within each gene was selected as the representative sequence (Unigene). Gene function was annotated against databases, including NR and GO. Gene expression changes between treatments were evaluated based on log₂ fold change values, and genes with an absolute log₂ fold change ($|\log_2\text{FoldChange}|$) > 1 were retained as candidate differentially expressed genes (DEGs) for downstream analyses. KEGG enrichment analysis of these candidate DEGs was conducted using the Goseq package in R to identify major enriched pathways and pathways associated with transcriptional variation. KEGG metabolic pathways involving the DEGs were subsequently categorized, and a comprehensive pathway map covering all functions within nitrogen metabolism was constructed.

2.7 Molecular response of key nitrogen metabolism genes to seawater acidification

To evaluate the molecular responses of key genes involved in nitrogen metabolism to seawater acidification, nitrate reductase (NR), nitrite reductase (NiR), and glutamine synthetase (GS) were selected based on the assembled transcriptome data of *P. globosa*. Gene-specific primers were designed using Primer 5.0 (Table 1), and quantitative real-time PCR (qPCR) was performed. Actin was used as the internal reference gene to normalize target gene expression. The RT-qPCR program consisted of an initial incubation at 50°C for 2 min, followed by pre-denaturation at 95°C for 10 min; amplification was then performed with 40 cycles of 95°C for 15 s and annealing at 61°C for 1 min.

Table 1
Primer information for key nitrogen-metabolism genes of *P. globosa*

Gene Name	Abbreviation	Primer Sequence (5'-3')	Product Size (bp)
Actin	ACT	TCCTCTCCTCGCTCTCCACCTT	187
		CGTCCATCACTCGGCTGTCTCA	
Nitrate reductase	NR	CCTGGCGGCTCCTCTTCAATTC	269
		AAGCGGAAGCGACGAGTGTTG	
Nitrite reductase	NiR	GCTCGGCTGATGTGGCTCATTG	172
		CCTCCTGTGCCTGCTTGTGAAC	
Glutamine synthetase	GS	GCGTCAAGGCAGTCAAGGCATT	192
		CGGAGATGAAGCGACCGAAGGA	
Relative gene expression levels under different treatments were determined from C _t values using the 2 ^{-ΔΔC_t} method to ensure accurate comparisons of differential expression and primer amplification efficiency (E) (Livak and Schmittgen 2001; Bustin et al. 2009). Calculations were performed according to formulas (2)–(4):			

Differential expression multiples = 2^{-ΔΔC_t} (2)

$\Delta\Delta C_t = (C_{t \text{ objective gene}} - C_{t \text{ internal reference gene}})_{\text{treatment}} - (C_{t \text{ objective gene}} - C_{t \text{ internal reference gene}})_{\text{control}}$ (3)

$E = 10^{-\frac{1}{k}} - 1$ (4)

where k is the slope.

3 Results

3.1 Changes in the carbonate system during incubation

The carbonate system parameters of the four experimental groups (PG48, PG47, PG18, and PG17) at the beginning and end of incubation are summarized in Table 2. Initially, the $p\text{CO}_2$ values of the four groups were 418 ± 6 ppm, 428 ± 2 ppm, 1050 ± 4 ppm, and 1076 ± 3 ppm, respectively. At the end of incubation, the corresponding values were 401 ± 4 ppm, 422 ± 16 ppm, 1037 ± 4 ppm, and 1044 ± 3 ppm, showing varying degrees of decline compared to the initial levels. The initial pH values were 8.15 ± 0.01 , 7.81 ± 0.01 , 8.07 ± 0.01 , and 7.77 ± 0.01 , while the final pH values were 8.13 ± 0.01 , 7.85 ± 0.01 , 8.07 ± 0.01 , and 7.82 ± 0.01 , indicating slight variations of 0–0.05 units. Throughout the incubation, carbonate system parameters remained within a controlled and reasonable range (Shi et al. 2015), confirming that the experimental conditions were stable and met the design requirements.

Table 2
Carbonate chemistry of *P. globosa* grown for each $p\text{CO}_2$ -pH treatment

Incubation period	Groups	$p\text{CO}_2$ /ppm	pH	DIC/($\mu\text{mol/kg}$)
Start	PG48	418 ± 6	8.15 ± 0.01	2587 ± 10
	PG47	428 ± 2	7.81 ± 0.01	1146 ± 17
	PG18	1050 ± 4	8.07 ± 0.01	5380 ± 23
	PG17	1076 ± 3	7.77 ± 0.01	2636 ± 7
End	PG48	401 ± 4	8.13 ± 0.01	2378 ± 15
	PG47	422 ± 16	7.85 ± 0.01	1215 ± 9
	PG18	1037 ± 4	8.07 ± 0.01	5317 ± 13
	PG17	1044 ± 3	7.82 ± 0.01	2888 ± 8

3.2 Differences in the growth of *P. globosa* cells under different culture conditions

The effects of different treatments on the growth of *P. globosa* are shown in Fig. 1. As indicated by the trends in cell abundance and specific growth rate (SGR, μ), the algal cells were still acclimating to the

experimental conditions during the first 3 days; and after this adaptation period, they entered a relatively stable growth phase. From day 4 to day 8, the cell abundances ($\times 10^5$ cells mL⁻¹) of PG48 and PG18 remained consistently higher than those of PG47 and PG17, with ranges of 2.39–2.59, 2.59–2.79, 2.04–2.21, and 2.03–2.14, respectively. Further analysis of the abundance revealed distinct environmental modulation patterns. Under the same pH condition, PG18 showed a significantly higher cell abundance than PG48 ($p_{\text{adj}} < 0.05$), whereas PG17 exhibited no significant difference and was slightly lower than PG47, indicating that elevated CO₂ concentration did not produce a consistent standalone effect on cell accumulation. In contrast, at the same CO₂ level, cultures grown at pH 7.8 (PG47 and PG17) exhibited significantly lower cell abundances than those at pH 8.1 (PG48 and PG18). When $p\text{CO}_2$ was 400 ppm, the cell abundance of PG47 was significantly lower than that of PG48 ($p_{\text{adj}} < 0.01$); when $p\text{CO}_2$ was 1000 ppm, the cell abundance of PG17 was also significantly lower than that of PG18 ($p_{\text{adj}} < 0.001$). Under the coupled conditions of elevated $p\text{CO}_2$ and declining pH, PG17 showed significantly lower cell abundance ($p_{\text{adj}} < 0.001$) than PG48, indicating that OA suppressed *P. globosa* growth.

3.3 Chlorophyll fluorescence parameters

The daily variations in the photosynthetic parameters of *P. globosa* under the four treatments are shown in Fig. 2.

F_v/F_m in all treatments increased during the first 48 h and then stabilized, indicating rapid cellular acclimation (Fig. 2a–d). After adaptation, F_v/F_m peaked on day 7 in PG48, PG47, and PG18 and on day 8 in PG17 (0.637), followed by slight declines. The 8-day mean F_v/F_m values were 0.550 (PG48), 0.548 (PG47), 0.543 (PG18), and 0.558 (PG17). Pairwise comparisons showed only minor differences among treatments, and none of these differences were statistically significant ($p_{\text{adj}} > 0.05$). However, when examining the directional responses, a distinct pattern emerged: under the same pH, elevated CO₂ decreased F_v/F_m at pH 8.1 (PG48 > PG18) but increased it at pH 7.8 (PG47 < PG17); under the same CO₂ concentration, reduced pH decreased F_v/F_m at 400 ppm (PG48 > PG47) but increased it at 1000 ppm (PG18 < PG17). This crossover pattern suggests that the effects of elevated $p\text{CO}_2$ and reduced pH on F_v/F_m are interdependent rather than independent. Overall, short-term acidification did not significantly affect F_v/F_m , suggesting that neither elevated $p\text{CO}_2$ nor reduced pH altered the maximum photochemical efficiency of photosystem II (PSII).

