

Original Article

Physiological, morphological, and molecular adaptations of *Pseudo-nitzschia multistriata* to phosphorus limitation

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ABSTRACT

Phosphorus is a key driver of marine primary production, and its increasing scarcity due to imbalanced anthropogenic nutrient inputs is a growing concern. The widespread diatom genus *Pseudo-nitzschia* includes several toxigenic species in which phosphorus limitation can induce domoic acid production; however, the responses of *Pseudo-nitzschia multistriata*, a common coastal species with extensive genomic resources, remain poorly characterised.

Here, we provide the first integrated physiological, transcriptomic, and chemical characterisation of *P. multistriata* under phosphorus limitation. Using a multidisciplinary approach, we assessed growth, cell viability, chloroplast and nuclear biovolumes, elemental stoichiometry, transcriptional responses and domoic acid quantification.

Under phosphorus deprivation, growth arrest occurs after three days while cell viability remains high, indicating a shift towards a survival strategy. This is accompanied by reduced chloroplast and nuclear size and marked alterations in C:N:P ratios. Transcriptomic analyses revealed strong induction of phosphorus acquisition genes and recycling pathways, including the SPX–PSR1 regulatory module, and suggested accumulation of TAGs. Intracellular domoic acid content increased under phosphorus limitation, consistent with upregulation of the domoic acid biosynthetic (*dab*) genes.

Overall, *P. multistriata* displays coordinated metabolic, transcriptional, and morphological adjustments that support persistence under phosphorus limitation and may influence nutrient cycling and ecological interactions in coastal ecosystems.

1. Introduction

Phosphorus (P) is an essential macronutrient required for numerous fundamental cellular processes. It is a crucial component of nucleic acids, ATP, phospholipids, and phosphorylated signalling molecules, and thus contributes to the storage of genetic information, energy transfer, membrane integrity, and regulation of cell functions (Karl, 2000).

In aquatic ecosystems, bioavailable inorganic phosphate (Pi, PO₄³⁻) is mainly derived from the weathering of continental rocks – a geologically slow process that often cannot meet the high biological demand for P (Paytan and McLaughlin, 2007). Along with nitrogen (N) and iron (Fe), P is widely recognised as one of the limiting factors of marine primary production. In fact, P limitation has been identified in several marine environments (Thingstad et al., 1998; Ly et al., 2014).

The increasing concern about P limitation arises from changes in

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elemental stoichiometry due to anthropogenic activities: while N and carbon (C) inputs to the ocean have increased due to agricultural runoff and fossil fuel burning, P inputs have remained relatively stable or have even decreased due to environmental regulations (Solidoro et al., 2009). This has resulted in unbalanced nutrient inputs and potential shifts in ecosystem productivity and structure (Elser and Bennett, 2011; Mozetič et al., 2012; Peñuelas et al., 2020). This discrepancy highlights the need to understand how phytoplankton, the basis of marine food webs, respond to P scarcity, as these responses can propagate through the food web and influence biogeochemical cycling (Duhamel et al., 2021; Duhamel, 2025).

Among phytoplankton species, diatoms often dominate under fluctuating conditions due to their remarkable adaptability. Common and well-documented cellular responses to P limitation include the upregulation of high-affinity phosphate transporters and the secretion of alkaline phosphatases that can hydrolyse organic phosphorus compounds (Dyhrman and Ruttenberg, 2006; Matsui et al., 2024). Meanwhile, cells initiate internal recycling mechanisms that mobilise P from RNA, phospholipids, and other intracellular sources (Alipanah et al., 2018; Lovio-Fragoso et al., 2021). Recent findings suggest that, as in higher plants, P homeostasis in diatoms is transcriptionally regulated through a mechanism involving SPX proteins, which act as P sensors and negative regulators of the P starvation response transcription factor (namely PSR1 or PHR1) (Harrison et al., 2025; Zhang et al., 2021; Sharma et al., 2020). Under sufficient P, SPX proteins interact with PSR1 and inhibit its activity. When P levels drop, SPX proteins lose their inhibitory function, allowing PSR1 to enter the nucleus and activate strategies used during P deficiency, including the consumption of dissolved organic phosphorus (DOP) in the form of phosphoesters, phosphonates, and polyphosphates (Van Mooy et al., 2009; Zhang et al., 2021).

The nutrient-specific responses (i.e. those related to its uptake and assimilation) are often accompanied by broader metabolic reprogramming, such as the regulation of central carbon metabolism. For example, the suppression of photosynthetic activity and corresponding transcripts has been identified as a common response across algal species from different taxa under both N and P starvation (Harke et al., 2017; Tan et al., 2019).

In diatoms, recent studies have revealed a tight coupling between P and N metabolism mediated by Ca^{2+} signalling (Harrison et al., 2025 and references therein). However, this regulatory cross-talk has mainly been explored in the model diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, and it remains uncertain whether it is conserved in other species.

The diatom genus *Pseudo-nitzschia* is widespread and ecologically important in marine environments (Bates et al., 2018). Of the 62 species described (Guiry, and Guiry, 2025), more than half are known to produce domoic acid (DA), a neurotoxin responsible for amnesic shellfish poisoning and often associated with harmful algal blooms. Some *Pseudo-nitzschia* species can synthesise this biomolecule during all growth phases, with production often peaking in the stationary phase (Lelong et al., 2012). DA production is influenced by various environmental factors, including nutrient availability, temperature, and the presence of grazers (Trick et al., 2010; Lelong, 2012; Lundholm et al., 2018); in this context, P limitation has been recognised as a common trigger (Fehling et al., 2004; Lelong et al., 2012; Brunson et al., 2018). DA can either accumulate within diatom cells or be released into the surrounding environment, where it occurs as particulate DA (pDA) associated with cells or as dissolved DA (dDA); both forms are a risk for higher trophic levels. Recent studies have clarified the biosynthetic pathway of DA, which involves four key enzymes – dabA, dabB, dabC, and dabD – and begins with geranyl pyrophosphate produced via the methylerythritol phosphate (MEP) pathway (Brunson et al., 2018; Harðardóttir et al., 2019). Among DA-producing species, *Pseudo-nitzschia multistriata* is a widespread diatom and a model organism for life cycle studies, supported by extensive omics resources and detailed

knowledge of its growth and physiology. However, only preliminary insights into its DA production are currently available (Amato et al., 2010; Ferrante et al., 2023; Mager et al., 2025). Investigating the transcriptional and physiological responses of *P. multistriata* to P deprivation is therefore essential for understanding its survival strategies and elucidating the mechanisms underlying *Pseudo-nitzschia* blooms and their ecological consequences, including impacts on biogeochemical nutrient cycling under nutrient-poor conditions in the context of global climate change.

In this study, we present the first comprehensive characterisation of the molecular and physiological responses of *P. multistriata* to P limitation. We assessed whether its transcriptional profile corresponds with previously described responses in other diatoms, including the effect of this nutritional regime on DA production.

2. Materials and methods

2.1. Experimental setup

A stock culture of *P. multistriata* strain VD10, obtained by laboratory crosses of strains 1334_1 and 1334_5 from the MareChiara Long-Term Ecological Research station in the Gulf of Naples (Italy), was maintained in diluted 1:1 f/2 medium (i.e. f/4) at a salinity of 36 (Guillard and Ryther, 1962), prepared with natural seawater from C1, the Long-Term Ecological Research station in the Gulf of Trieste (Italy). The cultures were grown at 20 °C under an irradiance of 70 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a 12:12 h light-dark photoperiod under cool white fluorescent light. Prior to the experiment, a low-density stock culture was acclimatised in fresh control medium prepared with artificial seawater (RedSea Salt) at a salinity of 36 to match the medium used for the experimental controls. The medium contained 441 $\mu\text{M NaNO}_3$, 18.1 $\mu\text{M NaH}_2\text{PO}_4$, 53 $\mu\text{M Na}_2\text{SiO}_3$, and vitamins and trace metals as in f/4.

Experimental cell suspensions were prepared by filtering the stock culture through cellulose acetate filters (Sartorius, 47 mm diameter, 1.2 μm pore size) to obtain an initial density of approximately $2 \cdot 10^4$ cells mL^{-1} in a final volume of 1.5 L. Filters were washed three times with either control medium (the same as used previously) or P-free medium (noP), then resuspended in the respective media.

