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# Abiotic stress applied to *Tetraselmis subcordiformis* reveals enhanced metabolic responses and increased production of Omega-3 fatty acids: A Metabolomic approach

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## ABSTRACT

The appeal of high value algal metabolites is certain due to their sustainability, low energy and space requirements. Yet, commercial-level production of such products is limited and has the potential to be enhanced. In microalgae *Tetraselmis subcordiformis* (*T. subcordiformis*) was subjected to four different stressors and the stress response was recorded in terms of changes in the metabolic profile of the strain. The stressors applied were nitrogen (N-starvation) and phosphorus starvation (P-starvation), high salinity, and cold stress. Although growth of the strain was impacted by application of all stressors, an increase of 34 % and 14.9 % in the protein content was noted for 100 PSU and P-starvation stress. Moreover, nitrogen content increase by 20 % and 5.8 % for 100 PSU and N-starvation stress. While the most common lipids for algae in high salinity and N-starvation stressors were triglycerides with  $\alpha$ -linoleic (18:3), linoleic (18:2), and palmitic (16:0) fatty acids. Overall, compared to all stressors applied high salinity of 100 PSU enhanced the overall metabolic profile of *T. subcordiformis*.

## 1. Introduction

In recent years, microalgae have emerged as a promising alternative for producing energy and food to meet the increasing demand caused by global population growth [1]. It is a sustainable source and has been applied in various biotechnological applications such as the biosynthesis of nutraceuticals, biodiesel, and dietary supplements, as well as the bioremediation of wastewater [2].

Microalgal products include high-value pigments, lipids, polysaccharides, polyunsaturated fatty acids, polyphenols, peptides, and vitamins. These high-value algal metabolites (HVAMs) are a sustainable choice of production as extensive land area is not required for farming, less nutrient supply is needed, and wastewater can be used for growth [2]. Furthermore, the market for HVAMs has been growing successfully and is projected to increase in the coming years. For example, demand for algae-based carotenoids was recorded at 1.24 billion dollars in 2016 and was projected to increase to over 1.53 billion US dollars by 2021 [3]. Various research has been published showcasing the different products from algal sources such as astaxanthin production from *Haematococcus*

*pluvialis*, alginates from *Sargassum sinicola*, beta-carotene from *Dunaliella salina*, triglycerides and fatty acids from *Chlorella vulgaris*, and fucoxanthin sourced from *Undaria pinnatifida*.

However, optimization of the production of HVAMs is necessary as only a few products have been successfully commercialized due to constraints such as dependency on factors such as diurnal and seasonal temperature, light fluctuation, contamination, and loss of nutrients and water in open raceway ponds. While using photo bioreactors is a better approach, as growth and culturing conditions can be controlled, it can be an unattractive alternative due to higher production and maintenance costs [1].

It may be important to optimize the growth conditions and metabolite production of algae prior to large scale production. It can be essential to consider environmental factors such as nutrient availability, temperature, salinity, and light intensity. Any changes in these factors can significantly affect the growth and metabolite production rates and quality of a strain. For example, according to a study by Valledor et al. [4], N-starvation led to changes in membrane structure, the accumulation of oil bodies, and the reduction of putative enzymes involved in

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carbon-concentrating mechanisms. In another study, it was concluded that a decrease in nitrogen can result in a reduction in protein content and enhanced lipid or carbohydrate storage [5]. Additionally, according to Araujo et al. [6], limiting phosphate led to a significant reduction in the number of lipids, proteins, and carbohydrates in algae. Although nutrient stress approaches, especially nitrogen starvation, can lead to high lipid accumulation, it is mainly limited by the very low algae biomass productivity associated with low lipid productivity [7].

Salinity is an important factor to consider as it can significantly affect the growth rate of microalgae. It is widely known that fluctuations in salinity can affect the morphology and physiology of microalgae, usually negatively [8]. In a more recent study by Nedbalová et al. [9], it was proven that changes in salinity affected the proportion of unsaturated and saturated fatty acids in microalgae. Therefore, low salinity increased the concentration of asymmetric regioisomers while symmetric regioisomers dominated at higher salinity. Furthermore, Zhang et al., [10] conducted a study on the high starch marine algae *Chlorella sorokiniana* and found that increased salinity stress caused an increase in starch conversion to lipids and carbon redistribution. A decrease in saturated fatty acids was also noted in algae due to salinity stress.

The effect of temperature on microalgal growth and lipid production is like that of light intensity. Microalgal growth and lipid production exponentially increase as the temperature increases and reaches an optimal level. Furthermore, temperature is known to affect the uptake of nutrients, cell chemical composition, and CO<sub>2</sub> uptake [11]. In another study, Wei et al. [12] studied temperature effects on microalgae *T. subcordiformis* and *Nannochloropsis oculata* and found that increased temperature led to a decrease in polyunsaturated fatty acids and an increase in saturated and monounsaturated fatty acids.

Isolating species from the local environment can be important for ensuring the survival and optimized growth of the strain as it is already acclimated to the environmental stressors in the area of cultivation [13]. In this study, *T. subcordiformis* was isolated from Qatari coastal waters. In addition to its ability to cope with Qatar's harsh environment, it is known for several advantages, such as: fast growth, elevated production of proteins, lipids, essential fatty acids (omega 3, omega 6, and omega 9), and essential aminoacids, spontaneous settling capacity due to its large size, and tolerance of high salinity. To assess the metabolomics response of this strain to abiotic stressors, *T. subcordiformis* strain QUCCCM50 was subjected to 4 different stress cultivation regimes: N-starvation, P-starvation, 100 PSU salinity, and cold cultivation at 15 °C.

## 2. Materials and methods

### 2.1. Algae cultivation under different stress regimes

The microalgae isolate *T. subcordiformis*, QUCCCM50, used for the current study, belongs to the Qatar University Culture Collection of Cyanobacteria and Microalgae (QUCCCM) [14]. A pure colony of the strain was used to inoculate 10 mL of BG11 growth medium prepared with seawater of 40 PSU salinity and incubated for 12 days using an illuminated shaker (#76DG08PBBB, Eppendorf, USA) under a temperature of 30 °C, an agitation of 150 rpm and light intensity of 150 μmol m<sup>-2</sup> s<sup>-1</sup> with a 12:12 h light/dark cycle.