Across all treatments, α showed a general upward trend during incubation and peaked on day 6 or 7. PG48 and PG17 both reached maximum values on day 7, whereas PG47 and PG18 peaked on day 6 (Fig. 2e–h). The ranges of α overlapped across treatments, with PG17 exhibiting the highest peak (0.226). Notably, α exhibited the same crossover response pattern as F_v/F_m with respect to $p\text{CO}_2$ and pH: at pH 8.1, elevated CO₂ reduced α (PG48 > PG18), whereas at pH 7.8, it enhanced α (PG47 < PG17); at 400 ppm, reduced pH decreased α (PG48 > PG47), whereas at 1000 ppm, it increased α (PG18 < PG17).

rETR_{max} exhibited pronounced fluctuations throughout incubation (Fig. 2i–l). PG48 reached its maximum on day 6, whereas PG47 and PG18 peaked on day 4, and PG17 on day 8. The 8-day mean ETR_{max} values were 263.1 (PG48), 228.6 (PG47), 263.2 (PG18), and 239.4 (PG17). Unlike F_v/F_m and α , rETR_{max} exhibited a consistent and directional response to acidification. When pH was held constant, elevated CO₂ (1,000 ppm) had little effect on rETR_{max} in PG18 vs. PG48 (+ 0.03%) but increased in PG17 vs. PG47 (+ 4.73%). When CO₂ concentration was held constant, reduced pH from 8.1 to 7.8 decreased rETR_{max} (PG47 vs. PG48, – 13.12%; PG17 vs. PG18, – 9.05%).

3.4 Overview of transcriptomic characteristics and enrichment analysis of differentially expressed genes (DEGs)

A total of 60,634,610 (PG48), 42,245,570 (PG47), 36,381,426 (PG18), and 58,310,892 (PG17) raw reads were obtained under the four culture conditions. After removing low-quality and adapter-containing reads, the clean reads were assembled into 32,443 unigenes with a total length of 38,040,398 bp, ranging from 301 bp to 21,728 bp, and an average length of 1,172.53 bp. Functional annotation of the 32,443 unigenes was performed against six public databases, including KEGG and GO (Table S1). Among them, 15,145 unigenes (46.68%) were annotated in the NR database, representing the highest proportion. Additionally, 5,635 unigenes (17.37%) were annotated in GO, 7,999 (24.66%) in KEGG, 14,042 (43.28%) in Pfam, 13,534 (41.72%) in eggNOG, and 3,148 (9.70%) in SwissProt. A total of 3,148 unigenes (9.7%) were annotated across all six databases.

Functional annotation of unigenes against the eggnog, GO, and KEGG databases revealed broad coverage of biological functions (Fig. S1a–c). In the eggNOG database, posttranslational modification, protein turnover, and chaperones represented the largest functional category (1,515 genes, 11.19%), followed by signal transduction (1,046 genes, 7.73%) and metabolic functions including amino acid transport and metabolism (874 genes, 6.46%), energy production and conversion (834 genes, 6.16%), and lipid and carbohydrate transport and metabolism (759 and 752 genes, 5.61% and 5.56%, respectively). GO annotation assigned 5,635 unigenes of *P. globosa* across 57 subcategories classified into 3 major categories (BP, CC, and MF), cellular process and metabolic process dominating BP, cell and cell parts in CC, and binding and catalytic activity in MF. KEGG database annotated unigenes into 5 functional groups, carbohydrate metabolism (776 genes, 9.70%) and signal transduction (751 genes, 9.39%) being the most enriched pathways. These results indicate that *P. globosa* transcriptome functions are mainly enriched in basic metabolic and catalytic processes.

Candidate DEGs in *P. globosa* were identified based on an absolute log₂ fold-change threshold of > 1 (Fig. 3). Relative to PG48, PG47 (low pH) and PG18 (high pCO₂) exhibited 5950 (2692 down, 3258 up) and 5170 (2078 down, 3092 up) candidate DEGs, respectively. Notably, the combined-stress treatment (PG17) showed the largest number of DEGs, with 6095 genes exceeding the fold-change threshold, including 3062 down-regulated and 3033 up-regulated genes.

To further explore the metabolic pathways in which DEGs may function, a KEGG enrichment analysis was performed. In total, 5950, 5170, and 6095 DEGs were enriched in 129, 130, and 133 different metabolic pathways in the populations adapted to low pH, high CO₂, and its combination with low pH, respectively. Detailed information on all enriched pathways is provided in Tables S2-S4. Based on the KEGG database, the top 20 enriched pathways in each comparison were categorized according to Level 1 classification (Fig. 4a–c). Several pathways were commonly enriched across all 3 comparisons relative to PG48, although their expression profiles differed among treatments. Protein processing in endoplasmic reticulum (ER) was enriched in all 3 comparisons, with 40 up- and 20 down-regulated genes in PG47, 20 up- and 25 down-regulated genes in PG18, and 19 up- and 38 down-regulated genes in PG17. Fatty acid metabolism was also enriched across all 3 comparisons, with more up-regulated genes in PG18 (21 up, 13 down) but more down-regulated genes in PG47 (13 up, 23 down) and PG17 (8 up, 18 down). Besides the 2 pathways above, several other pathways were also commonly enriched across all 3 comparisons, including Metabolic pathways, Biosynthesis of secondary metabolites, Carbon metabolism, Biosynthesis of amino acids, Glycolysis/Gluconeogenesis, Ribosome, and Motor proteins. Other pathways showed treatment-specific enrichment patterns. PG47 uniquely enriched Glyoxylate and dicarboxylate metabolism (7 up, 15 down), Carbon fixation by Calvin cycle (6 up, 14 down), Plant-pathogen interaction (13 up, 11 down), and Plant hormone signal transduction (9 up, 11 down). PG18 showed enrichment of multiple amino acid and central carbon metabolism pathways, including Lysine degradation (14 up, 11 down), Tryptophan metabolism (13 up, 7 down), Pyruvate metabolism (19 up, 13 down), Citrate cycle (15 up, 6 down), and 2-Oxocarboxylic acid metabolism (20 up, 8 down). In PG17, DNA replication exhibited a pronounced up-regulation pattern (28 up, 2 down), accompanied by up-regulation of Base excision repair (26 up, 2 down), Mismatch repair (19 up, 1 down), and Nucleotide excision repair (19 up, 3 down). In contrast, Protein processing in ER (19 up, 38 down), Endocytosis (9 up, 20 down), Peroxisome (9 up, 16 down), and Ubiquitin mediated proteolysis (10 up, 17 down) showed predominantly down-regulated expression.

Since the impact of acidification on nitrogen metabolism is the focus of this study, the changes in nitrogen-related pathway under the 3 comparison conditions were further analyzed (Fig. 2S). Compared with PG48, Nitrogen metabolism (ko00910) was enriched in all 3 comparison groups, involving 11 genes in PG47 (45.5% up-regulated), 7 genes in PG18 (85.7% up-regulated), and 5 genes in PG17 (60.0% up-regulated). Carbon metabolism (ko01200) was also enriched in all 3 comparison groups, with 69 genes in PG18 (71.0% up-regulated), showing the strongest up-regulation trend, followed by 49 genes in PG17 (40.8% up-regulated) and 54 genes in PG47 (33.3% up-regulated). In addition to these 2 core metabolism pathways, several amino acid metabolism pathways closely related to nitrogen metabolism were also enriched across all 3 comparisons, including Arginine biosynthesis, Arginine and proline metabolism, Alanine, aspartate and glutamate metabolism, Cysteine and methionine metabolism, and Glycine, serine and threonine metabolism. The expression of these pathways was predominantly up-regulated in PG18, relatively balanced in PG47, and highly variable in PG17. Notably, in PG17, all 17 genes related to Arginine and proline metabolism were down-regulated, and only 16.7% of genes in the Arginine

biosynthesis were up-regulated. Glutathione metabolism in PG17 showed an up-regulated pattern, with 18 genes enriched, of which 72.2% were up-regulated.

3.5 Assembly of the cellular nitrogen metabolic pathway

A total of 15 nitrogen-metabolism-related enzymes (Table S5) were identified through functional annotation and analysis of the *P. globosa* transcriptome. Based on KEGG pathway analysis, the cellular nitrogen metabolic pathway of *P. globosa* was reconstructed (Fig. 5). Two key enzymes involved in nitrate assimilation-nitrate reductase (NR) and nitrite reductase (NiR), were annotated in *P. globosa*. The organism harbors two types of glutamate synthase, GLT1 (NADH-dependent) and GLU (ferredoxin-dependent), which utilize NADH and ferredoxin, respectively, as electron donors in glutamate synthesis. In addition to nitrate assimilation and the GS/GOGAT cycle, three major endogenous ammonium-supplementing mechanisms were identified in the nitrogen metabolic network of *P. globosa*: urea degradation, cyanate degradation, and the catalytic conversion of formamide. Cyanate lyase (CYN) catalyzes the conversion of cyanate into ammonia and CO₂. Carbonic anhydrase (CA) reversibly interconverts CO₂ and HCO₃⁻, indirectly supporting this reaction by modulating the bicarbonate pool. Formamidase (FMD) catalyzes the hydrolysis of formamide to produce ammonia, while the resulting by-product, formate, can subsequently enter methane and glyoxylate metabolism, thereby linking nitrogen metabolism to carbon metabolic pathways. Urease (URE) catalyzes the conversion of urea into HCO₃⁻ and ammonia, with the HCO₃⁻ entering the cyanate pool and the ammonia contributing to endogenous ammonium supplementation.