For both control and noP treatments, triplicate 1 L flasks containing 500 mL of culture were incubated at 20 °C, 70 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, under a 12:12 h light-dark cycle for 7 days.

Two independent experiments (hereafter Exp. 1 and Exp. 2) were conducted using the same experimental setup. In Exp. 1, samples for dissolved and particulate nutrient analyses were collected together with samples for transcriptomic analysis. In Exp. 2, samples were collected to assess cell viability, chloroplast and nuclear morphometric analyses, in addition to samples for DA analyses (both gene expression and toxin quantification).

2.2. Dissolved nutrients and particulate C, N and P

In the Exp. 1, samples for dissolved nutrients (nitrite - NO_2 , nitrate - NO_3 , ammonium - NH_4 , phosphate - PO_4 , and silicate - SiO_4) were collected on days 0, 3, 4, and 6 from both treatment and control cultures. Simultaneously, samples for particulate organic C (POC), N (PN), and P (TPP) were also taken.

Samples for dissolved inorganic nutrients were filtered over pre-combusted (450 °C for 4 h) Whatman GF/F filters (nominal pore size 0.7 μm , ϕ 25 mm) in acid-washed polyethylene vials and kept frozen (-20 °C) until laboratory analysis. Dissolved inorganic nutrients were determined colorimetrically using a segmented flow autoanalyzer (QuAatro - Seal Analytical) following the method of Hansen and Koroleff (1999). The detection limits for the procedure were 0.01, 0.01, 0.03, 0.01 and 0.02 μM for NO_2 , NO_3 , NH_4 , PO_4 , and SiO_4 respectively.

For POC, PN and TPP, aliquots between 3 and 25 mL were filtered on

pre-combusted (450 °C for 4 h) Whatman GF/F filters (nominal pore size 0.7 µm, ø 25 mm) and were then stored at –20 °C until analyses. The analyses for POC and PN were performed using a CHNS-O Costech elemental analyzer (mod. ECS 4010) as described in Quero et al. (2020). For POC determination, the filters were previously treated with HCl 1 M to remove carbonates (Nieuwenhuize et al., 1994). The analytical protocol of Aspila et al. 1976 was used for the determination of TPP. Phosphate was extracted from particulate P by acid treatment with 1 M HCl, after a 2 h combustion of the filters at 550 °C and its concentration was determined with the autoanalyzer as PO₄.

2.3. Cell density and viability

In both experiments, cell density was estimated on day 0 and then daily from day 3 to day 6 by counting at least 200 cells using a Sedgewick-Rafter counting chamber at 200 × magnification under a Leica MC170HD light microscope (Leica Microsystems, Milton Keynes, UK) after fixation in 2% neutralised Lugol's iodine solution.

Additionally, cell viability was assessed using fluorescein diacetate (FDA) staining during Exp. 2, as described by Steinberg et al. (2011). Samples were analysed at 200 × magnification within 30 min using a fluorescence microscope Leica DMI3000B (Leica Microsystems, Milton Keynes, UK) equipped with an FITC filter set.

2.4. Chloroplast and nuclear morphological changes

Morphometric analyses were conducted on samples collected from control and treatment cultures on days 0, 3, 4, and 6 of Exp. 2 after fixation with 4% formaldehyde and staining with the nuclear dye DAPI. A total of 68 Z-stack images of 50 single cells per condition and sampling point were acquired using an HC PL APO CS2 63 × /1.20 water-immersion objective on a Stellaris 8 confocal microscope (Leica Microsystems, Milton Keynes, UK) with two fluorescence channels (DAPI and chlorophyll a) and one transmitted light channel. Chloroplast sphericity, biovolume, and nuclear biovolume were measured and analysed following Walde et al. (2023).

2.5. RNA extraction, sequencing and bioinformatic analysis

RNA samples were collected from triplicate cultures in both experiments. RNA from Exp. 1 was used for sequencing and differential gene expression analysis, while RNA from Exp. 2 was used for qRT-PCR validation. Samples were taken from control cultures during the exponential phase (T4, day 4) and early stationary phase (T6, day 6), and from the early stationary noP cultures (T4, day 4). Samples were filtered using filters with a pore size of 1.2 µm (25 mm diameter, Millipore), immersed in TRIzol reagent (Invitrogen), and stored at –80 °C until RNA extraction. Total RNA was extracted according to the manufacturer's instructions, then treated with RNase-Free DNase Set (Qiagen) and purified using the RNeasy Plant Mini Kit (Qiagen). The resulting RNA samples were quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) and their integrity assessed using a High Sensitivity RNA ScreenTape assay (Agilent, Santa Clara, CA).

RNA-seq libraries were prepared using the Illumina Stranded mRNA Prep kit, including poly(A) mRNA enrichment and strand-specific library construction. Paired-end libraries were sequenced on an Illumina Novaseq X Plus platform (Illumina, San Diego, CA, USA).

Raw reads were initially quality checked with FASTQC (available at <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and then cleaned using Trimmomatic (Bolger et al., 2014), with the head-crop parameter set to 3. All cleaned reads were mapped to the reference genome of *P. multistriata* (Basu et al., 2017) using the STAR aligner (Dobin et al., 2013). Read count calculation for each replicate was performed using the FeatureCount tool (Liao et al., 2014).

Differential gene expression analysis was performed implementing two statistical approaches: DESeq2 (Love et al., 2014) and edgeR

(Robinson et al., 2010). For each gene, the mean of the log₂ fold change (FC) values obtained with the two tools was calculated. The thresholds for calling differentially expressed genes (DEGs) were FDR ≤ 0.05 for edgeR and P-adjusted ≤ 0.05 for DESeq2, with |log₂(FC)| ≥ 1.5.

Functional annotations and Gene Ontology (GO) terms for each gene were retrieved using the *P. multistriata* annotation file available at <https://bioinfo.szn.it/pmultistriata/>. GO enrichment analysis of DEG datasets was performed using the Ontologizer software (Bauer et al., 2008), with the “parent-child union” calculation method and a P-value threshold of P ≤ 0.05 to identify significantly enriched functional terms. *P. tricornutum* homologs were obtained by blasting *P. multistriata* against the PLAZA database (Osuna-Cruz et al., 2020).

2.6. Quantitative real-time PCR (qPCR)

cDNAs from samples collected during Exp. 2 at the same sampling point used for RNA sequencing were produced using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Primer sequences for *dabA* and *dabC* were obtained from Mager et al. (2025), and primers for the reference gene *CDK A* from Adelfi et al. (2014).

For three technical replicates for each sample, reactions were carried out in a final volume of 10 µL containing 1 µL of cDNA (diluted 1:5), primers at 300 nM, and 5 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The thermal profile included an initial denaturation (30 s at 94 °C), followed by 34 cycles with the following parameters: denaturation (20 s at 94 °C), annealing (20 s at 55 °C), and elongation (1 min at 68 °C). At the end of the amplification cycles, a final step of 5 min at 68 °C was included. Data analysis was performed using CFX Maestro software version 2.2 (Bio-Rad).

2.7. Domoic acid quantification

Samples for pDA and dDA were collected on days 0, 4, and 6 during Exp. 2 from the three biological replicates of both control and noP cultures. Cultures were filtered on GF/F filters (Whatman, 25 mm diameter, 0.45 µm pore size), and the corresponding filters and filtrates were stored at –80 °C until analysis. For pDA, bead mill homogenisation was selected as the cell disruption method, as it proved more reliable and reproducible than the sonication method used in Turk Dermastia et al. (2022).

Replicates were disrupted using 0.5 mm glass beads in a bead mill homogeniser (Vender, Model MM400, Retch GmbH, Germany) at a ratio of 0.25 g beads per 10⁶ cells and shaken for 5 min in a test tube with 2 mL of 50% v/v methanol (Pisapia et al., 2017). For dDA, filtrates were diluted in 50% v/v methanol and shaken for 5 min. All samples were then centrifuged at 4500 rpm for 30 min, and the resulting supernatant was filtered through a Millex 0.22-µm syringe filter (Merck-Millipore, Darmstadt, Germany) to remove debris. The filtrate was subsequently stored at –20 °C. All solvents used were HPLC grade (VWR, Radnor, USA).

