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RESEARCH

# Enhancement of heat tolerance by salt stress in *Tetraselmis striata* CTP4: impacts on HSP gene expression, pigments, and proximal composition

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**Abstract**
As the world average temperature is on the rise and heat waves are becoming more prevalent, microalgal producers have been facing significant challenges regarding the time periods during which they are able to grow less thermotolerant microalgae in outdoor production facilities. Therefore, it is urgent to explore how microalgae cope with thermal stress and under which growth conditions tolerance to temperatures close to or higher than their maximum thermal threshold is induced. For this purpose, *Tetraselmis striata* CTP4, a euryhaline microalga known for its thermotolerance, was selected. *Tetraselmis striata* belongs to the Chlorodendrophyceae, a clade that branched off early from other "core chlorophyte" clades, usually comprised of microalgae able to colonise freshwater habitats. Here, we present compelling evidence that the ability of this microalga to withstand otherwise lethal thermal upshifts to 40 °C is induced by exposure to higher salinity (35 ppt). In contrast, this response is abrogated at lower salinities. Concomitantly, the expression of genes encoding *HSP70* and *HSP100,* two heat shock proteins known to mediate thermotolerance and tolerance to other stresses (e.g., salt stress) in fungi, animals and plants, was enhanced when exposed to both heat stress and higher salinities. This suggests that cross-protective mechanisms against abiotic stress appeared early during the evolution of the core chlorophytes and of Archaeplastida in general. This knowledge can be used to select novel strains and growth conditions that promote thermotolerance in microalgae that are grown in outdoor industrial production facilities in environments where heat waves are expected.

**Keywords** Chlorophyceae · Cross-acclimation · Abiotic stress · Thermotolerance · Industrial production

## Introduction

As the global surface temperature has been rising ≈0.2 °C per decade since the 20$^{th}$ century (Min 2024), and this trend seems to have been accelerating over the past 60 years (Storto and Yang 2024), it is crucial to understand how thermotolerance is regulated in industrial microalgae, as it can have a very significant impact on the revenues of microalgal producers. Microalgae can be cultivated on a large scale in either open (e.g., raceway ponds) or closed (photobioreactor) systems which can undergo wide thermal shifts during the day and throughout the year as they are usually placed outdoors (Pereira et al. 2018). Temperature is critical for producing a given microalgal strain because too low or too high temperatures might prevent growth or even promote culture collapse. Because of this, very much like farmers, microalgal producers are often forced to have "winter" (e.g., *Phaeodactylum tricornutum*) and "summer" (e.g., *Tisochrysis lutea*) strains.

Recently, a thermotolerant microalga, *Tetraselmis striata* CTP4, has been isolated from the Ria Formosa, a lagoon on the Algarve coast, using a fluorescent-activated cell sorting-based pipeline to isolate lipid-rich strains. This strain was shown to grow from 5 to 100 ppt salinity and to be

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an interesting lipid hyperproducer (~50% of dry weight) at both high light intensities (400 µmol photons $m^{-2}$ $s^{-1}$) and high temperatures (35 °C) (Pereira et al. 2016; Schulze et al. 2017; Monteiro et al. 2023). *T. striata* CTP4 showed high resilience against wide thermal fluctuations under industrial settings despite the occurrence of known grazers of microalgae in the photobioreactors (Pereira et al. 2018). *T. striata* belongs to the Chlorodendrophyceae, a class of green microalgae that had previously been classified as belonging to the early diverging, flagellated prasinophytes, whose cell body is often covered with scales (Lewis and McCourt 2004). However, similarly to other "core chlorophytes," such as the Trebouxiophyceae (e.g., *Chlorella)* and the Ulvophyceae (e.g., *Ulva*), *Tetraselmis* cells exhibit closed mitosis and microtubule arrays along the plane of cell division, i.e., a "phycoplast" (Massjuk and Lilitska 2006; Leliaert et al. 2012). Despite this resemblance with the core chlorophytes, *Tetraselmis* belongs to an early branching clade in the cusp between the prasinophytes and the more evolved Chlorophyta clades (Leliaert et al. 2012, 2016). Moreover, *Tetraselmis* cells are often euryhaline and eurythermal due to their tolerance to a broad spectrum of salinities and temperatures (Fabregas et al. 1984; Arora et al. 2013). Interestingly, this flexibility might be due to their intermediate evolutionary position between mainly marine species (prasinophytes) and microalgae that were able to colonize freshwater ecosystems (core chlorophytes), which might also explain why *Tetraselmis* is known to dominate mixed cell cultures, being able to maintain fast growth rates even under challenging industrial conditions (Pereira et al. 2018).