The culture was scaled up gradually to 50 mL using a 250 mL Erlenmeyer, followed by 500 mL using a 1 L flask. These cultures were cultivated under the same previously described conditions. Consequently, an adequate volume of the last *T. subcordiformis* was used to inoculate a 1 L photobioreactor (DASGIP, Eppendorf, USA) where the initial optical density at 750 nm (OD<sub>750nm</sub> = 0.4). After reaching the stationary phase (6 days), the biomass was harvested, washed, and used to perform the cultivation under the 4 stress conditions listed below: (i) N-starvation, (ii) P-starvation (iii), salinity stress (100 PSU salinity), and (iv) cold stress. This latter consists of decreasing the temperature from 30 °C to 15 °C by changing bioreactor settings for 2 days. Cold stress was applied for two days to give enough time for the algae to turn biomass

metabolites into high value products.

This temperature corresponds to the lowest annual temperature in Qatar. Marine algae cultured in f/2 medium was used as a control. All cultivation regimes were performed under the same physical parameters of light intensity (400 μmol m<sup>-2</sup> s<sup>-1</sup> with a 12:12 h light/dark cycle), CO<sub>2</sub> (5 %), and temperature (30 °C). All cultures were performed in duplicates, and the experiment was performed twice. In total, four experimental values were recorded for each cultivation regime.

### 2.2. Assessment of microalgae growth parameters

1 mL of culture samples were collected daily from each photobioreactor to perform an OD<sub>750nm</sub>. For dry weight assessment, a 10 mL aliquot of culture was filtered through a 0.47 mm cellulose nitrate membrane filter (microfiber filters Whatman, Germany). The filtrate was washed successively with 0.5 M ammonium formate and distilled water. The collected filter was dried at 80 °C until a constant weight was reached, then transferred to a desiccator over silica gel for complete dehydration until a stable weight was produced. The weight of the dry mass was evaluated in relation to the relative biomass of the cultures. Guillard, [15] calculation of the growth rate (μ) and doubling time (dt) with the following equations:

$$\mu = (\ln x_2 - \ln x_1) / (t_2 - t_1) \quad (1)$$

where  $x_1$  is initial and  $x_2$  are cell densities at initial time  $t_1$  and final time  $t_2$

$$dt = 0.6931 / \mu \quad (2)$$

### 2.3. Metabolomic characterization of algae biomass

#### 2.3.1. Total lipid extraction

Total lipids were extracted from algae biomass using the method of Folch et al. [16] with modifications from Saadaoui et al. [14]. The total lipid content was determined gravimetrically. The lipid content (LC (%)) was determined using the following equation:

$$LC (\%) = (TL/DM) \times 100 \quad (3)$$

where TL and DM are total lipids and dry microalgal biomass respectively in grams.

#### 2.3.2. Total protein extraction

The total protein was extracted after hydrolysis with sodium hydroxide (NaOH 0.1 M). 8 mL of NaOH was added to 50 mg of dried algae biomass, and the lysate was centrifuged (10 min/14000 rpm) after being incubated overnight at 60 °C. The extraction was repeated until the pellet had decolorized.

The total protein content was determined for the supernatant by using the Lowry assay [17], using Folin ciocalteu reagent and bovine serum albumin as a standard.

#### 2.3.3. Chlorophyll content determination

On alternative days for chlorophyll extraction, 1 mL of algae culture was collected. The sample was harvested, and the biomass was suspended in 1 mL of methanol 90 % and kept in a 60 °C water bath until it was colorless. Following extraction, the mixture was cooled until it reached room temperature, and the tube was centrifuged at 15000 rpm for 5 min. The OD of the supernatant was measured to determine the chlorophyll content.

A Spectrophotometer (Jenway, 6305, UK) was used to measure the absorption of the extract at two different wavelengths (650 and 665 nm) to determine the chlorophyll concentration. The following equations, described by Porra et al., [18] were used to calculate the chlorophyll concentration in mg L<sup>-1</sup>:

$$Chl a = (16.5 \times OD_{665nm}) - (8.3 \times OD_{650nm}) \quad (4)$$

$$\text{Chl } b = (33.8 \times OD_{650\text{nm}}) - (12.5 \times OD_{665\text{nm}}) \quad (5)$$

$$\text{Total Chl : Chl } a + b = (4 \times OD_{665\text{nm}}) - (25.5 \times OD_{650\text{nm}}) \quad (6)$$

where Chl a and b are chlorophyll a and chlorophyll b respectively, and  $OD_{665\text{nm}}$  and  $OD_{650\text{nm}}$  are the optical density at wavelengths 665 nm and 650 nm respectively.

## 2.4. Lipidomic profiling of *T. subcordiformis* under the different stress conditions

### 2.4.1. Sample preparation and analysis

The metabolites were extracted in chloroform, methanol (1:2), and water on the same day they were homogenized by the tissue lyser protocol (10 min, 30 Hz). The organic phase was collected, evaporated in a vacuum centrifuge, and reconstituted for injection on an ultra-high-pressure liquid chromatography system coupled to an Orbitrap Fusion Lumos Tribrid Mass Spectrometer. The analysis was conducted in positive and negative polarity modes separately along with identification and quality control samples.

### 2.4.2. Data processing

The identification samples, where MS2 was triggered, were input as raw files into LipidSearch (version 4.2.21). The precursor mass and fragment mass were checked against the database with a mass tolerance of 5 ppm and 10 ppm respectively. Lipids in which all fatty acyl chains were identified, called Grade A identification, were annotated from the lipid classes, sphingolipids, neutral lipids, glycolipids, fatty acyls, and phospholipids. The data was exported to a mass list. The sample files were processed using Compound Discoverer (version 3.1), where features were aligned and grouped with a mass tolerance of 5 ppm and a retention time maximum shift of 0.2 min. Features not able to be identified by LipidSearch were annotated with the following online databases: mzCloud, Metabolika pathways, BioCyc pathways and ChemSpider (AnalytiCon Discovery, AraCyc, Baoji Herbest Bio-Tech, Extrasynthase, Golm Metabolome DB, Indofine, LipidMAPS, PlantCyc and Sequoia Research Products). All discovered compounds were integrated.