The determination of DA content in the extracted samples was carried out following chromatographic separation on a reversed-phase C18 column (5 µm, 250 mm × 4.6 mm) under isocratic conditions (5% acetonitrile with 0.1% v/v TFA). Ten microlitres of sample was injected and analysed using an HPLC with a UV–VIS detector set at 242 nm. The amount of DA was calculated by the external standard method. A certified DA standard from NRC–Canada was used to prepare six standard solutions to calibrate the HPLC system.

2.8. Statistical analyses

All physiological data were analyzed using GraphPad Prism version 9.5.1. A repeated measures two-way ANOVA with Geisser–Greenhouse correction was applied. Šidák's multiple comparisons test was used to assess the effects of treatment, time, and their interaction. Differences

were considered statistically significant at $P \leq 0.05$.

3. Results and discussion

3.1. Cell growth and physiological changes

The growth of *P. multistriata* followed a similar pattern in both experiments. Cell density in noP cultures remained comparable to controls until day 3, after which growth arrest became evident. These cultures stabilised at approximately $1.2 \cdot 10^5$ cells mL^{-1} , while controls continued to grow, reaching an average of $3.5 \cdot 10^5$ cells mL^{-1} (Fig. 1a, c). This pattern was directly linked to P availability, as nutrient analyses confirmed that no other nutrient was limiting in noP cultures throughout the experiments (Supporting Information Fig. S1). In controls, the concentration of all nutrients decreased sharply throughout the experiment, most likely as a consequence of continuous assimilation associated with algal growth. In contrast, in the noP medium, where P was already depleted at the start of the experiment, the concentrations of the other macronutrients declined sharply during the first three days and then decreased only marginally thereafter. As a result, by the end of the experiment, the concentrations of most nutrients were higher in the noP medium than in the replete conditions. Such growth cessation under P stress is consistent with previous observations in other microalgal species, including diatoms, reaffirming the pivotal role of P in sustaining photosynthetic microorganisms (Dyhrman and Palenik, 1999; Bertilsson et al., 2003; Lovio-Fragoso et al., 2021). Despite the growth arrest, cell viability remained high ($\sim 80\%$) throughout Exp. 2 (Fig. 1f), indicating

that P limitation triggers a survival-oriented physiological state rather than immediate mortality, consistent with observations in other phytoplankton species such as *T. pseudonana*, *P. tricornutum*, and *Amphidinium carterae* (Dyhrman et al., 2012; Cruz de Carvalho et al., 2016; Li et al., 2016).

Phenotypic and biochemical remodelling preceded growth arrest in both experiments. By day 3, when cell densities were still similar, noP cultures appeared visibly paler than controls (Fig. 1b), coinciding with marked intracellular changes. The intracellular C:N ratio increased significantly in treated cultures on days 3 and 4, reaching a final value of ~ 10 , while remaining around 6 in control cultures throughout the experiment (Fig. 1d). This difference, due to the culture medium and exacerbated by time in culture, is a common response to P starvation and may be related to C fixed by non-dividing photosynthetic organisms. Increased C:N ratios, for example, have been observed in *A. carterae*, *Prochlorococcus*, *Synechococcus*, and *Chaetoceros wighamii* under similar limitation (Bertilsson et al., 2003; Spilling et al., 2015; Li et al., 2016). The N:P ratio did not exceed 23 in control cultures, whereas noP cultures exhibited values approximately fivefold higher by day 6, reaching ~ 127 (Fig. 1e). This resulted in final elemental ratios of 129:23:1 in control cultures and 1256:127:1 in noP cultures. Although local deviations from the Redfield ratio are commonly observed and do not necessarily imply specific physiological constraints (Geider and La Roche, 2002), the extremely elevated ratios observed in noP cultures indicate severe P limitation and suggest a low P demand for survival in *P. multistriata*.

Consistently, DAPI staining, which preferentially binds DNA with minimal contribution from RNA, revealed a significant reduction in

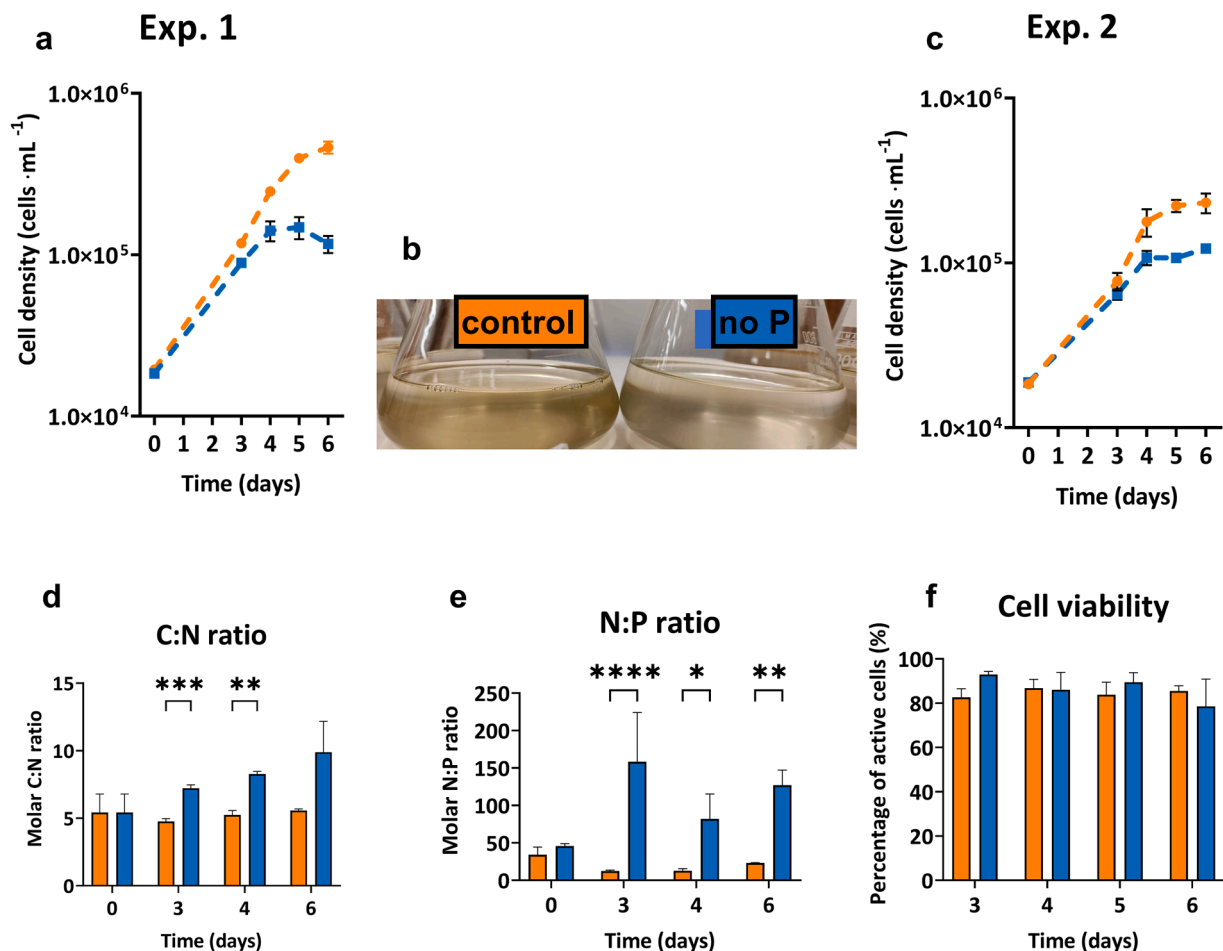


Fig. 1. Effects of P limitation on *P. multistriata* cultures (control in orange, noP in blue): a) growth curves of Exp. 1; b) representative image of experimental cultures after 3 days in control and noP media; c) growth curves of Exp. 2; d) molar C:N ratio in Exp. 1; e) N:P ratio in Exp. 1; f) percentage of live cells during Exp. 2. All data are presented as average $\pm$ st. dev. $N = 3$. Statistically significant P values are reported with asterisks (* ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001).

nuclear volume in noP cells at the single-cell level during Exp. 2 (Fig. 2a). This reduction indicates decreased DNA-related activity, such as mitosis and possibly transcription, and correlates well with the aforementioned changes in growth rates and the transcriptional downregulation of the GO term “DNA replication” observed in Exp. 1 (see Section 2, Fig. 3d).