Notably, components of a urea cycle were also identified in *P. globosa*. Within the urea cycle-related pathway, Argininosuccinate synthase (ASS), Argininosuccinate lyase (ASL), and Nitric oxide synthase (NOS) were annotated. ASS and ASL catalyze the sequential conversion of citrulline to argininosuccinate and subsequently to arginine and fumarate. The production of fumarate from this reaction provides a direct link to the TCA cycle. NOS mediates the interconversion between citrulline and arginine. However, ornithine transcarbamylase (OTC) and arginase (ARG) were not detected. In addition, transcriptome data revealed the presence of aspartate aminotransferase (AAT), which is involved in the conversion of L-glutamate to L-aspartate. L-aspartate is then condensed with citrulline via ASS to form L-argininosuccinate, which enters the urea cycle and supplies substrates to the incomplete pathway. Oxaloacetate and α-ketoglutarate, which participate in this conversion, also serve as intermediates linking this process to the TCA cycle.

3.6 Expression analysis of key nitrogen metabolism genes by RT-qPCR

Based on the annotated nitrogen metabolism pathway of *P. globosa* described above, 3 key genes representing core steps in nitrogen assimilation, nitrate reductase (NR), nitrite reductase (NiR), and glutamine synthetase (GS), were selected for RT-qPCR analysis to further validate the transcriptional regulation of critical nodes in nitrogen metabolism under acidification stress. Assessment of RNA quality showed that the extracted RNA was of high purity and suitable for downstream analyses.

Picodrop measurements yielded A_{260}/A_{280} ratios of approximately 2.0, indicating intact RNA without detectable protein or organic solvent contamination. Primer gel electrophoresis (Fig. 6a) produced single, intense bands with amplicon sizes consistent with the expected lengths, and no nonspecific or smeared bands were observed. BLAST analysis further confirmed that the PCR products corresponded to the target *P. globosa* gene sequences. Evaluation of primer performance revealed that all primer pairs exhibited acceptable amplification efficiencies, ranging between 80% and 110%, falling within the recommended theoretical range of 80% ~ 120% (Joseph and Villareal 1998) (Table 3). These results collectively demonstrate that the RNA, cDNA, and primers used in this study met the quality and specificity requirements necessary to support reliable quantitative PCR analyses.

Table 3
qPCR performance parameters for key nitrogen-metabolism genes
of *P. globosa*

Gene Name	Abbreviation	Efficiency (%)	R ²
Actin	ACT	89	0.99
Nitrate reductase	NR	86	0.99
Nitrite reductase	NiR	101	0.98
Glutamine synthetase	GS	113	0.98

Relative expression levels of the 3 nitrogen-metabolism genes, NR, NiR, and GS, were quantified using PG48 on day 2 as the reference (Fig. 5b-d). The 3 genes exhibited distinct temporal dynamics and transcription response differences under varying pH and $p\text{CO}_2$ conditions.

For the NR gene, transcriptional sensitivity to environmental factors was prominently observed during the early and mid-stages. On day 2, under 400 ppm $p\text{CO}_2$, reducing pH from 8.1 to 7.8 (PG47 vs. PG48) led to a significant down-regulation of NR expression; however, under elevated $p\text{CO}_2$ (1000 ppm), this pH-induced decrease was reversed, with PG17 showing significantly higher expression than PG47. By day 4, elevated $p\text{CO}_2$ combined with reduced pH (PG17) drove a significant synergistic increase in NR transcription compared to ambient controls (PG48) and high- $p\text{CO}_2$ and ambient-pH treatments (PG18). By day 8, a residual pH-driven difference was maintained solely between the ambient $p\text{CO}_2$ groups (PG47 vs. PG48).

The transcription of NiR demonstrated strong sensitivity to $p\text{CO}_2$ elevation during the first half of the experiment. On day 2, high $p\text{CO}_2$ significantly stimulated NiR expression, as evidenced by PG18 being significantly higher than PG48 and PG47, and PG17 exceeding PG47. This pronounced positive response to elevated $p\text{CO}_2$ was also evident on day 4, where PG18 maintained significantly higher expression than all other treatment groups, indicating a dominant $p\text{CO}_2$ -driven transcriptional enhancement. Conversely, by day 6, this trend inverted, PG18 showing a significant reduction in NiR expression compared to PG48 and PG47.

Regarding the GS gene, distinct factor-specific responses emerged during the mid-stage. On day 4, PG47 provoked a robust up-regulation, showing significantly higher GS transcription than PG48, PG18, and PG17, while control PG48 significantly surpassed the combined stress treatment PG17. By day 6, the transcriptional hierarchy shifted, and the combined stress PG17 exhibited a significant compensatory increase, surpassing both PG47 and PG18.

4 Discussion

4.1 Physiological trade-offs between carbon fertilization and H⁺ toxicity of *P. globosa*

This study demonstrated that elevated $p\text{CO}_2$ and decreased pH exert distinct, and potentially opposing, effects on the growth of *P. globosa*. The enhanced cell abundance observed in PG18 suggests a positive effect of CO_2 enrichment under ambient pH. Such stimulation may be associated with increased inorganic carbon availability and enhanced photosynthetic electron transport, as reflected by the higher ETR_{max} under elevated $p\text{CO}_2$ in the present study. Although *P. globosa* possesses a constitutive carbon concentrating mechanisms (CCM), elevated CO_2 may nevertheless alleviate constraints on inorganic carbon acquisition and support carbon fixation, thereby contributing to enhanced biomass production. Previous studies have similarly reported that elevated CO_2 can stimulate the physiological performance of *P. globosa*, although the magnitude and persistence of this response depend on acclimation conditions (Chen et al. 2014).

In contrast, the reduced cell abundance in PG47 indicates that decreased pH imposed a physiological constraint on growth. Consistent with the growth response, ETR_{max} was also lower under reduced pH, whereas F_v/F_m and α remained relatively stable across the treatments. This differential sensitivity suggests that short-term acidification did not cause pronounced impairment of the maximum photochemical efficiency of PSII or photosynthetic efficiency, but may have constrained the capacity for photosynthetic electron transport. This finding is consistent with those of Wu et al. (2022), who reported that *P. globosa* is capable of regulating its photoprotective system under various environmental stresses including low temperature, high light intensity and iron limitation (Wu et al. 2022). Such a response is consistent with the short-term inhibition of photosynthetic performance reported for *P. globosa* under lower pH conditions (Chen and Gao 2011; Chen et al. 2014). The energetic costs associated with maintaining intracellular pH homeostasis may further divert cellular resources from growth and other biosynthetic processes. Importantly, Chen et al. (2014) showed that the growth inhibition induced by lower pH could be alleviated following acclimation over 1–19 generations, accompanied by enhanced pigment content and photochemical quantum yield. In this study, however, the inhibitory effect on PG47 abundance persisted throughout the 8-day culture period, suggesting that the duration of the experiment may have been insufficient for full physiological acclimation.

When elevated $p\text{CO}_2$ and reduced pH occurred simultaneously, the stimulatory effect of CO_2 enrichment did not fully compensate for the negative effect of acidification. The significantly lower abundance in PG17 than that in PG48 indicates that the growth-promoting effect associated with elevated CO_2 was at least partially offset by proton stress under reduced pH. This pattern is consistent with the opposing responses of ETR_{max} to the two environmental divers, with elevated $p\text{CO}_2$ enhancing electron transport whereas reduced pH exerted an inhibitory effect. This pattern is consistent with the findings of Liang et al. (2023), who also observed a decline in photosynthetic performance in *P. globosa* under combined warming and acidification, as evidenced by the light-harvesting complex (LHC) genes and reduced electron transport rates, although their experimental conditions included an additional warming stress (Liang et al. 2023).