### 2.4.3. Normalization and scaling

The experimental process has the potential to introduce non biological variations. To ensure that both non-biological and biological variations do not affect the results, the data was normalized. This was accomplished using a Pareto scaling technique that involves subtracting the mean of each lipid column and dividing it by the square root of its standard deviation.

### 2.4.4. Log transformation

A log2 transformation was used to remove any potential heteroscedasticity and to correct for the skewness of the data distribution.

### 2.4.5. ANOVA analysis

Statistical Package for the Social Sciences (IBM SPSS Statistics) was adopted for the analysis of variance. Data was expressed as the mean of two independent parallel experiments with two repetitions per experiment (four values per variable). ANOVA analysis using Duncan's multiple range was used to assess the differences among the treatments. The standard error of the mean was calculated at  $p < 0.05$  level of significance.

### 2.4.6. Volcano plots

This plot combines the fold change differences and the statistical significance for each lipid: the x-axis displays the log2 fold change, while the y-axis displays the negative log10 of the  $p$  value from a robust two-sample  $t$ -test. The  $p$ -values displayed in the plots have been corrected

using the Benjamini-Hochberg (BH) procedure [19]. The lipid is considered significant if the absolute fold change is  $>4$  and the BH-corrected  $p$ -value is  $<0.05$ .

### 2.4.7. Principal component analysis (PCA) and heat map clustering

These multivariate techniques were used for extracting important information from data by replacing original features with new ones called "principal components", which are a linear combination of the original features. Significant lipids were defined as those with an absolute fold change greater than four and a corrected  $p$ -value  $<0.05$  after running univariate analyses comparing each pair of groups. The data corresponding to this lipid group was subtracted, and the PCA was performed. For heat map construction, the Euclidean distance algorithm was applied, and samples were clustered in terms of lipid profiles while also providing information on the upregulation or downregulation of specific lipid species. Data was analyzed using R software ver 4.2.1 and plots were generated using Phyloseq and ggplot2 (3.4.1).

## 3. Results

### 3.1. Morphology and growth properties of *T. subcordiformis* under various stresses

The morphology of *T. subcordiformis* differed according to the cultivation condition (Fig. 1). Similar morphology was evidenced under N-starvation, but lipid vesicles were enhanced (Fig. 1B). P-starvation led to slight changes in cell shape and a reduction in the number of intracellular lipid vesicles. However, high salinity (100 PSU) led to a much higher number of lipid vesicles compared to control and N-starvation, with a complete change in cell shape from oval to spherical. Finally, it was also noticed that applying cold stress after 6 days of cultivation in control and stress conditions (Fig. 1AC, BC, CC, DC), the shape was not affected, but a dramatic reduction of the intracellular lipid vesicles was noted in all conditions.

Regarding the growth properties' analysis, results proved that *T. subcordiformis* growth was heavily affected by the three different stresses applied (Fig. 2). Under control conditions, the strain showed an interesting  $\mu$  of  $0.38 \text{ day}^{-1}$  (Table 1). However, a significant decrease was observed in the case of the three stress conditions used. High salinity and N-starvation showed the lowest growth rate of *T. subcordiformis*, followed by P-starvation.

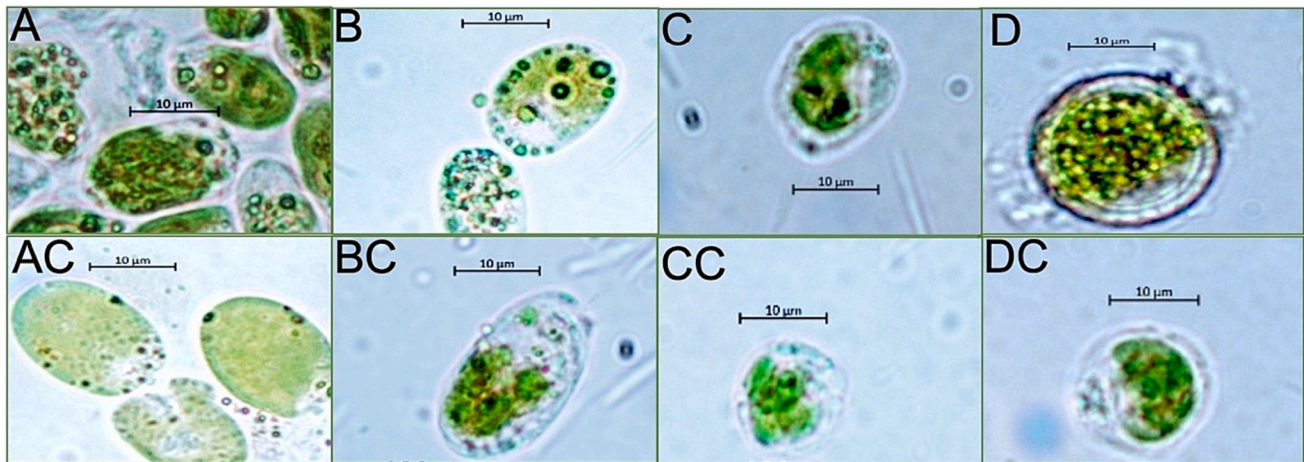
### 3.2. Chlorophyll content determination

The chlorophyll b content was noted to be higher than chlorophyll a for the control culture, on the other hand, all other cultures showed an inverse relation where chlorophyll a content was higher compared to chlorophyll b. For example, P-starvation ( $2.18 \text{ mg L}^{-1}$ ), N-starvation ( $0.946 \text{ mg L}^{-1}$ ) and 100 PSU ( $2.18 \text{ mg L}^{-1}$ ) chlorophyll a was higher for day 6 compared to P-starvation ( $2.13 \text{ mg L}^{-1}$ ), N-starvation ( $0.934 \text{ mg L}^{-1}$ ) and 100 PSU ( $1.83 \text{ mg L}^{-1}$ ) chlorophyll b. The most significant decrease of approximately 70 % in chlorophyll content was observed due to N-starvation ( $p = 0.026$ ), followed by decreases of almost 37 % and 32 % due to 100 PSU salinity stress and P-starvation, respectively when comparing to control. The survey of the chlorophyll content at days 3, 5 and 6 showed that the chlorophyll content of P-starvation was similar to the level of the control until day 5, then it decreased at day 6. However, the 100 PSU salinity stress led to early reduction of the chlorophyll compared to control that was maintained as stable with  $4 \text{ mg L}^{-1}$ . Finally, N-starvation led to a significant decrease in the lipid content from almost  $3 \text{ mg L}^{-1}$  at day 3 to  $<2 \text{ mg L}^{-1}$  by days 6. (Fig. 3A).