Additionally, the same cells exhibited smaller, more compact chloroplasts under noP conditions compared to the elongated and voluminous chloroplasts observed in controls (Fig. 2b, c, d). These findings are consistent with transcriptomic evidence of stress adaptation (see Section 2) and resemble those reported in *Leptocylindrus aporus* and *L. hargreavesii*, where P-starved cells had smaller chloroplasts and a tendency to form longer colonies compared to P-replete conditions (Kuzat et al., 2022). Although we did not quantify the length of *P. multistriata* colonies in our study, a similar tendency was observed in both experiments, suggesting that this may represent a shared adaptive strategy at both the single-cell and population levels among chain-forming species.

Finally, some cells under starvation also showed intracellular rounded structures, presumably lipid droplets, whose identity requires further investigation (Fig. 2d) (see Section 2.3).

3.2. Transcriptional changes

Out of a total of 12,008 predicted genes (Supporting Information Table S1), 2839 were differentially expressed, with 1123 upregulated and 1716 downregulated in noP samples compared to the control at the exponential phase. There were 2500 differentially expressed genes, with 1083 upregulated and 1417 downregulated, when comparing noP and control cultures during the stationary phase (T6) (Fig. 3a, Supporting Information Table S2). The comparison between noP and the control samples at T4 revealed significantly higher and lower FC values than the comparison with T6 (Supporting Information Table S2).

Approximately half of these DEGs (1748) were shared between the two comparisons, highlighting a possible nutrient-specific physiological

response (Fig. 3a).

At the transcriptional level, the most evident changes related to P deprivation are reflected in the analysis of enriched GO terms, particularly in biological processes (Fig. 3, Supporting Information Table S3). P starvation resulted in the downregulation of “Photosynthesis” and “Pigment metabolic processes”, regardless of the growth phase of control cultures used for comparison (Fig. 3d, e), in line with the evidence obtained at the single-cell level (Fig. 2). Other biological processes expected to be reduced during this type of stress are those involving the P-rich nucleic acids (Lovio-Fragoso et al., 2021). In fact, DNA replication and DNA biosynthesis processes, as well as translation, were downregulated compared to exponentially growing cultures. An opposite trend was observed for categories related to “RNA metabolic processes” (Fig. 3b, c). While the downregulation of “Protein translation” is clearly observed among the enriched GO terms, categories related to mRNA, rRNA, tRNA, and ncRNA are instead enriched in the set of upregulated genes (Fig. 3b and c). This apparent contrast may be explained by the fact that, on one hand, cells initiate immediate responses to starvation by reducing protein synthesis; on the other, they begin preparing for long-term adaptations that will enable them to cope with prolonged nutrient limitation experienced in the following experimental days.

3.2.1. SPX-PSR1 regulatory system of P homeostasis

Despite advances in omics technologies, the metabolic pathways involved in phosphate sensing and uptake remain only partially understood.

SPX proteins can be classified into four subfamilies based on the presence or absence of additional domains; those lacking additional domains typically respond to P starvation in both *Arabidopsis* and *P. tricornutum* (Zhang et al., 2021). In our study *P. multistriata* presented two SPX genes differentially expressed: 0019820, the homolog of the SPX gene characterised by Zhang and co-authors in *P. tricornutum* (PtSPX, Phatr3_J47434) was upregulated, as in their study, while the other, 0027680, showed negative FC values (Table 1, Fig. 4a and

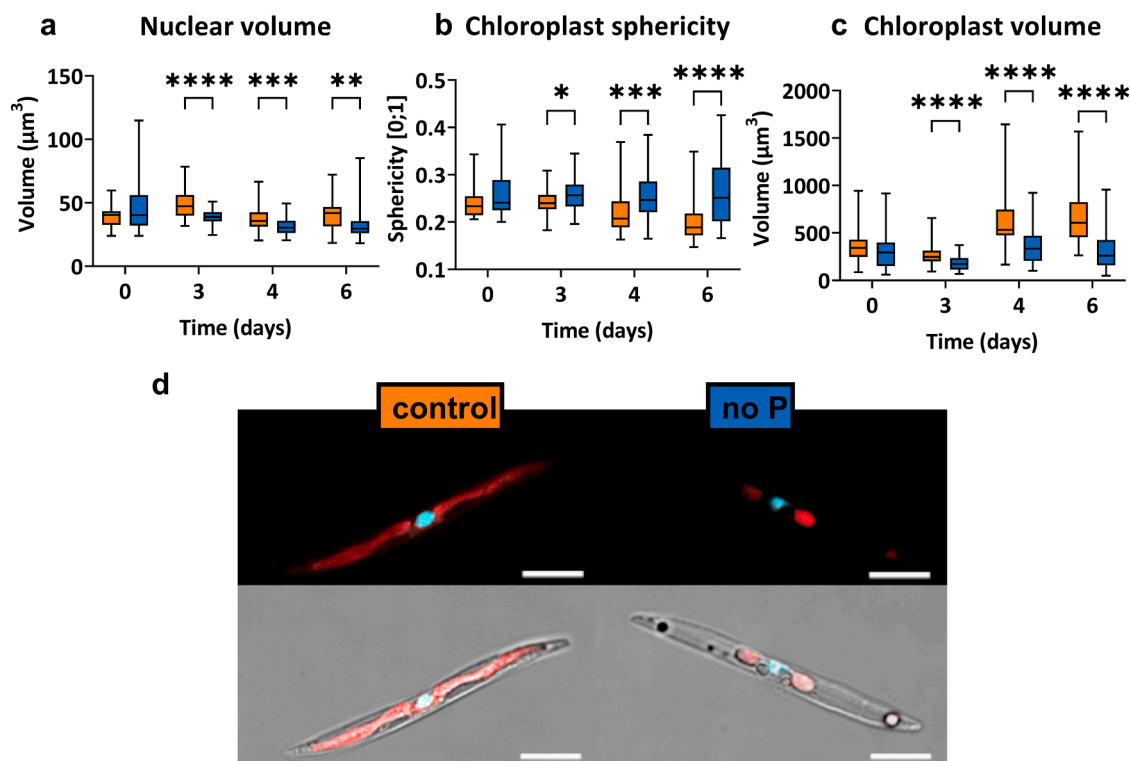


Fig. 2. Morphological changes at the single-cell level during Exp. 2 (control in orange, noP in blue): a) nuclear volume; b) chloroplast sphericity; c) chloroplast volume; d) representative images of cells analysed in confocal microscopy (CLSM); red is the chlorophyll autofluorescence and blue is DAPI. All data are presented as average $\pm$ st. dev. $N = 50$. Scale bars = 10 μm . Statistically significant P values are indicated with asterisks (* ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001).