4.2 pH and $p\text{CO}_2$ induce distinct transcriptional responses

Transcriptome analysis revealed extensive molecular reprogramming of *P. globosa* under single and combined stresses. Based on $|\log_2\text{FC}| > 1$, 5950, 5170, and 6095 genes were identified as candidate DEGs under low pH (PG47 vs. PG48), elevated CO_2 (PG18 vs. PG48), and combined stress (PG17 vs. PG48), respectively. Notably, low pH and elevated CO_2 predominantly induced transcriptional up-regulation with up/down ratios of 1.21 and 1.49, respectively, whereas the combined treatment showed a nearly balanced response (3033 vs. 3062; ratio = 0.99). This transcriptional response suggests that acidification resulted in a non-additive molecular response rather than simple addition of either single-stressor response. Similar non-additivity has been reported in other diatoms, albeit with different manifestations. For instance, combined warming and elevated CO_2 in *Th. weissflogii* significantly amplified DEG counts compared to single stressors, indicating a synergistic rather than additive interaction (Zhou et al. 2024). This complexity aligns with the framework proposed by Li et al. (2023), which categorizes stressors into “energy overload” and “metabolic resource limitation” types that induce opposing transcriptomic directions (Li et al. 2023), which may explain why combined stress responses are often non-additive.

The 3 stress treatments induced extensive reprogramming, with Protein processing in ER and Fatty acid metabolism enriched across all comparisons, implicating shared response mechanisms related to protein homeostasis and lipid metabolism. The ER is the site of secretory protein folding and quality control, and an imbalance between unfolded protein load and folding capacity activates the unfolded protein response (UPR), which initially reduces protein load and subsequently up-regulates chaperones, folding enzymes and ER-associated degradation (ERAD) components to restore proteostasis (Ron and Walter 2007). Although UPR activity was not directly measured in this study, the transcriptional profiles of ER protein processing genes provide indirect evidence for changes in ER proteostasis. The predominance of up-regulated genes in PG47 suggests an enhanced transcriptional response of the ER protein processing machinery under low-pH stress, whereas the relatively balanced pattern in PG18 and the predominant down-regulation in PG17 indicate distinct transcriptional regulatory states under elevated $p\text{CO}_2$ and combined stress, rather than a simple additive or strengthened low-pH response.

These treatment-specific patterns are consistent with the established role of ER stress responses in regulating ER protein folding, quality control and degradation capacity (Ron and Walter 2007). Fatty acid metabolism showed more up-regulated genes in PG18 but more down-regulated genes in PG47 and PG17. The co-enrichment of Fatty acid metabolism with ER protein processing is mechanistically coherent, because the ER is a principal site of phospholipid biosynthesis, and the UPR up-regulates genes involved in lipid and inositol metabolism and promotes phospholipid biosynthesis and membrane biogenesis (Ron and Walter 2007). The down-regulation of fatty acid metabolism genes in PG47 and PG17 may reflect a shift of cellular energy toward maintaining intracellular pH homeostasis under low pH, which imposes substantial energetic costs in phytoplankton (Zhang et al. 2022a; Lin 2023), whereas the up-regulation in PG18 under elevated $p\text{CO}_2$ is consistent with reduced energy demand for CCM, freeing resources for lipid biosynthesis (Lin 2023).

Separate pH reduction (PG47) and separate $p\text{CO}_2$ elevation (PG18) induce markedly different transcriptional responses, indicating that these 2 acidification-related factors do not act through entirely identical transcriptional modules. Previous studies on diatoms have shown that reduced pH shares part of the transcriptional response with energy/resource-limitation stresses (Li et al. 2023); consistent with this, carbon acquisition genes were downregulated under OA in *Karenia mikimotoi* (Zhang et al. 2022b). At the transcriptional level, these responses suggest that low pH primarily affected carbon assimilation. In contrast, elevated $p\text{CO}_2$ mainly reshaped central carbon metabolism and multiple amino acid metabolism pathways, both showing predominantly up-regulated patterns. In the microalgae *Coccomyxa subellipsoidea* C-169, studies have shown that elevated $p\text{CO}_2$ can enhance glycolysis, the TCA cycle, and anaplerotic carbon reactions, and can drive nitrogen assimilation and amino acid metabolism (Peng et al. 2016), providing support for understanding the metabolism remodeling observed in PG18. The changes in amino acid metabolism in PG18 further indicate a close link between carbon and nitrogen metabolism; the discussion of its impact on nitrogen metabolism is provided below.

Compared with either single-stress treatment, combined stress PG17 exhibited a distinct transcriptional response: DNA replication and multiple DNA repair pathways were coordinately up-regulated, whereas protein processing, endocytosis, and ubiquitin-mediated proteolysis were predominantly down-regulated. This suggests that under combined stress, cells do not simply amplify the response intensity of a single stress, but instead initiate a resource reallocation program that invests more heavily in genome maintenance. OA perturbs intracellular pH homeostasis, and maintaining this homeostasis itself imposes a sustained energetic burden (Lin 2023). Under combined stress, this burden is likely exacerbated, further tightening the cellular energy budget and forcing trade-offs among functional modules. Because genome integrity determines whether downstream stress responses can be executed correctly, it is prioritized; protein processing and degradation, which have relatively greater short-term tolerance for error, become the targets of resource reduction. A comparable pattern has been reported under acidification alone: in *Sargassum vulgare* at volcanic CO_2 vents, transcripts related to DNA repair and RNA processing, together with genes encoding histones and chromosome structural maintenance proteins, were up-regulated, suggesting that genome maintenance contributes to acidification tolerance

in algae (Kumar et al. 2017). Of course, the above inference is based solely on transcriptomic observations and requires functional validation targeting DNA repair and protein homeostasis.

4.3 Nitrogen assimilation and endogenous ammonium replenishment in *P. globosa*

Transcriptomic and physiological studies on *P. globosa* have demonstrated that this species exhibits a strong preference for nitrate over ammonium, with high-affinity nitrate transporters (HNRT), nitrate reductase (NR), and nitrite reductase (NiR) actively expressed during bloom development (Zhu et al. 2023). Quantitative ^{15}N isotope tracer experiments further revealed that *P. globosa* assimilates nitrate at rates substantially higher than many other phytoplankton taxa, and that nitrate assimilation cannot be completely suppressed even at elevated ammonium concentrations (Guo et al. 2024). In the present study, a complete nitrate assimilation pathway (NR and NiR) and a functional GS-GOGAT cycle were identified from transcriptomic data.

To determine whether these pathway components are dynamically regulated under stress, the temporal expression pattern of NR, NiR, GS were examined across days 2, 4, 6, and 8. Distinct temporal response profiles emerged: NR expression differences were most pronounced at early to mid-stages (day2 and 4) and primarily modulated by pH, suggesting that nitrate reduction represents an early response to acidification stress. NiR expression exhibited significant differences from days 2 through 6, with $p\text{CO}_2$ as the dominant regulatory factor, indicating that nitrite reduction is sensitive to CO_2 availability. In contrast, GS expression diverged among treatments primarily at mid-stage (days 4 and 6), with the direction of regulation varying depending on the specific stressor. NR is recognized as the rate-limiting enzyme in nitrate assimilation (May et al. 2011), and its early response aligns with its role as the initial entry point of the pathway. GS functions as the central enzyme in the GS/GOGAT cycle, catalyzing the incorporation of ammonium with glutamate to form glutamine, representing a critical step in ammonium assimilation (Suzuki and Knaff 2005). The delayed GS response may reflect additional post-translational regulation via reversible phosphorylation and 14-3-3 protein interactions (Finnemann and Schjoerring 2000), as well as its role in integrating NH_4^+ from multiple internal sources, consistent with observations in diatoms that GSII is induced during nitrate assimilation rather than direct ammonium uptake (Takabayashi et al. 2005).

Beyond canonical nitrate assimilation, the transcriptome also revealed 3 potential endogenous ammonium-replenishment pathways: urea degradation, cyanate degradation, and formamide hydrolysis. The presence of cyanate hydratase (Cyns) and formamidase in *P. globosa* has been previously documented (Zhu et al. 2023), corroborating these annotations. This tripartite replenishment strategy substantially expands the nitrogen supply repertoire of *P. globosa*, potentially conferring an adaptive advantage when exogenous inorganic nitrogen sources are limited or when environmental stressors disrupt transmembrane ion exchange.