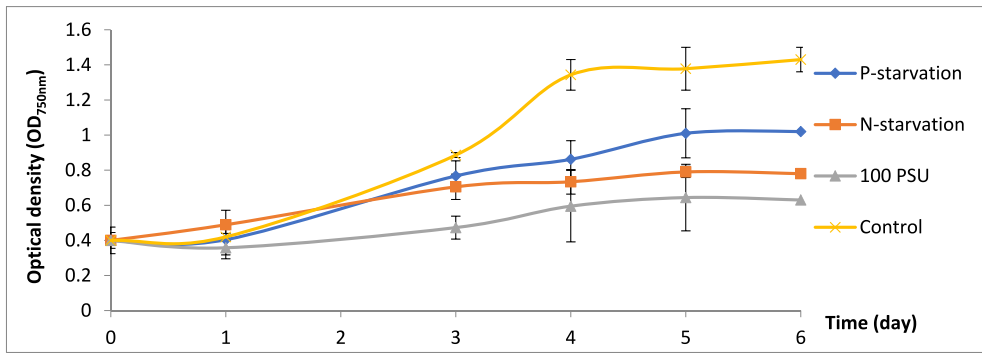
### 3.3. Analysis of the total protein and lipid content

The lipid content was around 24.5 % for *T. subcordiformis* at control conditions when the temperature was  $30^\circ\text{C}$ . The strain's lipid content





**Fig. 1.** Morphology of the *T. subcordiformis* cultivated under different stress conditions under light microscopy with a magnification of 100×. A: Control; B: N-starvation; C: P-starvation and D: 100 PSU. The strain was subjected to cold stress at 15 °C on day 6 of cultivation in the mentioned stresses. AC: Control strain in cold stress; BC: N-starvation strain in cold stress; CC: P-starvation strain in cold stress; DC: High salinity stressed strain in cold stress.



**Fig. 2.** The growth rate analysis of *T. subcordiformis* under different stressors over time. All measurements were taken in duplicates and error bars show confidence interval.

**Table 1**  
Growth rate and doubling time under the different stress conditions.

	$\mu$ (day <sup>-1</sup> )	Doubling time (day)
P-starvation	0.25 ± 0.02 b	0.36 ± 0.28 b
N-starvation	0.13 ± 0.0 c	0.18 ± 0.04 c
100 PSU	0.17 ± 0.07 c	0.24 ± 0.32 c
Control	0.38 ± 0.04 a	0.54 ± 0.25 a

a–c: same letters indicate no significant differences between means at the confidence level 99.9 % according to the Duncan's multiple range test (at  $p \leq 0.05$ ).

increased after 6 days of exposure to high salinity stress conditions. The lipid content increased by 5 %, rising from 24.5 % in control conditions to 29 % at 100 PSU salinity. The strain with the lowest lipid content was noted to have been cultivated in P-starvation conditions, with an overall decrease of almost 4 % compared to the control, followed by cold stress (15 °C), where the lipid content decreased by approximately 3 %. Furthermore, N-starvation stress resulted in a 1.42 % increase in lipid production in the microalgae (Fig. 4).

There was an increase in protein content due to high salinity stress (26.88 %) and P-starvation stress (23.41 %) compared to control conditions. Protein content decreased under N-starvation (17.27 %) and cold stress conditions (17.05 %).

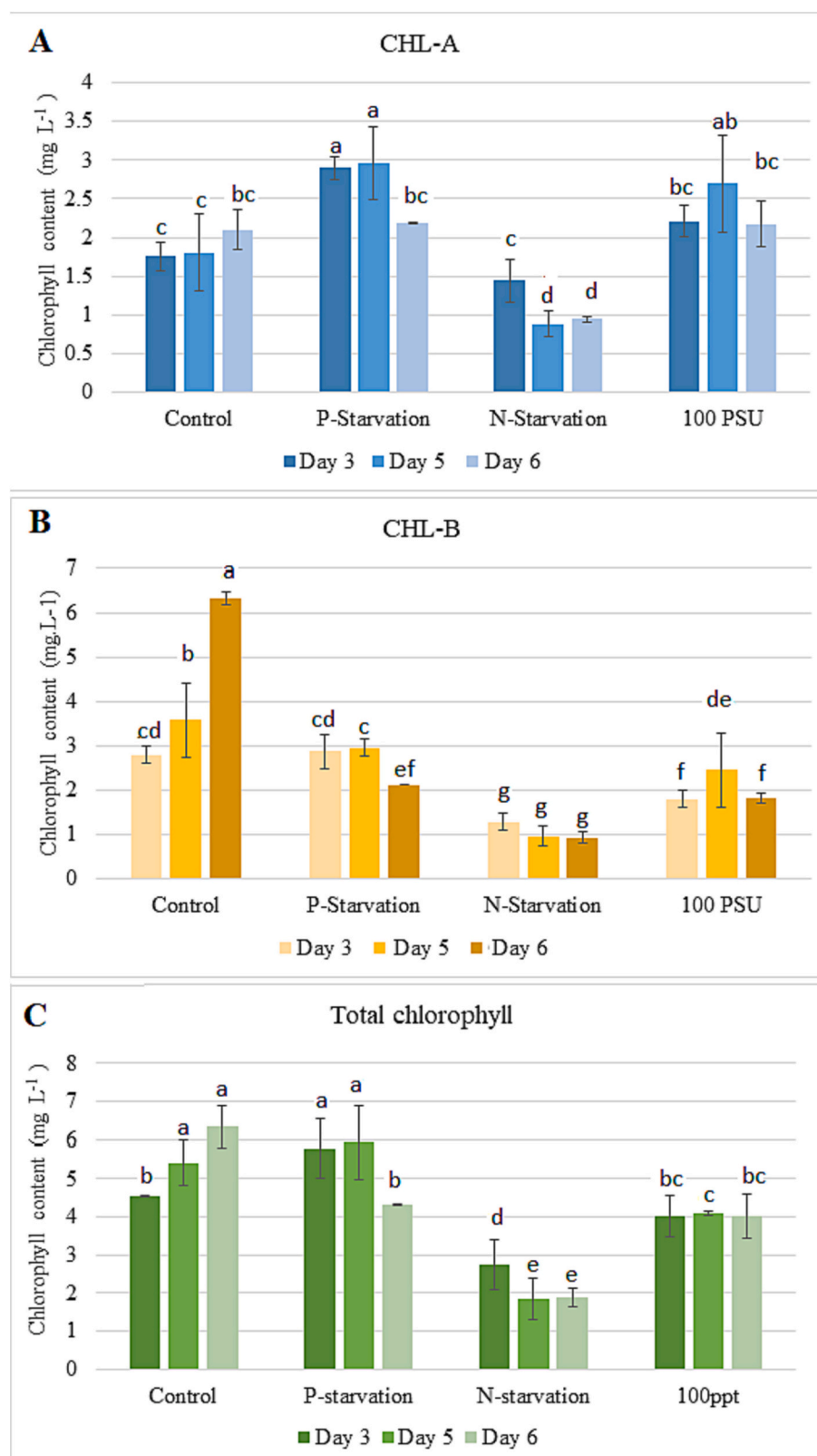
3.4. Lipidomic analysis of *T. subcordiformis* under N-starvation and high salinity stress conditions