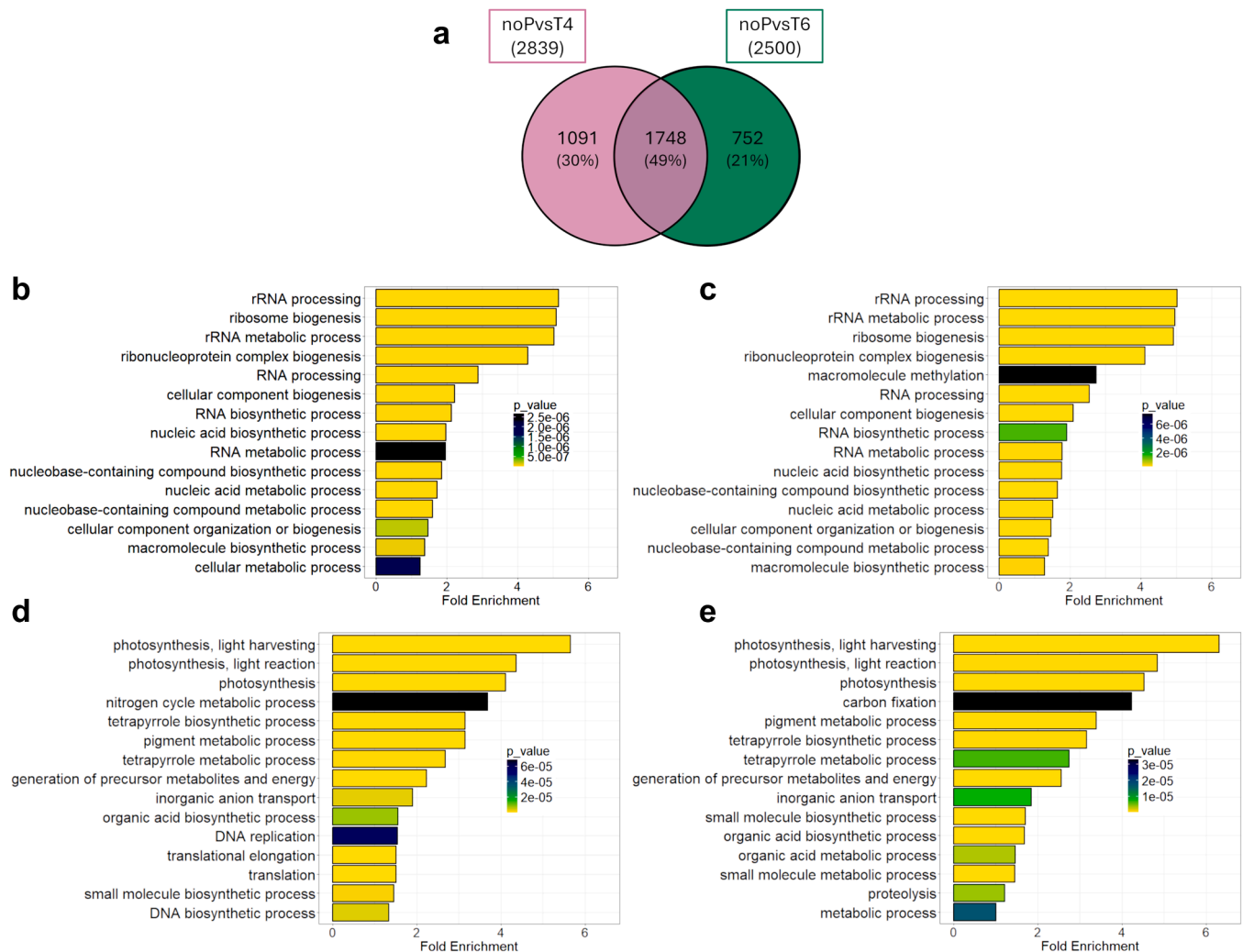


Fig. 3. Differential expression analysis and Gene Ontology (GO) enrichment between cultures in noP and in exponential (T4) and stationary (T6) controls. a) Number of differentially expressed genes (DEGs) exclusive to and shared between the two comparisons; b) biological processes upregulated in the comparison noP vs control T4; c) biological processes upregulated in the comparison noP vs control T6; d) biological processes downregulated in the comparison noP vs control T4; e) biological processes downregulated in the comparison noP vs control T6. All GO terms statistically significant are listed in supplementary table S2.

Supporting Information Table S4). This, together with the fact that 0019820 is the only DEG presenting an SPX domain exclusively, suggests that this gene may have a similar function in *P. multistriata*. Additionally, PSR1 (Phatr3_J47256 in *P. tricornutum*), which in diatoms belongs to the MYB transcription factor family, has a putative homolog in *P. multistriata* that was also upregulated under P starvation (Table 1 and Fig. 4a), further supporting a broader role for the SPX-PSR1 system in P sensing and regulation in diatoms.

Although our understanding of the interactions among SPX proteins is still incomplete, the detection of SPX domain-containing transcripts in environmental datasets highlights their potential ecological significance in *P. multistriata*. Specifically, *P. multistriata* transcripts associated with SPX domains were identified in seven Metagenomic-based Transcriptomes (MGTs): MGT-v1–15, MGT-v1–24, MGT-v1–50, MGT-v1–57, MGT-v1–95, MGT-v1–228, and MGT-v1–574 (Zhang et al., 2021), suggesting that SPX-mediated regulation could be active in natural assemblages, plausibly during P-limited conditions. Such evidence supports the idea that mechanisms of P sensing and regulation uncovered under laboratory conditions may play an ecologically relevant role in shaping planktonic population structure in the field.

In our dataset, several calcium-related genes were differentially expressed, including two Ca^{2+} /calmodulin-dependent protein kinases (CAMKs) that were downregulated and a Ca^{2+} -dependent kinase that

was upregulated (Supporting Information Table S5), suggesting a possible role for the latter in the P-Ca^{2+} signalling pathway, which is especially important when P is resupplied, as previously proposed for *P. tricornutum* and *T. pseudonana* (Helliwell et al., 2021).

3.2.2. P metabolism under P limitation

As mentioned in the introduction, the uptake of P, either directly or following its release by extracellular enzymes, represents the most straightforward strategy for phosphorus acquisition, as reflected by the induction of P transporters. In *P. multistriata*, both organic and inorganic P transport across the plasma membrane appeared to be activated, as indicated by the upregulation of a putative *glycerol-3-phosphate transporter*, a *sodium-dependent phosphate transport protein*, and an *inorganic phosphate transporter* in starved cells (Table 1, Fig. 4a and Supporting Information Table S4).

In our experiment, two alkaline phosphatases were highly upregulated: a *phytase-like alkaline phosphatase* and a *PhoD-type enzyme* (Table 1, Fig. 4a and Supporting Information Table S4). In *P. tricornutum*, transcription of these proteins appears to be controlled by external P concentration (Dell'Aquila et al., 2020). As suggested for *PtPhos2* (Phatr3_J47612), the phytase-like protein produced by *P. multistriata* could be secreted into the extracellular space, where it hydrolyses organic compounds before the products are imported into

Table 1

Genes related to P homeostasis in *P. multistriata*. Gene name, Uniprot ID, and function are reported, along with log₂ fold changes (FCs) in the comparison between noP and exponential (T4) and stationary (T6) controls, the ID in *P. multistriata* (note that only the numerical part of the ID is reported after the gene name prefix PSNMU-V1.4.AUG-EV-PASAV3), and the ID for the *P. tricornutum* homolog. Asterisks indicate genes with FC below 1.5, the cutoff used in this study. The “+” symbol indicates a low identity percentage.

Gene name	UniProt ID	Function	FC noPvsT4	FC -noPvsT6	<i>P. multistriata</i> gene ID	<i>P. tricornutum</i> homolog
SPX domain-containing protein 2	O48781	P uptake regulator	3.1	1.9	0019820	Phatr3_J47434 (PtSPX)
Bifunctional protein HldE (SPX domain)	Q1R1M6	P uptake regulator	−8.2	−6.1	0027680	Phatr3_J54257 (PtSPX2)
Transcription factor BOA- (putative PSR1)	Q9LTH4	P uptake regulator	2.1	2.1	0119090	Phatr3_J47256
Calcium-dependent protein kinase 3 (CPK31)	Q9S9V0	P sensing	2.2	3.7	0102250	Phatr3_J47797
Inorganic phosphate transporter 1–1	Q8VYM2	P transport	2.1	-	0109750	N/A ⁺
Sodium-dependent phosphate transport protein 2A	Q28620	P transport	4.8	3	0083590	Phatr3_J33266
Putative glycerol-3-phosphate transporter 3	Q9SA71	P transport	5.8	4.8	0124090	N/A ⁺
Alkaline phosphatase (ALP, phytase-like)	UP10003B66ED6	DOP hydrolysis	7.7	-	0038700	Phatr3_J47612 (PtPhos2)
Alpha/beta hydrolase domain-containing protein 17B (PhoD)	Q5VST6	DOP hydrolysis	4.0	3.6	0048210	Phatr3_J45757 (PtPhos6)
SPX domain-containing membrane protein At4g22990 (VPT1)	Q93ZQ5	P transporter into the vacuole	1.2*	-	0024480	Phatr3_J19586
Vacuolar transporter chaperone 2 (VTC2)	O13718	P storage	1.7	-	0117880	Phatr3_J15281
Vacuolar transporter chaperone 4 (VTC4)	P78810	P storage	2.3	-	0054630	Phatr3_J50019