Although the responses of NR, NiR, and GS revealed distinct temporal pattern and factor-specific regulation of key steps in nitrogen assimilation, pathway-level transcriptomic analysis provided a broader perspective on whether these gene-level responses extended to the nitrogen metabolic network. Nitrogen metabolism (ko00910) exhibited distinct response pattern under the 3 stress conditions. PG18 showed the highest proportion of up-regulated genes (85.7%), consistent in direction with the coordinated up-regulation of carbon and nitrogen metabolism reported in *Coccomyxa subellipsoidea* under elevated CO₂, which was proposed to contribute to maintaining cellular C/N balance (Peng et al. 2016). PG47 involved the largest number of genes but showed the lowest proportion of up-regulated genes (45.5%), indicating a mixed response that was partly consistent with the differential reprogramming of distinct nitrogen metabolic processes observed in *Symbiodinium kawagutii* under long-term low-pH exposure (Wen et al. 2026). PG17 showed an intermediate proportion of up-regulated genes but involved the fewest genes, suggesting that the combined effects of low pH and elevated pCO₂ were not simply additive. Interactions between acidification and nutrient limitation can be additive, synergistic, or antagonistic (Lin 2023); the response pattern observed in PG17 may indicate a potentially antagonistic interaction.

Carbon metabolism (ko01200) showed distinct response patterns across the 3 comparisons, which, together with the nitrogen metabolic responses, revealed distinct patterns of C-N metabolic coordination under the 3 stress conditions. PG18 showed the strongest up-regulation of carbon metabolism-related genes (71.0%), consistent with the coordinated transcriptional responses of carbon and nitrogen metabolism and the maintenance of C/N balance reported under elevated CO₂ (Peng et al. 2016). The proportions of up-regulated genes in carbon metabolism were lower in PG47 and PG17 (33.3% and 40.8%, respectively). In PG47, both carbon and nitrogen metabolism showed predominantly down-regulated patterns, whereas PG17 exhibited divergent responses, with nitrogen metabolism biased toward up-regulation and carbon metabolism toward down-regulation. These contrasting patterns further suggest non-additive transcriptional reprogramming of C-N coupling under low pH and elevated pCO₂. Dai et al. (2025) found that the negative effect of acidification on primary production was strongest under nitrogen limitation and could be alleviated by nitrate addition, highlighting the important role of nitrogen availability in modulating responses to acidification (Dai et al. 2025). This provides a useful reference for interpreting the divergent carbon and nitrogen metabolic responses observed in PG17.

Beyond these 2 core metabolic pathways, several amino acid metabolism pathways closely related to nitrogen allocation were also enriched across the 3 comparisons. These pathways were predominantly up-regulated in PG18, relatively balanced in PG47, and highly heterogeneous in PG17. Notably, all 17 genes associated with Arginine/Proline metabolism were down-regulated in PG17, whereas 72.2% of genes involved in Glutathione metabolism were up-regulated. Glutathione, a hydrophilic antioxidant and thiol-based redox regulator, plays an important role in the response of microalgae to environmental stress (Tamaki et al. 2021). This pattern suggests that the transcriptional response under combined stress may be biased toward glutathione-dependent redox defense, indicating that acidification-induced

transcriptional reprogramming extends beyond primary nitrogen assimilation to downstream nitrogen allocation.

4.4 A truncated urea cycle and its ecological implications

In addition to the complete nitrate assimilation and ammonium replenishment pathways, a partially annotated ornithine-urea cycle (OUC) was identified in the transcriptomic dataset. AAT, GDH, and ASS were present, whereas OTC and ARG were not annotated. The presence of AAT supports the conversion of L-glutamate to L-aspartate via transamination (Suzuki and Knaff 2005), which subsequently serves as the substrate for ASS, linking core nitrogen assimilation to the truncated urea cycle. Thus, despite OTC and ARG may not be identified because of their low expression levels, the pathway appears metabolically connected to upstream amino acid metabolism, with L-aspartate potentially feeding into L-argininosuccinate generation.

In mammals, a key physiological function of the urea cycle is the detoxification of neurotoxic ammonia by converting it into urea for excretion, thereby preventing hyperammonemia and associated neurological damage (Lee et al. 2000). In contrast, in marine diatoms, the complete ornithine-urea cycle serves a biosynthetic role, functioning as a carbon-nitrogen repartitioning hub that facilitates rapid recovery from nitrogen limitation and contributes to their competitive success in nitrogen-variable environments (Allen et al. 2011; Dagenais-Bellefeuille and Morse 2013). Interestingly, this functional perspective is challenged by observations in the bloom-forming haptophyte *P. globosa*. Despite forming massive blooms in nitrate-replete waters, metatranscriptomic analyses of *P. globosa* bloom in the Beibu Gulf revealed that none of the genes involved in the urea cycle were highly expressed during bloom development (Zhu et al. 2023), further implying that a complete OUC may not be transcriptionally or functionally required for bloom formation. Instead, *P. globosa* appears to rely on alternative nitrogen acquisition strategies, including high-affinity nitrate transporters and active glycan synthesis for colony formation (Zhu et al. 2023), which may compensate for the reduced urea cycle capacity under nitrate-replete conditions.

Similar to the results of Zhu et al. (2023), the OUC annotated by transcriptome of *P. globosa* strain in this study is notably truncated, and the key enzymes OTC and ARG were not detected, possibly owing to their low expression levels. The undetected expression of OTC, which catalyzes the first committed step of the cycle. As reviewed by Shi et al. (2018), carbamoyl phosphate (CP), the product of carbamoyl phosphate synthetase (CPS), is a highly labile and energy-rich molecule. In a complete cycle, CP is rapidly consumed by OTC to form citrulline. When OTC is not detected or only weakly expressed, any CP produced might be prone to decomposition, representing a futile drain of energy and carbon, or could be partitioned towards alternative pathways such as pyrimidine biosynthesis (Shi et al. 2018). Furthermore, the low expression of ARG indicates an inability to regenerate ornithine and release urea from arginine, precluding the classical nitrogen-excretion function. Consequently, this truncated OUC likely does not confer the same metabolic flexibility or competitive advantage observed in organisms with a complete cycle (Allen et al. 2011; Dagenais-Bellefeuille and Morse 2013).

Notably, although CPS is also undetected in this study organism, downstream components of the urea cycle, including ASS, and ASL, are retained. The widespread occurrence of these enzymatic components is not limited to organisms with a complete urea cycle; across diverse eukaryotic lineages, these downstream enzymes remain highly conserved even when the cycle is incomplete, suggesting that they possess independent metabolic roles beyond classical urea synthesis (Horák et al. 2020). In *P. globosa*, these retained components may primarily function in the turnover and recycling of endogenous nitrogen: when intracellular proteins or nucleic acids are degraded, they can be efficiently transformed into arginine from L-argininosuccinate through the catalytic action of ASL. Of particular importance is the bridging role of this incomplete cycle in energy metabolism: fumarate produced by ASL-catalyzed reactions is a key intermediate of the TCA cycle and can feed directly into the central metabolic hub (Horák et al. 2020). This mechanism channels the products of nitrogen metabolism directly into carbon metabolic pathways, achieving efficient coupling between nitrogen recycling and central carbon metabolism.

This “modular” metabolic architecture reflects the remarkable flexibility of the urea cycle. Comparative genomic and phylogenetic analyses have shown that the OUC is present in stramenopiles and haptophytes but absent in green algae and land plants, indicating that it is not a universally conserved pathway among photoautotrophs (Allen et al. 2011). The distinctive modular configuration observed here, characterized by the apparent loss of CPS and OTC with retention of downstream components such as ASS and ASL, is highly congruent with its ecological niche of forming blooms in eutrophic marine waters. This architecture enables physiological synergy between the metabolic network and ecological strategy: rather than relying on a complete cycle for ammonia detoxification, the truncated pathway facilitates rapid nitrogen recycling from endogenous sources and couples this process to energy production via TCA cycle intermediates. More importantly, under the ocean acidification scenarios simulated here by lowered pH/elevated $p\text{CO}_2$ and combined stress, the transcriptional reprogramming of these nitrogen metabolic modules suggests that *P. globosa* may adjust its nitrogen handling strategy to maintain internal ammonium homeostasis and C-N balance. This metabolic plasticity could be central to its ecological success, allowing this bloom-forming species to sustain growth and competitiveness not only when nitrogen is replete, but also when acidification alters the availability, form, or intracellular routing of nitrogen. Thus, the modular nitrogen metabolic architecture may represent a key adaptive trait that underpins the resilience of *P. globosa* to future acidified ocean conditions.