3.4.1. Volcano plot analysis

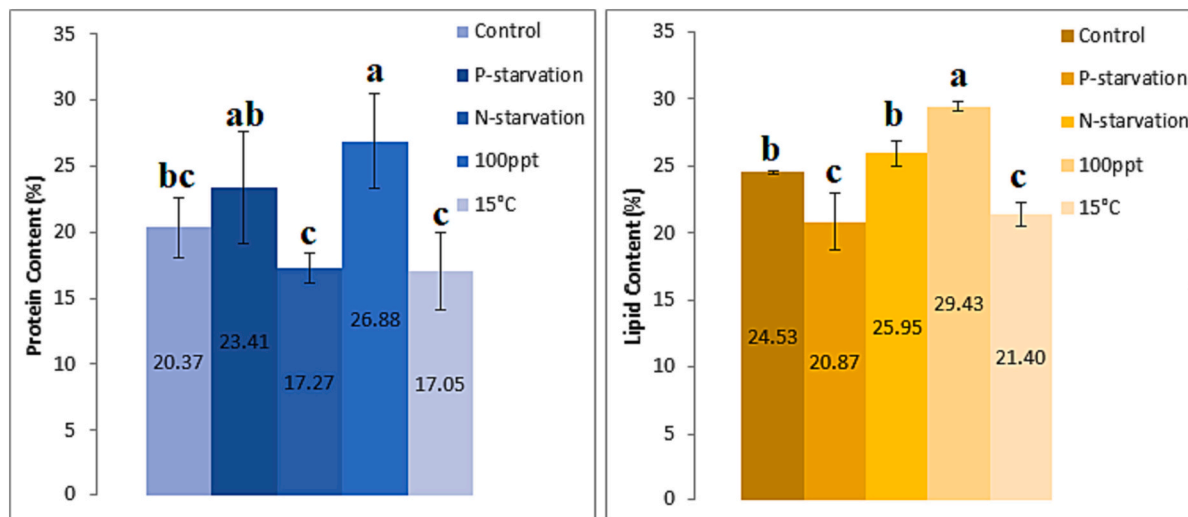
Three plots were constructed with the following condition combinations: Control vs high salinity, control vs N-starvation and high salinity vs N-starvation (Fig. 5). Overall control had a higher expression of significant lipids compared to N-starvation and high salinity stress conditions. One of the more interesting results is when comparing *T. subcordiformis* cultivated in both stress conditions; high salinity and N-starvation. It was noted that a higher volume of significant lipids was expressed in N-starvation compared to high salinity.

3.4.2. Principal components analysis (PCA) and heat map

To further investigate the various lipids that were expressed differently in the various cultivation regimes, a PCA biplot and heat map were constructed (Fig. 6). These plots show the significant lipids that were expressed in the various groups. The heat map shows the activation and deactivation of significant lipids produced in all three conditions. The red color signifies upregulation, while the blue color signifies down-regulation of specific lipids. For N-starvation, the two key lipids with high expression were triglycerides (TG) (16:0\_16:0\_18:2) and TG (16:0\_14:1\_18:3) (Fig. 6A). Although, a lot more lipids were lowly expressed, in *T. subcordiformis* cultivated in N-starvation conditions, lipids that were highly expressed were evidenced by more blue clusters than red clusters in the heat map (Fig. 6B). Lipids that were least expressed in this group include Coenzyme Q6 (CoQ6), which is a 6



**Fig. 3.** Survey of the chlorophyll content ( $\text{mg L}^{-1}$ ) of the *T. subcordiformis* at control and different stress conditions. The chlorophyll content was recorded for biomass collected on day 3, 5 and 6. A: Chlorophyll A; B: Chlorophyll B; C: Total Chlorophyll. a-g: bars with the same letters indicate no significant differences between means at the confidence level 99.9 % according to the Duncan's multiple range test (at  $p \leq 0.05$ ). Errors bars indicate  $\pm$  SE.



**Fig. 4.** Metabolites Content of *T. subcordiformis* cultivated under different cultivation regimes. A: Protein content; B: Lipid content measured in percent per gram weight. a-c: bars with the same letters indicate no significant differences between means at the confidence level 99.9 % according to the Duncan's multiple range test (at  $p \leq 0.05$ ). Errors bars indicate  $\pm$  SE.