the cell. Internal recycling activity can also be hypothesised, since the metallophosphatase D homolog of *PtPhos6* localises to the endomembrane system (Dell'Aquila et al., 2020; Li et al., 2022). Other enzymes involved in phosphate acquisition during P deficiency include 5'-nucleotidases, which may facilitate both intra- and extracellular phosphate recycling from RNA and DNA molecules. Several of these enzymes were differentially expressed in our dataset (Supporting Information Table S4); however, the most upregulated 5'-nucleotidase identified in *P. tricornutum* (Phatr3_J43694), which is predicted to act extracellularly, was not differentially expressed in *P. multistriata*, despite being present in its genome (Alipanah et al., 2018). This lack of expression may reflect the low mortality observed during the experiment, which would limit nucleic acid release and thus reduce the functional relevance of this enzyme under the tested conditions.

The active growth observed during the first three days, despite undetectable P levels in the treatment, is plausibly attributed to polyphosphates, storage compounds that accumulate in vacuoles when environmental P is present in excess (Lin et al., 2016). In diatoms, the transport of these compounds is thought to be mediated by vacuolar transporter chaperones. *P. multistriata* expresses homologs of the vacuolar transporter complex, VTC2 and VTC4, as well as two pyrophosphate-energised vacuolar membrane proton pumps involved in moving phosphate into the vacuole (Table 1 and Fig. 4a). VTC4 localisation has not yet been confirmed and shows bimodal expression: it is weakly downregulated in the early days of P limitation but upregulated under severe P starvation in both *P. tricornutum* and *T. pseudonana* (Yang et al., 2014; Cruz de Carvalho et al., 2016; Alipanah et al., 2018; Dell'Aquila et al., 2020). Additionally, gene 0024480, a putative *P. multistriata* homolog of the vacuolar phosphate transporter VPT1, which is upregulated during P limitation in *P. tricornutum* (Dell'Aquila et al., 2020; Alipanah et al., 2018), was not present in our DEGs dataset because its FC value was slightly below our cutoff (FC = 1.2) (Table 1). The strong upregulation of this gene in *P. tricornutum* after 48 h of P deprivation suggests an early role in the P starvation response. Consistent with these observations, the expression patterns in our dataset indicate that *P. multistriata* had already progressed beyond the initial stages of P starvation by day 4. Together, these results indicate that both extracellular and intracellular phosphate recycling are activated in *P. multistriata* under P limitation, as observed in other microalgae (Dyhrman et al., 2012; Shi et al., 2017; Kalinina et al., 2025).

3.2.3. Influence of P limitation on other nutrient metabolisms

P limitation often results in reduced N metabolism due to lower protein synthesis and, at times, a general decrease in transcriptional activity (Lovio-Fragoso et al., 2021). Only recently has a hierarchical mechanism, in which P metabolism is finely coordinated by N availability, been highlighted in diatoms, with the latter inhibiting the physiological response to P limitation when both are colimiting (Harrison et al., 2025).

In addition to PSR1, which plays a central role in integrating P signaling with N availability, a novel role in sensing and coordinating N and P uptake has been attributed to trypsin, particularly TryP2 in *P. tricornutum*, whose expression increases under P depletion (You et al., 2022; 2023). In our dataset, only one trypsin-like serine protease shows a slightly positive FC, but due to its low identity scores with *PtTryP2*, no definitive conclusions can be drawn about its involvement in N–P regulatory system. Nonetheless, in agreement with findings in *P. tricornutum*, P limitation led to the downregulation of most genes related to N uptake and assimilation (Fig. 4b, Supporting Information Table S4) (Helliwell et al., 2021).

Carbon metabolism is also affected, as indicated by the increase in intracellular C content and the enrichment of “Carbon fixation” and “Photosynthesis” biological terms among the downregulated genes (Figs. 1d, 3d and e). Central pathways for carbon metabolism are glycolysis and gluconeogenesis, as they break down and synthesise glucose, respectively. Furthermore, glycolysis is closely interconnected with the pentose phosphate pathway (PPP) and the TCA cycle: beyond producing ATP and pyruvate, glycolysis generates intermediates that can be diverted into the PPP, supporting NADPH and nucleotide biosynthesis, or into the TCA cycle, which drives further ATP production. Key glycolytic enzymes are differentially expressed: two pyruvate kinases (PK7 and PK8) show opposing trends, while a phosphofructokinase (PFK) is upregulated, suggesting increased glycolytic flux (Fig. 4c). In contrast, gluconeogenesis appears repressed: five fructose-1,6-bisphosphatases (FBPs) are mostly underexpressed, as are a phosphoenolpyruvate carboxykinase (PEPCK) and a pyruvate carboxylase (PC.2) (Fig. 4c, Supporting Information Table S4). Furthermore, pyruvate from glycolysis may not enter the TCA cycle, as components of the pyruvate dehydrogenase complex are downregulated, consistent with the downregulation of TCA enzymes (Supporting Information Table S5).

The oxidative branch of the PPP is also downregulated, with transcripts such as ribulose-5-phosphate 3-epimerase and ribose-5-phosphate