Here, we present data showing that thermotolerance in *T. striata* cells to otherwise lethal thermal upshifts (20→40 °C) is observed at salinities closer to seawater (35 ppt). In contrast, this response is abrogated at lower salinities (5 and 20 ppt). This salt-induced cross-protection against an otherwise lethal heat shock was observed regarding biomass growth and cell viability as scored by SYTOX Green staining. Interestingly, this cross-protection is accompanied by the induction of the expression of *T. striata HSP70* and *HSP100* genes, which code for two proteins crucial for acquired thermotolerance in bacteria, fungi, animals, and plants (Queitsch et al. 2002; Beckham et al. 2008; Kim et al. 2013; Mishra and Grover 2016). The term "stress" has been defined by Borowitzka (2018) as the disturbance of homeostasis caused by a stressor; this state is reversed once the cells restore homeostasis either by acclimation or adaptation, hence being no longer stressed. Both HSP70 and HSP100 can be induced under various stress conditions, such as heat, oxidative stress and inflammation, indicating their broad role in stress response mechanisms (Gul et al. 2021; Zhang et al. 2022a). Moreover, salinity, temperature, and their combination significantly affect the biochemical composition of microalgae, resulting in variations in the levels of fatty acids, pigments, and proteins. Because these changes are crucial to better understand the effect and the response to thermal stress (Pugkaew et al. 2019; Arena et al. 2021), data on the proximal composition and pigment profile of cells under mesophilic and thermal stress conditions at different salinities are also presented and discussed.

## Methods

### Culture conditions and growth

*T. striata* CTP4 was isolated from the Ria Formosa, Algarve, Portugal (Pereira et al. 2016). The strain was initially cultivated under mesophilic growth conditions at a temperature of 20 ºC, with a light intensity of 100 µmol photons $m^{-2}$ $s^{-1}$, in diluted seawater (20 ppt) supplemented with Modified Algal Medium (MAM) (Pereira et al. 2016; Schüler et al. 2017). Continuous aeration was provided during the culture growth.

To initiate the experiments, *T. striata* colonies were inoculated into 50 mL test tubes and the culture was allowed to grow exponentially. Subsequently, the culture was scaled up sequentially to a 1-L Schott flask and then to a 5-L Schott flask, with a working volume of 4 L. Growth was measured by optical density readings at 750 nm ($OD_{750}$) and cell counts using a Neubauer chamber. Upon calibration between $OD_{750}$ and dry weight (DW), the latter was calculated using Eq. (1):

$$DW\ [g\ L^{-1}] = 1.61 \times OD_{750} \quad (1)$$

### Growth experiment to assess salt-induced thermotolerance

The initial culture was grown under mesophilic conditions until reaching a concentration of 0.8 g $L^{-1}$. Subsequently, the culture was divided into 12 treatments in 1-L reactors. To induce stressful experimental conditions, the inoculum was subjected to temperature shifts from 20 °C to 10 °C, 30 °C, and 40 °C, in addition to salinity variations of 5 ppt, 20 ppt, and 35 ppt. As a result, a test grid consisting of twelve unique combinations of temperature and salinity was established with three replicates each.