isoprenyl unit coenzyme Q, Phosphatidylethanolamine (18:3\_18:3), Monogalactosyldiacylglycerol (20:2\_20:5) and some TGs. Moreover, two important lipids that were highly expressed in N-starvation conditions were TGs containing polyunsaturated fatty acids (PUFAs) linoleic acid (LA 18:2) and  $\alpha$ -linoleic acid (ALA 18:3) and saturated fatty acid (SAFA) palmitic acid (16:0) were found. Some of the fatty acids that were deficient in the nitrogen starved strain include docosahexaenoic acid (DHA 22:6), stearidonic acid (18:4) ALA (18:3) and eicosapentaenoic acid (EPA 20:5), and docosaentaenoic acid (DPA 22:5). On the other hand, major lipids produced by the strain in high salinity stress were phosphatidylcholine (PC 16:0\_18:3), PC (20:1\_20:5), PC (20:5\_18:2), hexocylceramides (Hex1Cer 18:2\_24:1), Hex1Cer (18:2\_24:0 + O), and other glycolipids (Fig. 6A). The most common fatty acids were LA (18:2), PA (16:0), and ALA (18:3). The least expressed lipids were triglycerides with fatty acids including EPA (20:5), arachidonic acid (ARA 20:4), DHA (22:6), and Oleic acid (18:1). However, in the control group, high concentrations of TG and diacylglycerols (DG) were found with high expression of the fatty acids EPA (20:5) and ALA (18:3). This group had the least number of weakly expressed lipids, which included some PC lipids. Fig. 6B also shows a shift from red to blue, which signifies the decrease of upregulation of lipids from control to high salinity to N-starvation.

Furthermore, control and high salinity cultivation samples were clustered together, which proves that the lipid profile is not compromised due to high salinity while N-starvation cultivation conditions were clustered separately based on lipid profile. An interesting trend that can be noted is the formation of a small blue cluster for control and salinity stress groups, while the pattern for these lipids was red in N-starvation, showing upregulation of unique lipids not expressed in control or high salinity stress conditions.

#### 4. Discussion

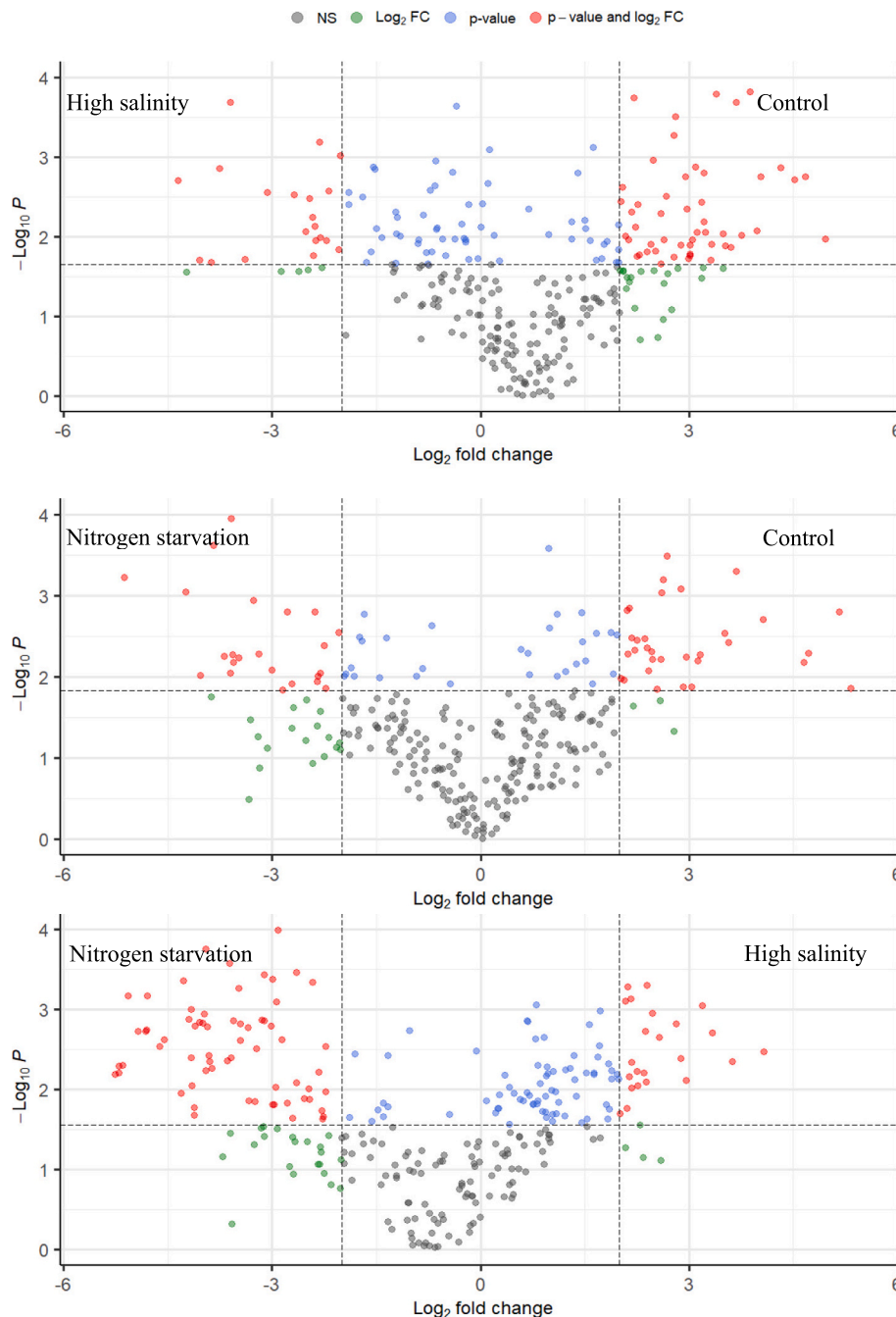
The key aim of stressing microalgae is to explore the changes in the strains' capability to produce valuable products that can be extracted. With *T. subcordiformis*, a lot of factors affected the growth rate and other metabolomic factors. Among the stress conditions chosen, high salinity was appropriate as the natural local habitat contains high salinity, while nutrient stress conditions were explored as limiting key nutrients in cells and can be vital regulators for generating high-quality compounds in algal cells [20].

Under control conditions, *T. subcordiformis* grew at a  $\mu$  of  $0.38 \pm$

$0.04 \text{ day}^{-1}$ . However, in the case of all stress conditions applied, a decrease was noted. The lowest growth rate was obtained through N-starvation, followed closely by salinity stress compared to other stress conditions. According to Zhang et al., [10] salinity can affect phytoplankton in three ways: 1) ion stress, 2) changes in ionic ratio due to selective membrane; and 3) osmotic stress. It is widely known that algae can withstand some degree of salinity by maintaining turgor pressure using vacuoles. In this study, high salinity caused the growth rate to increase for the first three days, followed by a decrease in growth until a lag phase was reached on the fifth day of the experiment. The initial increase in growth rate could be possible due to facilitation of photosynthesis by sodium ions, alkalotolerance, and internal pH regulatory mechanisms [21] followed by reduction in growth due to unfavorable growth conditions. The biomass production was lowest under high salinity stress and may be due to negative effects on the physiology of the cells from the excessive salt present, as was noted in the study of Zhang et al., [10].