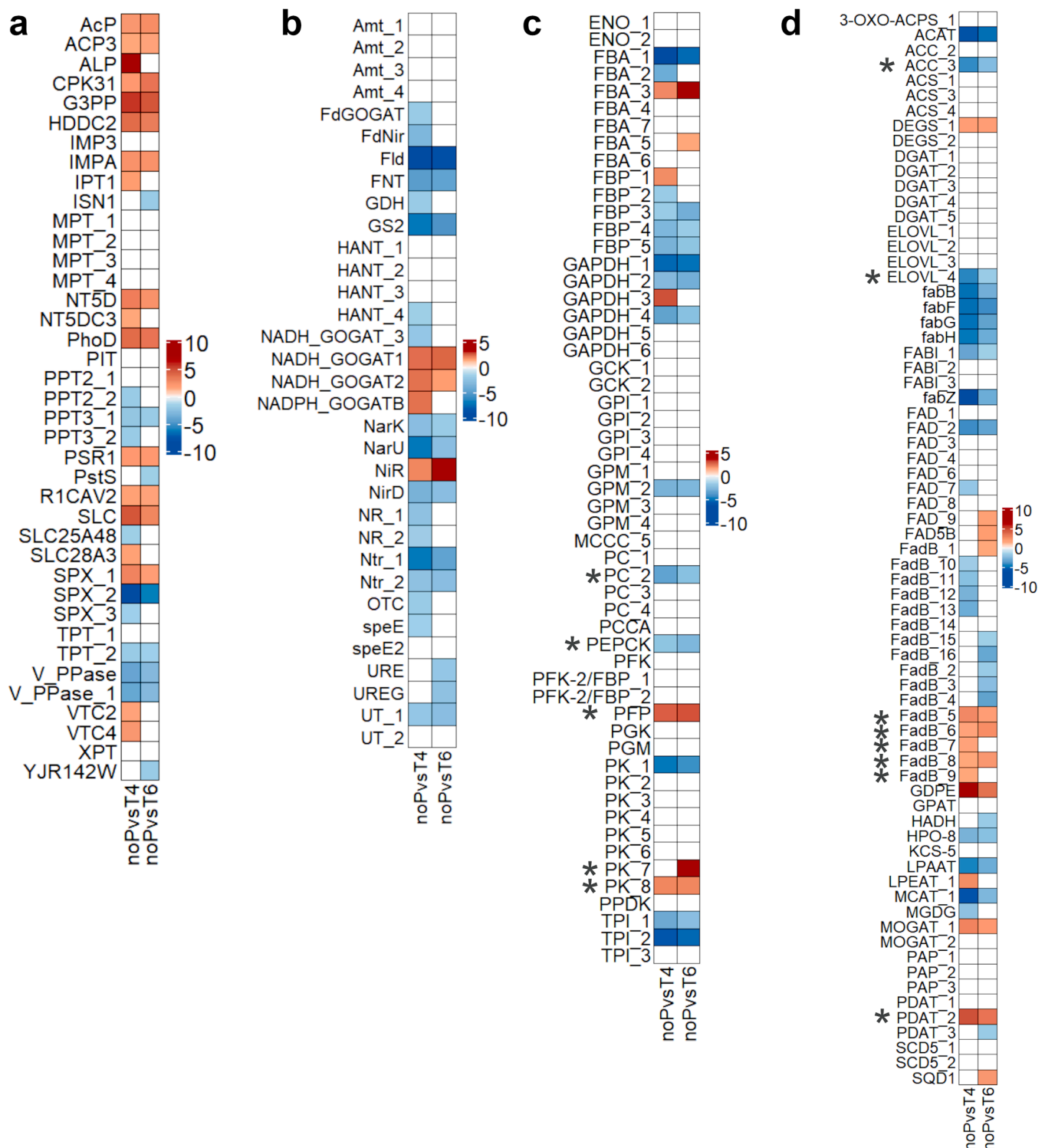


Fig. 4. Heatmaps showing genes differentially expressed in noP cultures compared to control in exponential (T4) and stationary (T6) phases. The genes reported are related to a) phosphate; b) nitrogen; c) glycolysis and gluconeogenesis; d) fatty acid and triacylglycerol metabolism. Data are shown as the mean of FCs. N = 3. In c and d, genes discussed in the text are marked with asterisks; all gene abbreviations are listed in supplementary table S4.

isomerase showing strong repression, indicating limited nucleotide biosynthesis under P stress (Supporting Information Table S5). This is consistent with observations in other *Pseudo-nitzschia* species and *P. tricornutum* under similar growth conditions (Alipanah et al., 2018; Lema et al., 2019). Altogether, these transcriptional patterns are coherent with the idea of maintaining short-term energy balance while reducing anabolic processes. To determine whether the observed

intracellular carbon was derived from lipids or carbohydrates, we also examined DEGs related to lipid or carbohydrate pathways.

De novo fatty acid biosynthesis and elongation appear generally reduced, as *acetyl-CoA carboxylases* (ACCs) and *elongation of very long chain fatty acids* proteins (ELOVLs) are mostly downregulated or not differentially expressed (Fig. 4d, Supporting Information Table S4). Although this transcriptional pattern does not necessarily correlate with

actual fatty acid production in diatoms (Dunahay et al., 1996), it suggests that their concentration may follow a similar trend, as observed in *P. tricornutum*, where both transcriptomic and biochemical analyses were performed (Alipanah et al., 2018).

In contrast, triacylglycerol (TAG) accumulation driven by the recycling of membrane lipids appears enhanced: this is indicated by the strong upregulation of *glycerophosphodiester phosphodiesterase* (GDPE), the most induced gene related to lipid metabolism in our dataset, together with a *phospholipid:diacylglycerol acyltransferase* (PDAT), which catalyses TAG biosynthesis using phospholipid-derived acyl groups. In addition, five of the sixteen phospholipases (FadBs) identified in our dataset were upregulated (Fig. 4d), further supporting enhanced phospholipid degradation as a source for TAG synthesis and P as in *P. tricornutum* (Alipanah et al., 2018). Collectively, these transcriptional signatures support the interpretation that the intracellular bodies observed by CLSM correspond to lipid droplets (Fig. 2d).

Diatoms also store energy as chrysolaminarin, a light-regulated polysaccharide whose metabolism responds to environmental conditions (Kwasiborski et al., 2025). Its synthesis is mediated by β -1,3-glucan synthases, while endo-1,3- β -glucanases and β -glucosidases are involved in its degradation. In our dataset, one β -1,3-glucan synthase was differentially expressed only relative to stationary-phase cells, whereas two *phosphoglucomutases* involved in UDP-glucose synthesis, the main substrate for chrysolaminarin, were upregulated under noP conditions compared to both controls (Supporting Information Table S5), suggesting a potential shift of carbon flux towards carbohydrate biosynthesis (Yang et al., 2020). Genes involved in chrysolaminarin degradation showed variable and inconsistent expression patterns, making conclusions uncertain. Unlike *T. pseudonana* and *P. tricornutum*, where degradation pathways are strongly induced under P limitation (Brembu et al., 2017), this response appears less pronounced in *P. multistriata*.

The putative resulting biochemical changes during P starvation, particularly in lipid content and quality, could affect carbon export, as suggested for other phytoplankton species under nutrient-starved conditions (Wu et al., 2025; Zhang et al., 2023).

3.3. Domoic acid production

P limitation can trigger domoic acid (DA) production in *Pseudo-nitzschia* (reviewed in Lelong et al., 2012), and in *P. multiseriata* this condition was instrumental in the discovery of the DA biosynthetic pathway (Brunson et al., 2018). Nonetheless, the correlation between DA levels and *dab* gene expression is not always linear (Lema et al., 2019). We observed strong upregulation of the genes *dabA*, *dabC*, and *dabD*, as well as genes involved in the synthesis of their isoprenoid precursors via the MEP pathway (Table 2).

In plants as in algae, the MEP pathway is localised in the plastid, where it modulates isoprenoid production in response to carbon status, reducing power, ATP availability and environmental stress (Lohr et al., 2012; Ruiz-Sola et al., 2016; Hoshino and Gaucher, 2018; Harðardóttir

et al., 2019).

The *dab* genes exhibited some of the highest FCs in the dataset, and these FCs were consistent regardless of whether noP cultures were compared to exponential (T4) or stationary (T6) control conditions. Similarly, *isopentenyl diphosphate delta-isomerase* (IDI) and *4-hydroxy-3-methylbut-2-enyl diphosphate reductase* (HDR) displayed lower but stable fold changes across both noP vs T4 and noP vs T6 comparisons. The *dabB* gene model is missing from the *P. multistriata* gene models dataset but the coding sequence can be found when manually inspecting the genomic region (Mager et al., 2025). Visualization of RNA-seq reads mapped to the reference genome shows many reads in P depleted samples mapping to the relative region, indicating that this gene follows the same trend of the others, albeit having always lower expression levels with respect to the other three *dab* genes (Supporting Information Fig. S2). These transcriptional signals, coming from Exp. 1, were further confirmed by qPCR analysis of *dabA* and *dabC* in Exp. 2. Our results clearly show that six days of P limitation are sufficient to significantly increase the transcription of DA genes in *P. multistriata* (Fig. 5a). Additionally, the expression of *dab* genes was downregulated in the stationary phase (T6) relative to the exponential phase (T4) in control cultures.

DA synthesis begins with GPP, a P containing molecule, yet, as observed for other *Pseudo-nitzschia* species, DA production is induced when P is limiting. This could be explained by considering that P does not end in DA but is released from the precursor by *dabA* and recycled back into the cell (Harðardóttir et al., 2019), making DA synthesis sustainable under P starvation.

Chemical analysis of intracellular DA (pDA) was performed at the same sampling point for both noP and control cultures in Exp. 2, with an additional sampling point at T6 for noP cultures. This analysis revealed that pDA production under P limitation was detected in only one replicate at the onset of stationary phase (T4) and in all replicates at the end of the experiment (Fig. 5b). In contrast, no pDA was detected under control conditions at the same sampling points, differing from patterns reported for other *Pseudo-nitzschia* species (Lelong et al., 2012). Additionally, no dissolved DA (dDA) was detected in the culture medium, which may be related to the low cell mortality observed in the same experiment. The measured pDA concentration was 0.01 pg cell⁻¹, a value within the range reported for the species (0.001–0.044 pg cell⁻¹, Mager et al., 2025). Similar values have been reported for other low DA-producing species, such as *P. galaxiae* and *P. fraudulenta* (Cerino et al., 2005; Sahraoui et al., 2011). We cannot exclude the possibility that dDA was present at concentrations below the detection limit of our chemical assay at those sampling points. Previous data suggest that the dDA released by cells depends on growth conditions and that under P limitation, only about 23% of DA is released into the medium, with most remaining particulate (Fehling et al., 2004). If this trend holds true for *P. multistriata*, the amount of DA released as dDA would be too low to be detected with our current analytical method (LOQ = 0.1 µg/mL).