5 Conclusion

This study investigated the physiological responses and nitrogen metabolic regulation of *P. globosa* under ocean acidification by explicitly decoupling the respective effects of elevated $p\text{CO}_2$ and reduced pH. The results demonstrate that reduced pH represents the primary controlling factor suppressing the growth of *P. globosa* under ocean acidification, overriding the positive effects of elevated $p\text{CO}_2$. Nitrogen-metabolism genes (NR, NiR, GS) are reprogrammed by single or combined pH/ $p\text{CO}_2$ drivers, with

temporal shifts from acute to acclimation phases. The truncated urea cycle (CPS/OTC undetected, ASS/ASL retained) may enable endogenous nitrogen recycling coupled with TCA-mediated energy production, a metabolic configuration that likely contribute to the ecological resilience of *P. globosa* in its eutrophic bloom niche.

Declarations

Author Contribution

Yuting Zhou and Yuping Lv conducted the experiments and wrote the original manuscript. Xiaohuan Li curated the data and prepared Figs. 1–6. Yu Zhen designed the experiments and provided funding. Zhigang Yu reviewed and edited the manuscript. All authors reviewed and approved the final manuscript.

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Figures

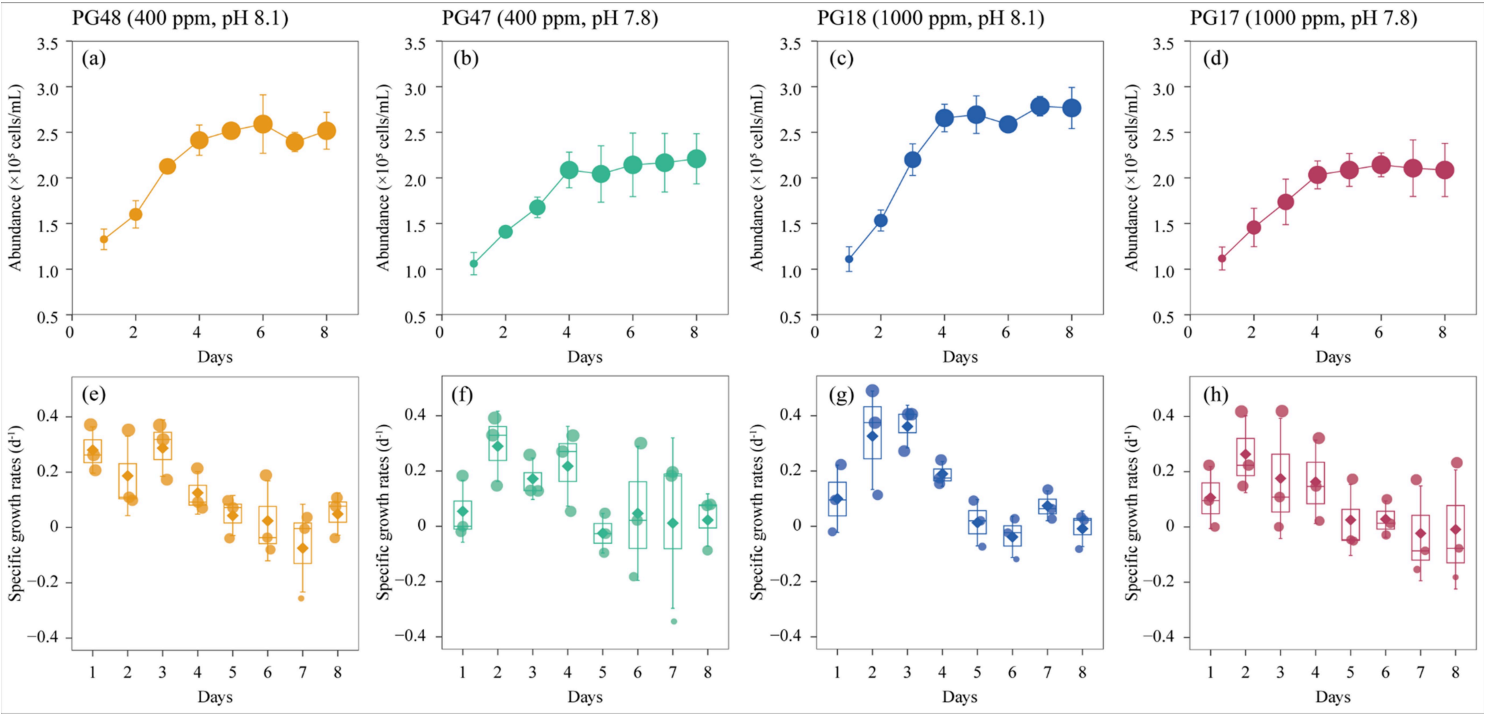


Figure 1

Growth curves and specific growth rate of *P. globosa*

Note: (a)–(d) show the growth curves of PG48, PG47, PG18, PG17, respectively; (e)–(h) show the specific growth rates of PG48, PG47, PG18, and PG17, respectively. Orange represents PG48, green represents

PG47, blue represents PG18, and magenta represents PG17; the same color scheme is applied in subsequent figures.

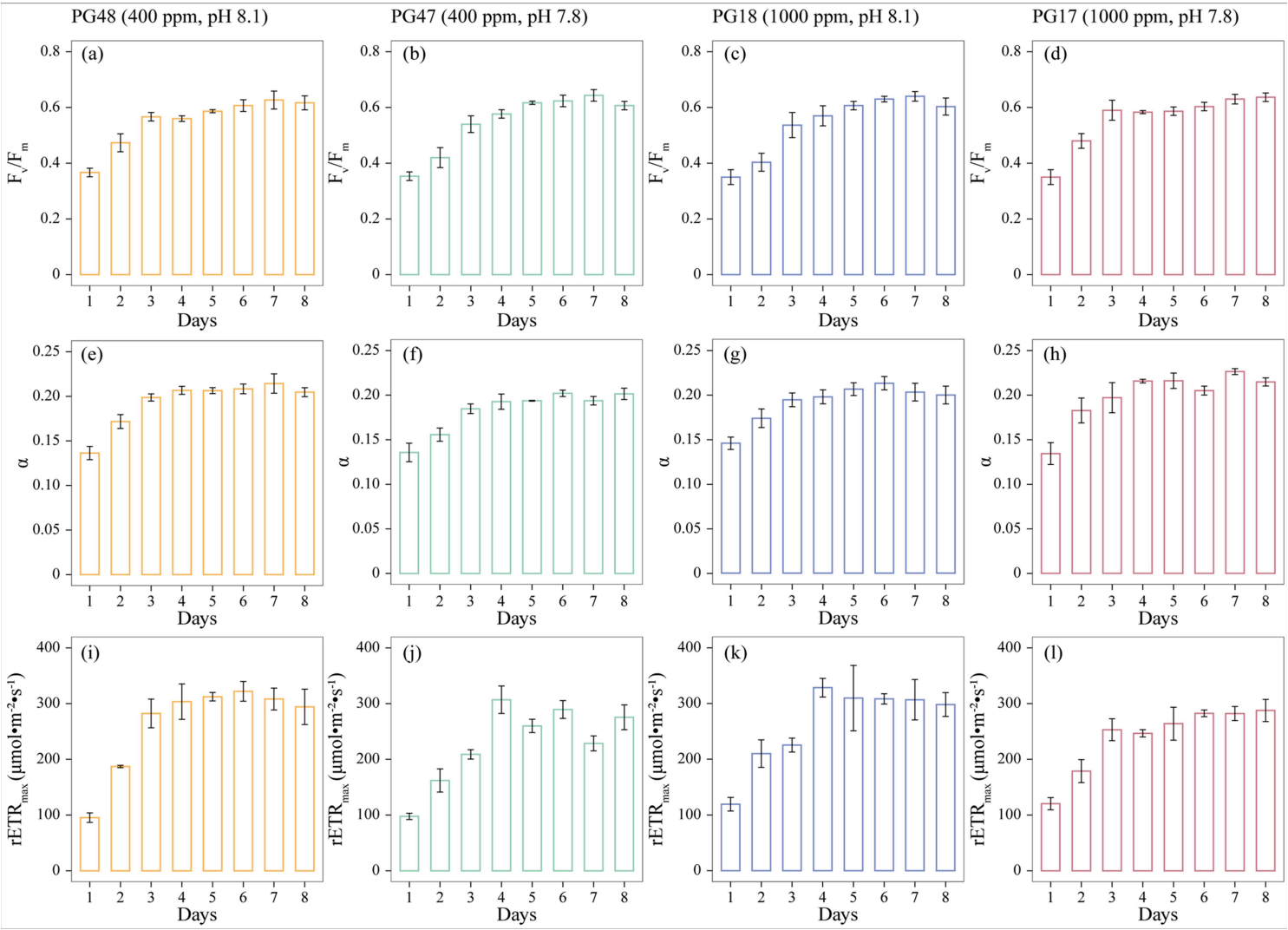


Figure 2

Photosynthetic parameters of *P. globosa*

Note: (a)–(d) show the maximum photochemical quantum yield F_v/F_m of PG48, PG47, PG18, and PG17, respectively; (e)–(h) show the light energy utilization α of PG48, PG47, PG18, and PG17, respectively; (i)–(l) show the maximum electron transfer efficiency $rETR_{max}$ of PG48, PG47, PG18, and PG17, respectively.