On the other hand, in N-starvation conditions, the algae grew for the first three days and then the growth rate slowed down, eventually reaching a stationary phase. This may be due to the depletion of the available nitrogen in the media or in the internal nitrogen reserves in the algal cells themselves. This was also noted in the study of Fernandes et al., [22], where low nitrogen samples reached a stationary phase on the 6th day of the experiment. Nitrogen is an essential nutrient that is used by cells to form amino acids, which are then used as building blocks to produce proteins [23]. As nitrogen depletes, the production of proteins ceases, which in turn reduces the biomass as noted in this study and in the study of Breuer [24]. Additionally, the results of this study align with the findings reported by Jerez et al. [25], proving that N-starvation triggered more than a 50 % reduction of the total chlorophyll. The decrease in chlorophyll content of N-starved microalgal cultures was most likely associated with the lack of nitrogen that forms part of the chlorophyll structure. Chlorophyll is composed of four pyrrole groups, which contain nitrogen, forming a ring around a single magnesium atom. Since chlorophyll, stabilized by four nitrogen atoms in each pyrrole group, a lack of nitrogen led to the lowering of chlorophyll as well [24,25].

Phosphorus is used by cells to make a variety of cell components such as RNA, DNA, and ATP, and it also plays an important role in the cell membrane. Limiting phosphorus caused a decrease in growth rate, just not as significantly as the other stressors [26]. According to [27], the growth rate of a freshwater alga cultivated in P-starvation conditions,



**Fig. 5.** Volcano plot analysis of A: High salinity vs control; B: N-starvation vs control; C: N-starvation vs high salinity. High salinity corresponds to 100 PSU. The lipids that are statistically significant and highly expressed in each condition was plotted in red points on the left and right corners of the volcano plot. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

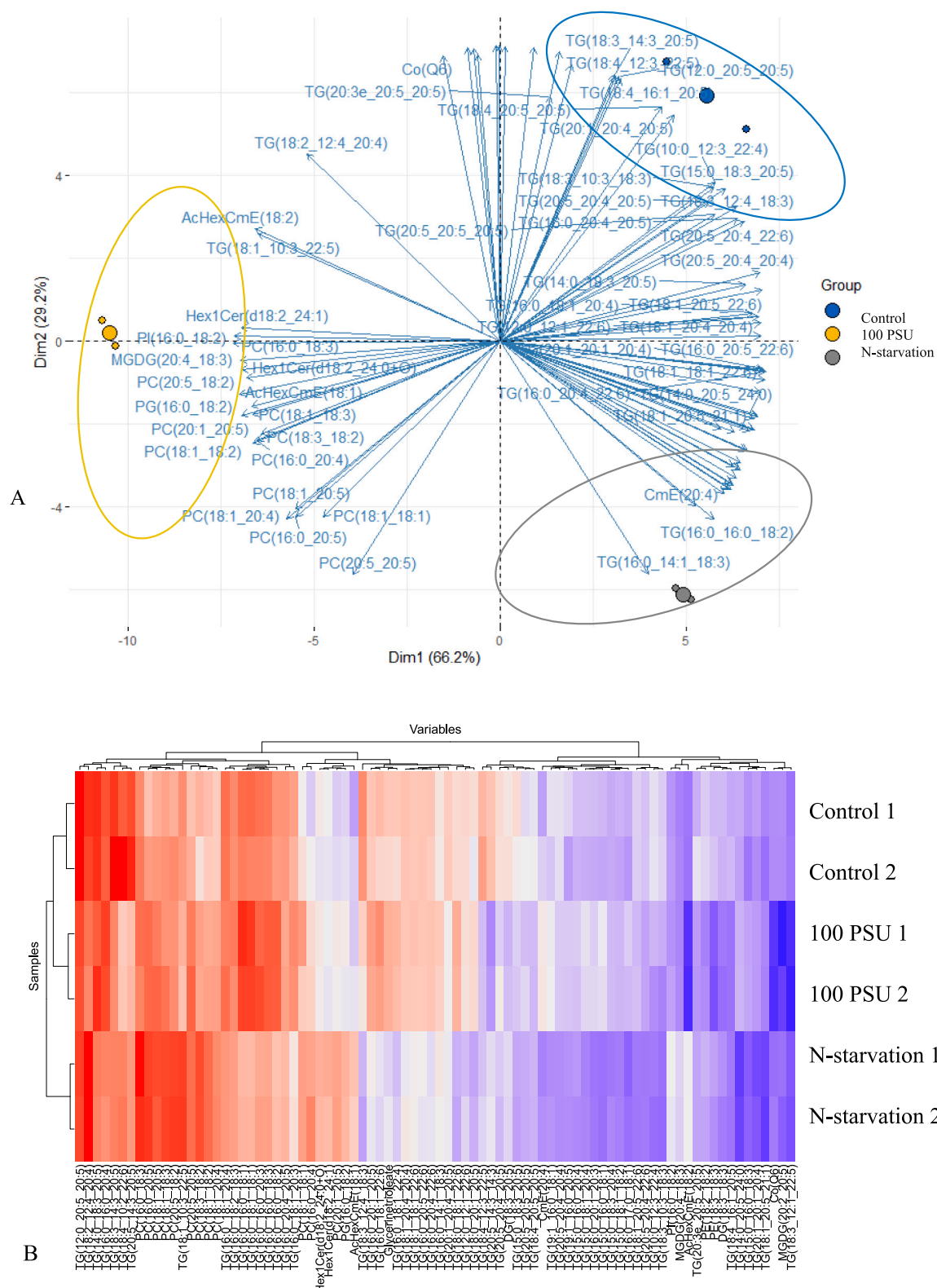
increased for 4 days, signifying that intracellular phosphate was sufficient on these days. A deficiency in growth was noticed when the cells ran out of phosphorus reserves. These results are in line with this study's results, where there was a steady increase in the growth rate for the first five days, followed by a stationary phase, where the supposed phosphorus reserves declined.