Our results suggest a temporal lag between *dab* gene expression and effective toxin production, consistent with what has been hypothesised for *P. fraudulenta* and *P. pungens*, in which DA was released although *dab* genes were not differentially expressed (Lema et al., 2019).

One of the putative functions attributed to DA is its role as a chelator of iron (Fe) and copper (Maldonado et al., 2002; Silver et al., 2010; Trick et al., 2010). Consistent with this hypothesis, both intracellular and dissolved DA production have been reported to respond to Fe availability in laboratory experiments, although the direction of this response varies among species and environmental conditions, suggesting a complex regulation involving nutrient status and acclimation history (Bates et al., 2001; Maldonado et al. 2002; Wells et al., 2005; Sobrinho et al., 2017). In *Pseudo-nitzschia multiseriata*, for example, early exposure to Fe stress reduces pDA, likely due to rapid release into the medium, whereas prolonged acclimation reverses this pattern, leading to increased pDA under decreasing Fe availability even when growth recovers (Wells et al., 2005; pDA range: 0.04–0.3 pg cell⁻¹).

Table 2
Genes related to the biosynthesis of domoic acid (DA). Gene IDs, gene names, and annotations are provided, along with Log₂ fold changes (FCs) in the comparison between noP and exponential (T4) and stationary (T6) controls.

Gene ID	Gene name	Annotation	FC-PvsT4P	FC-PvsT6P
0118210	<i>dabA</i>	Terpene cyclase nonplant C1	7.7	7.2
0118200	<i>dabC</i>	Flavonol synthase/flavanone 3-hydroxylase	8.4	7.5
0118190	<i>dabD</i>	Cytochrome P450 4C1	5.8	5.3
0102590	IDI	Isopentenyl-diphosphate Delta-isomerase	2.6	2.0
0049430	HDR	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	3.1	5.0

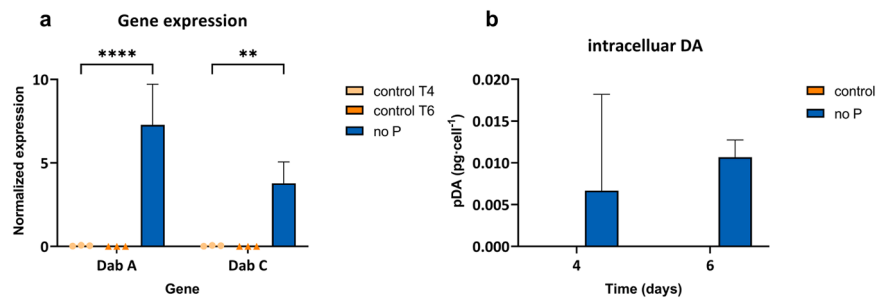


Fig. 5. Comparison of domoic acid (DA) production in both experimental conditions (orange control, blue noP): a) gene expression of *dabA* and *dabC* in noP and control during exponential (T4) and stationary (T6) phases; b) concentration of particulate domoic acid (pDA). Data shown as average $\pm$ st. dev. $N = 3$. Statistically significant P values are indicated with asterisks (* ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001).

In our noP treatment, however, iron limitation is unlikely, as indicated by the strong downregulation of three Iron Starvation-Induced Proteins (ISIPs), which were among the most repressed transcripts in our dataset (Supporting Information Table S5). These observations do not support a role for DA production linked to iron limitation under the conditions tested. Alternatively, the observed transcriptional patterns may be indirectly associated with the P limited physiological state of the cells rather than with Fe availability *per se*. Similar gene expression responses have been reported in *P. tricornutum* under P-limiting conditions (Helliwell et al., 2021; Zhang et al., 2021; Li et al., 2022), suggesting that DA-related genes could be part of broader regulatory adjustments that accompany P stress and contribute to the coordination of intracellular metabolism, including processes such as photosynthesis and pigment synthesis, which have high Fe requirements. However, further experimental evidence is required to establish a direct link between DA regulation and P sensing mechanisms.

DA has also been proposed to mediate ecological interactions, functioning as a defence against grazers (Tammilehto et al., 2015; Harðardóttir et al., 2019). Furthermore, the substantial release of dissolved DA into the surrounding medium indicates that it may participate in chemical signalling or interactions with associated microbial communities (Hagström et al., 2007; Delegrange et al., 2018). Although our data do not allow us to discriminate among these alternative functions, they clearly demonstrate a strong association between P stress, activation of the *dab* biosynthetic pathway and increased DA production in *P. multistriata*.

4. Conclusions

Overall, *P. multistriata* employs an elaborate strategy to cope with P limitation. This strategy relies on the integration of early transcriptional regulation with metabolic remodelling and recycling, organelle reorganisation, and secondary metabolite production. Cellular and biochemical changes, including altered C:N:P ratios and pronounced phenotypic remodelling, occur prior to growth arrest and are already evident by day 3, indicating a pre-emptive, survival-oriented response. These adaptations appear to be conserved strategies across phytoplankton species (Bertilsson et al., 2003; Li et al., 2016; Kužat et al., 2022).

The ability of *P. multistriata* to reach relatively high cell densities in the absence of external P reflects adaptation to fluctuating nutrient conditions. This capacity is supported by the ability to scavenge all available P sources, a low intracellular P quota, and internal carbon storage, likely in the form of triacylglycerols.

Although our transcriptomic data suggest a preferential use of carbohydrates, further biochemical analyses, including direct assessment of lipid and carbohydrate content, are required to fully determine carbon allocation strategies under this stress.

The observed differential expression of *SPX* and *PSR1* homologs supports the hypothesis of their contribution to P homeostasis across

multiple taxa (Harrison et al., 2025; Kalinina et al., 2025). This system, likely activated earlier than the time point sampled for transcriptomic analysis, remained operative in maintaining cellular P balance.

In the case of *P. multistriata*, the transcriptional signatures of *dab* genes may serve as early indicators of toxic potential, offering predictive insight into bloom dynamics.

Variability in toxin production among *Pseudo-nitzschia* species, and even strains, has been previously documented, especially in species with low toxicity (Lema et al., 2019) such as *P. multistriata*. However, the consistency of transcriptional and physiological responses observed across the two experiments indicates a consistent response to P limitation in our model species. P starvation strongly induced the transcription of both *dab* genes and DA precursor genes, confirming that this stress is a potent trigger for their activation. The temporal delay between gene expression and measurable intracellular DA suggests an uncoupling between transcription and toxin production, indicating that DA synthesis may be regulated downstream of transcription or involve yet unidentified regulatory processes.

Additionally, by refining knowledge of the transcriptional and metabolic state of *P. multistriata* under variable environmental conditions, this work is projected toward a system-level understanding of diatom responses and adaptation. The data produced will contribute to a fine reconstruction of gene regulatory networks, support genome-based modelling efforts and aid in the interpretation of the increasing meta-omics data being collected worldwide.

Overall, the adaptive response observed may influence nutrient cycling, trophic interactions and bloom toxicity in marine ecosystems, underscoring the ecological and biogeochemical relevance of physiological and transcriptional plasticity in *P. multistriata*.

CRediT authorship contribution statement

Angela Pelusi: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Luca Ambrosino:** Writing – review & editing, Visualization, Formal analysis. **Sonia Dall'Ara:** Writing – review & editing, Formal analysis. **Martina Kralj:** Writing – review & editing, Formal analysis. **Federica Relitti:** Writing – review & editing, Formal analysis. **Tiziano Mattia Vollaro:** Writing – review & editing, Formal analysis. **Cosimo Solidoro:** Writing – review & editing, Funding acquisition. **Maria Immacolata Ferrante:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Federica Cerino:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.hal.2026.103165](https://doi.org/10.1016/j.hal.2026.103165).

Data availability

Raw sequence data are available at NCBI's SRA depository under the accession number PRJNA1381538. All other data supporting the findings of this study are available on <https://doi.org/10.6084/m9.figshare.31412642>.

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