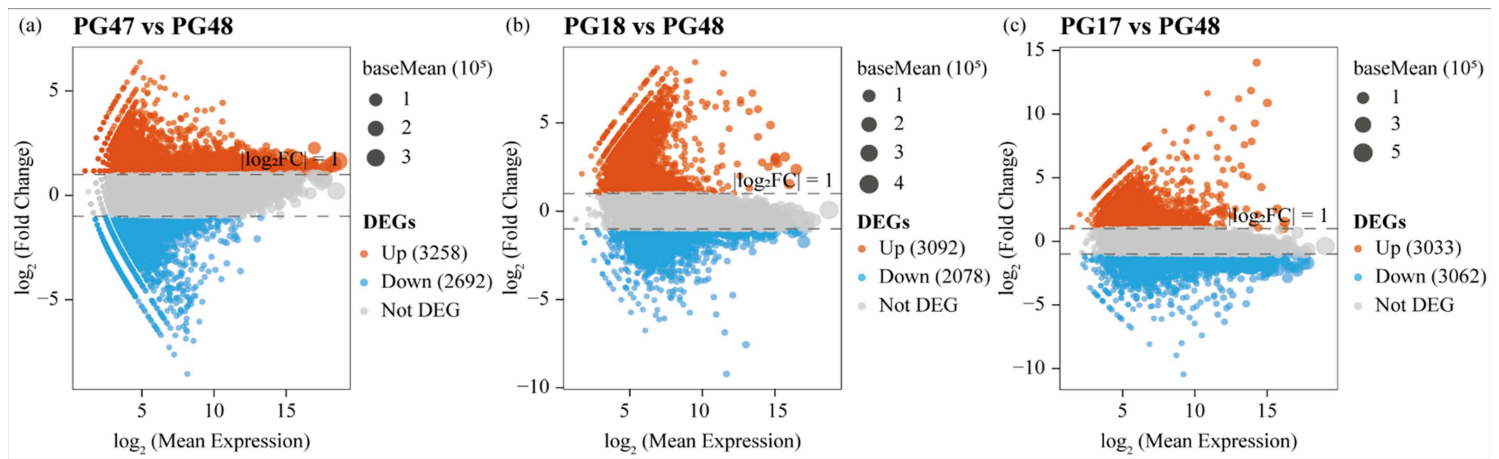


Figure 3

MA plot of candidate DEGs for 3 pairwise comparisons.

Note: (a) PG47 vs. PG48, (b) PG18 vs. PG48, (c) PG17 vs. PG48. The x-axis represents the $\log_2$ -transformed mean expression level ($\log_2$ baseMean), and the y-axis represents the $\log_2$ -transformed fold change. Red and blue dots indicate genes with $|\log_2FC| > 1$ (up-regulated and down-regulated, respectively), which were defined as candidate DEGs based solely on fold-change magnitude. Gray dots represent non-DEGs. Dashed lines indicate the fold-change threshold ($|\log_2FC| = 1$).

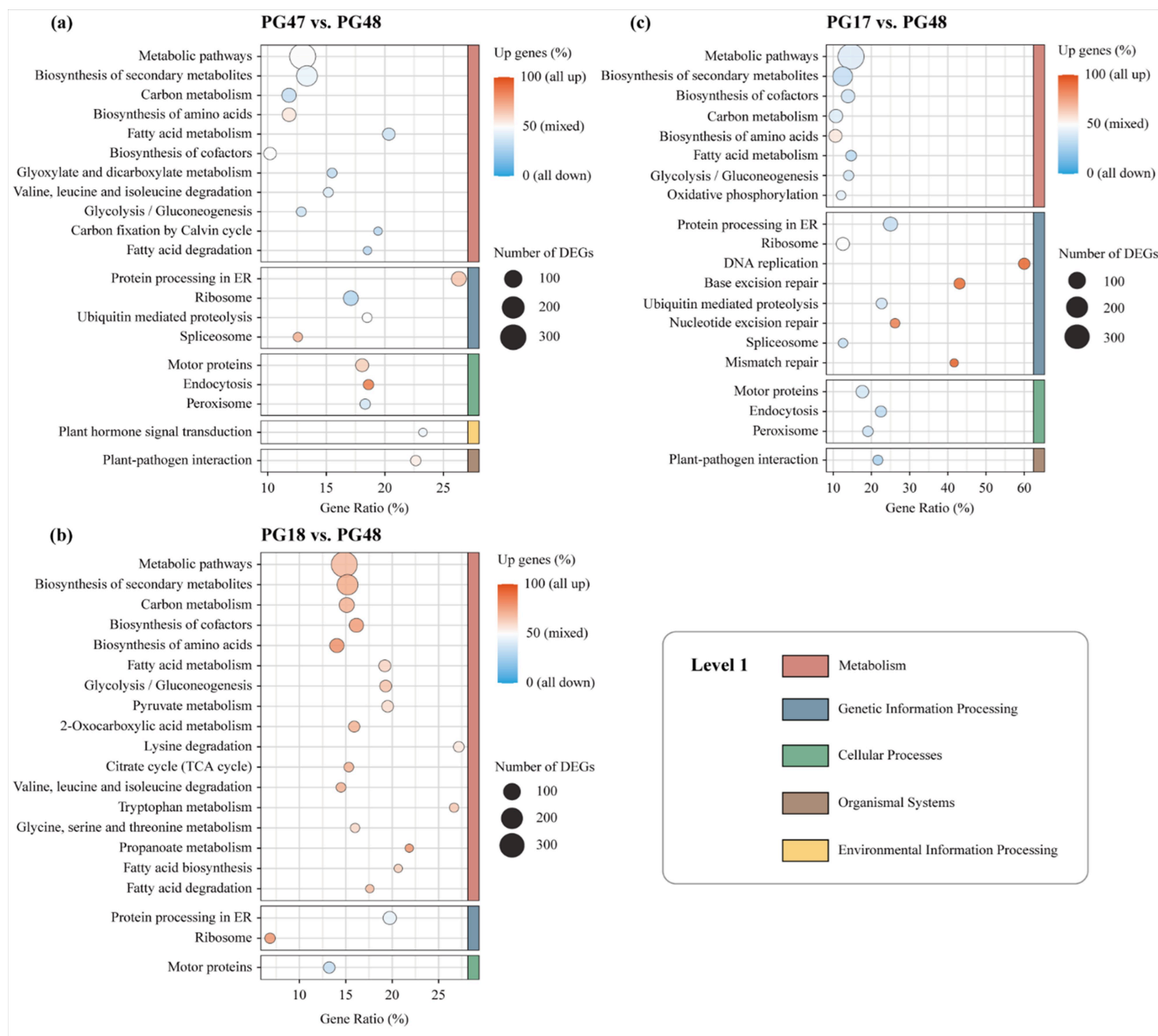


Figure 4

KEGG enrichment trends for 3 pairwise comparisons.

Note: (a) PG47 vs. PG48, (b) PG18 vs. PG48, (c) PG17 vs. PG48. Top 20 pathways ranked by DEG count in each comparison. The x-axis represents the gene ratio (DEG number/total number of genes in the pathway). Bubble size represents the number of DEGs per pathway. Bubble color indicates the proportion of up-regulated genes within each pathway (red: predominantly up-regulated; blue: predominantly down-regulated; white: mixed).