Protein content differs widely across groups of algae. Most microalgae contain all the essential amino acids (EAA) that humans cannot synthesize and must obtain through food. Nitrogen is an essential nutrient for microalgae and is critical for the synthesis of proteins, nucleic acids, and chlorophyll molecules [28]. However, a lower nitrogen concentration is known to increase the lipid content and decrease the protein concentration [29]. In this study, high salinity showed the

highest protein productivity, while P-starvation led to an increase in the protein content with (15 %) reaching 23.4 %. Such results could be explained by the fact that the presence of nitrate in the growth medium led to an increase in the number of proteins.

Nitrogen restriction is known to induce a wide variety of cellular responses in microalgae, including the accumulation of lipids intended for biodiesel production [30]. N-starvation increases the lipid and triglyceride content compared to the culture containing nitrate feed [29], which is also consistent with this study and its results compared to the control. The mechanism behind the increase in triglycerides due to nitrogen stress is that due to normal metabolic pathway restriction, a fatty acid synthetic pathway is generated, which continues the cells' metabolism while also accumulating TGs [31]. Phosphate deficiency, on the





**Fig. 6.** A: PCA Biplot for individuals and variables together. The size of the arrow gives an idea about the importance of the corresponding lipid in the structure of the observations. B: Heat map showing the clustering of lipid species for each cultivation condition samples based on Euclidean distance algorithm.

other hand, resulted in a 15 % decrease in lipid content because it is the primary source of lipid production.

The high salinity stress group produced the most lipids (30 %), followed by the N-starvation group (25 %), and the control group (23 %). According to Takagi et al., [32], high concentrations of salt resulted in

an increase in the intracellular lipid content of microalgae. This statement was true for the present study as well where the 100 PSU salinity stress induced the highest total lipid content 29.42 % recovery from the strain, as seen in other studies such as study of Zhang et al., [10]. Furthermore, in a study by Pancha et al., [33] showed a higher lipid

production in high salt stress conditions compared to nutrient starvation. It was suggested that this may be due to cells synthesizing more lipids to aid in the membrane rigidity needed to withstand osmotic pressure induced by high salinity.

Although the high salinity group had the highest amount of lipid production, the variation in lipids expressed in N-starved cultivated algae was greater. As evidenced in Fig. 6B, N-starvation led to the production of unique lipids that are not normally expressed by the strain. On the other hand, high salinity stress produced a larger quantity of significant lipids overall compared with low salinity stress.

The FA profile of N-starvation and high salinity stress conditions included high expression of PA (16:0), ALA (18:3), EPA (20:5), and LA (18:2). Similar results were also concluded in the studies of Xu et al., [34] and Salama et al., [21] where stressed cell cultures fatty acid profile consisted mainly of palmitic acid, ALA and LA. Algal cells in optimum control conditions were able to synthesize complex PUFAs such as EPA, ARA, ALA and SDA. One explanation for these results can be deduced from a study on the effect of nitrogen reduction on enzymes needed for the conversion of ALA and LA to EPA and DHA [35]. Results showed that lack of N in cultures caused changes in the expression of fatty acid desaturases. These are precursor enzymes for the synthesis of very long chain PUFAs such as DPA and EPA from ALA and LA [36]. Furthermore, CoQ6 was discovered to be one of the most downregulated lipids for N-starvation. CoQ6 is a conserved polyprenylated lipid essential for mitochondrial respiration. CoQ is composed of a redox-active benzoquinone ring and a long polyisoprenyl tail that serves as a membrane anchor. A decrease in CoQ can indicate a change in the proteins or other molecules required for the synthesis pathway of this molecule [37].

Although some studies such as [38,39] cold stress can lead to production of high value fatty acids such as long chain PUFAs and enhancement of proteins, this was not the case in this current study. Cold stress was applied after 6 days as this is the end of the exponential phase of strain *T. subcordiformis*. On the 6th day it was expected the maximum biomass production, where it will be profitable to apply the cold stress to investigate the conversion of produced metabolites into high value products. While in this study, cold stress application led to the least amount of protein and lipid production overall.

Fatty acids like ALA are known as one of the most important fatty acids for regulating human lipid metabolism and lowering the risk of diseases like cardiovascular disease, Alzheimer's disease, and Parkinson's disease [40]. LA is also a vital PUFA, required for fetal neural development, and is also known to aid in reducing the chances of diabetes, ischemic heart disease, high cholesterol [41], and blood pressure issues [42]. Furthermore, ALA and LA cannot be directly synthesized by humans but are required as part of a healthy diet, making them essential fatty acids [43]. *T. subcordiformis* cultivated in high salinity also had high production of lipids such as hexosylceramides, phosphatidylinositol, and phosphatidylcholine. These lipids are known to have many commercial uses, such as the use of ceramide lipids in skincare and cosmetic products [44].

## 5. Conclusion

This study investigated the effect of various stress application on microalgal strain *T. subcordiformis* and the assessed the effect on the strain's metabolome. Overall, an increase was noted in the volume and variety of metabolites produced by stressing the microalgae *T. subcordiformis*. For example, P-starved cultures had higher growth rate and chlorophyll content while cultures with high salinity stress show cased higher protein and lipid production. It is also important to note that unique lipids and fatty acids were synthesized in cultures with N-starvation. However, choosing the culture conditions of any microalgae depends on the desired product as the various stressors applied produced different reactions from the strain. Hence, this study investigates these various stressors on microalgal species *T. subcordiformis*, providing valuable information on how the metabolomics are affected

through stress application. Furthermore the experimental work conducted can be used to assess the as a guide for investigating other microalgal strains prior to large scale cultivation for downstream applications.

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## CRediT authorship contribution statement

**I.S:** Conceptualization, Methodology, writing—original draft preparation, writing—review and editing and funding acquisition; **M.C:** data acquisition, writing—review and editing, **S.A.S:** writing—original draft, writing—review and editing. **M.E:** Statistical Analysis; **F.M:** Statistical analysis; writing—review and editing; **H.A.J:** writing—review and editing.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Imen Saadaoui reports financial support and article publishing charges were provided by Qatar National Library.

## Data availability

Data will be made available on request.

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