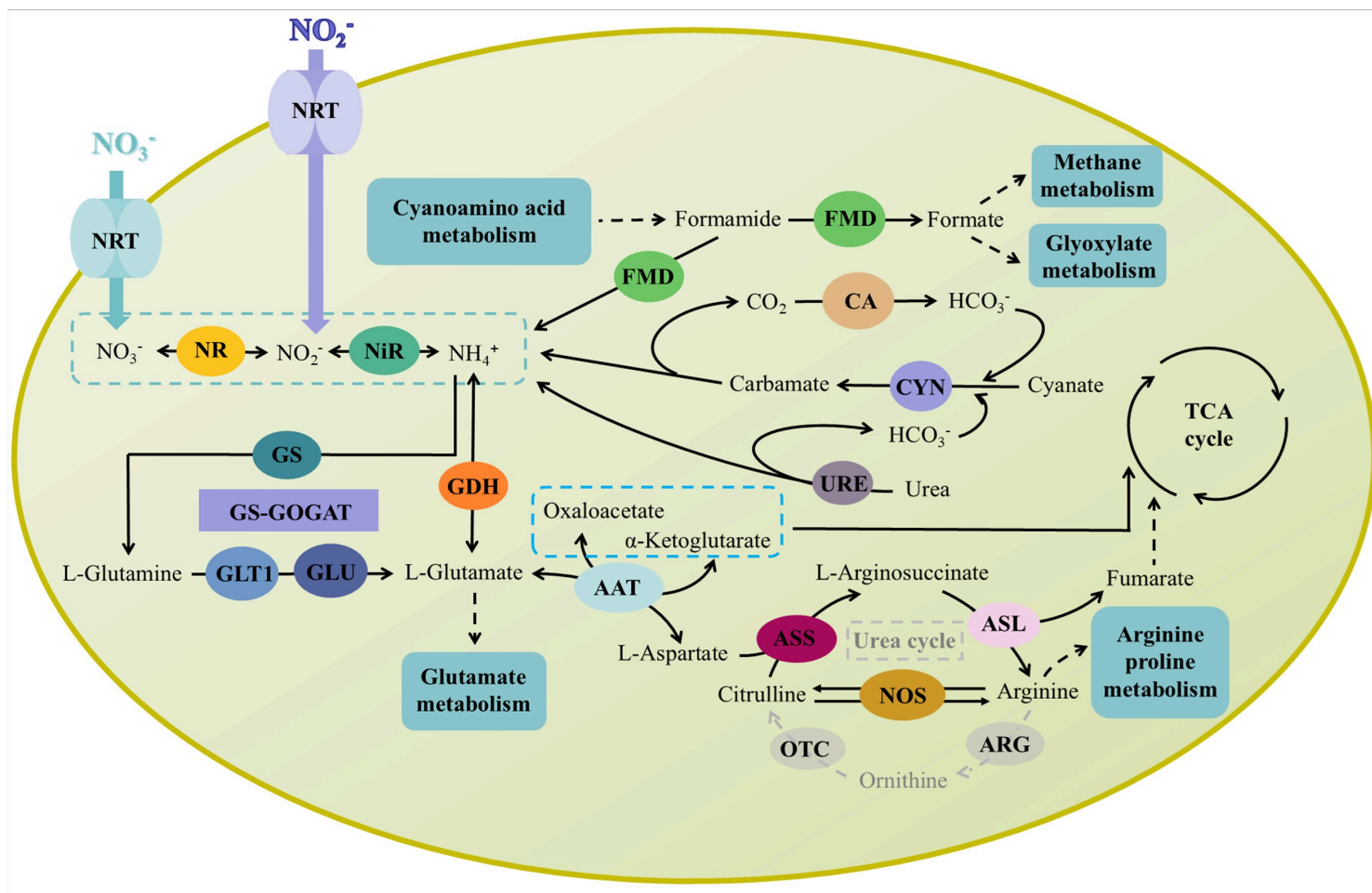


Figure 5

Nitrogen metabolism pathway of *P. globosa*

Note: NRT: Nitrate/nitrite transporter; NR: Nitrate reductase; NiR: Nitrite reductase; GLT1: Glutamate synthetase (NADH-GOGAT); GLU: Glutamate synthetase (fd-GOGAT); GS: Glutamine synthetase; GDH: Glutamate dehydrogenase; FMD: Formamidase; CA: Carbonic anhydrase; CYN: Cyanate lyase; AAT: Aspartate aminotransferase; OTC: Ornithine carbamoyltransferase; ASS: Argininosuccinate synthase; ASL: Argininosuccinate lyase; NOS: Nitric oxide synthase; ARG: arginase; URE: urease. OTC and ARG were not detected.

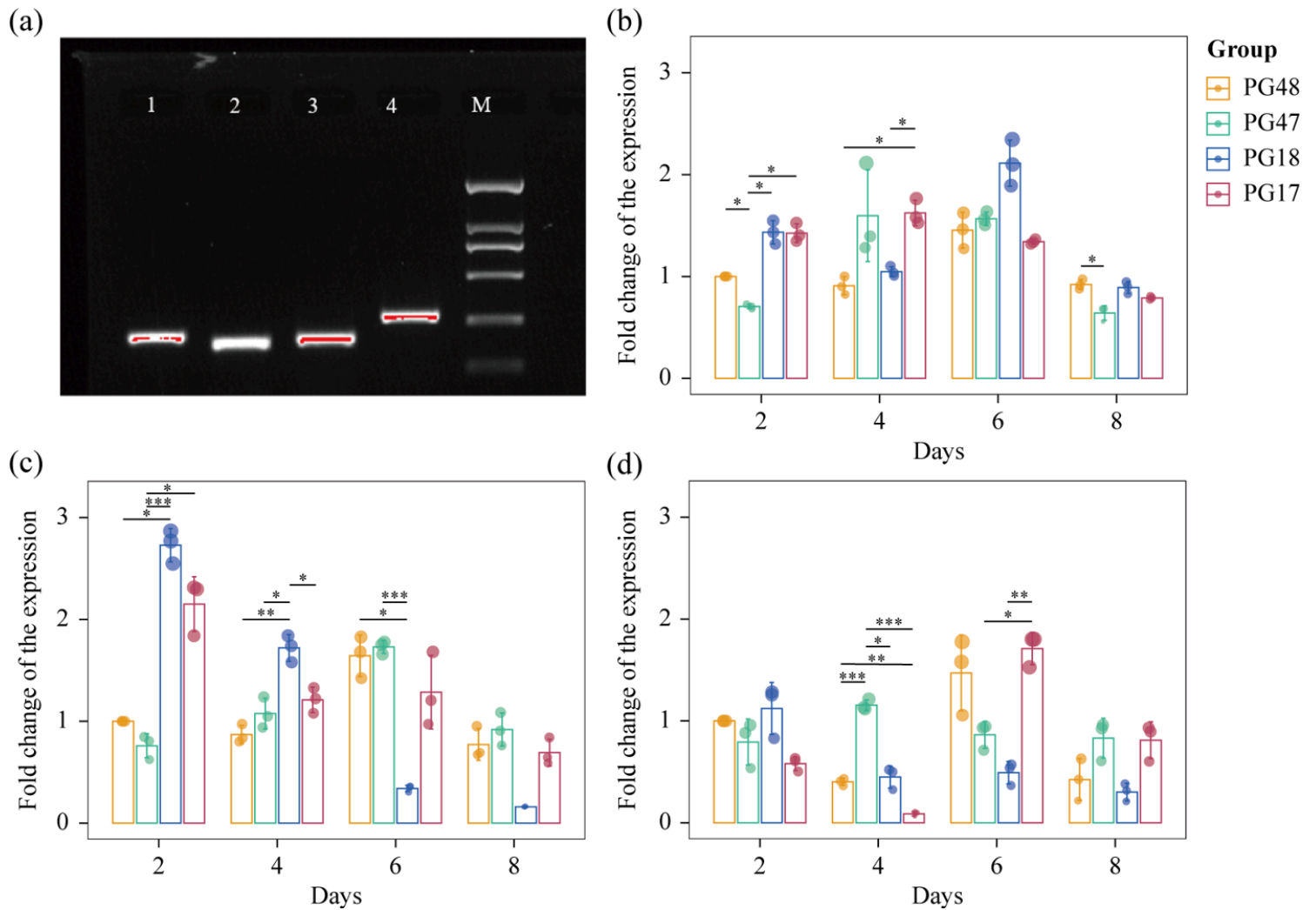


Figure 6

Primer validation and expression fold change of target genes.

Note: (a) Primer validation results, (b)–(d) trends in fold change in the expression of genes encoding NR, NiR, and GS. M: DL2000 marker; 1: GS gene; 2: NiR gene; 3: Actin gene; 4: NR gene. Significant difference between groups is indicated by asterisks: * $p_{\text{adj}} < 0.05$; ** $p_{\text{adj}} < 0.01$; *** $p_{\text{adj}} < 0.001$.

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