Two independent growth trials were conducted using this setup. In the first trial, the initial biomass concentration of the microalgae was set at 0.1 g $L^{-1}$ and the culture was allowed to grow for 15 days. Growth measurements were made at intervals of 3 days in order to follow the main phases of growth (lag, exponential and stationary phases), specifically on days 0, 3, 6, 9, 12, and 15. In the second trial, a higher initial biomass concentration (0.4 g $L^{-1}$) was

employed, and the growth of the cultures was monitored at shorter time intervals, 1, 6, 12, 24, and 48 hours after inoculation, to determine the early and late expression patterns of HSPs, as the proteins are known to be induced rapidly, being kept at high levels over long periods of time (Echevarria-Zomeño et al. 2016).

## Cell viability staining with SYTOX Green

The fluorescent dye SYTOX Green has been shown to selectively bind to the nucleic acids of cells with permeabilized membranes while leaving intact cells unstained (Schüler et al. 2017). In order to validate the use of SYTOX Green staining in flow cytometry (FC), control experiments were conducted by comparing cell counts obtained through microscopy with those obtained using the flow cytometer. Exponentially growing and dead cells were stained with SYTOX Green, and cell viability was analyzed. Microalgal cells emitting chlorophyll autofluorescence were initially counted using an optical microscope with fluorescence, where 100 cells were counted or gated using FC. Subsequently, cells emitting SYTOX Green fluorescence were counted to determine the percentage of dead cells (Schüler et al. 2017).

For the experimental procedure, cells from the inoculum and cells harvested after 24 and 48 h of growth were stained with SYTOX Green at a final concentration of 5 nM and incubated in the dark on ice for 10 min. Cell counts were performed using a Becton Dickinson FACS Calibur (BD Biosciences, Belgium) flow cytometer equipped with a blue laser (488 nm) and a red laser (633 nm), along with CellQuest Pro software (version 6.0). Fluorescence signals were obtained using the FL1 (630/30 nm) and FL2 (585/42 nm) filters upon excitation with the blue laser.

## RNA extraction and cDNA synthesis

RNA extraction was performed using a combination of the NZYol protocol (NZYTech, Portugal) and the RNeasy Mini kit (Qiagen, Germany) following a previously described method (Schüler et al. 2017). Briefly, frozen biomass was treated with NZYol, and cell disruption was achieved by bead-beating using an MM400 mixer mill (Retsch, Germany). The resulting mixture was centrifuged and the supernatant was collected and incubated at room temperature (RT) for 7 min. Subsequently, 0.2 volumes of chloroform were added to the supernatant and incubated for 3 min at RT. Phase separation was achieved by centrifugation, and the upper aqueous phase was collected. At this stage, 1 volume of 70% ethanol was added to the aqueous phase and the solution was transferred to the RNeasy Mini spin column following the manufacturer's instructions. On-column DNase digestion using RNase-free DNase sets (Qiagen, Germany) was performed to remove residual genomic DNA. The integrity and quantity of the extracted RNA were assessed using denaturing gel runs and NanoDrop One spectrophotometry (Thermo Fisher Scientific, USA). For cDNA synthesis, first strand cDNA synthesis was generated using superscript IV reverse transcriptase (Thermo Fisher Scientific) according to the manufacturer's protocol. A mixture of random hexamers and oligod(T)18 primers (1:10) was used for cDNA synthesis, with the following prolonged protocol: 23 °C for 10 min, 50 °C for 20 min, 55 °C for 20 min, and 65 °C for 10 min. Afterward, samples were incubated at 80 °C for 10 min to inactivate enzymatic activity. The obtained cDNA was diluted 1:50 and stored at -20 °C until further use.

## Real-time PCR

Transcript expression levels of genes involved in heat shock response were evaluated by quantitative real-time PCR using a CFX 96-well Real-Time PCR system (BioRad, USA). *HSP70* and *HSP100* transcripts encoding heat shock proteins (HSPs) were amplified with specific primers (Table 1), whereas 18S ribosomal RNA was used as an internal control. For the PCR reaction, NZYSpeedy qPCR Green Master Mix, ROX plus (NZYTech, Portugal) was used. Raw data were analyzed by the $2^{-\Delta\Delta Ct}$ method based on cycle threshold (Ct) values.

## Biochemical characterization

Microalgal biomass of the second growth trial was collected at the beginning of the experiment (0 h) and after 48 h of growth for all tested conditions. All samples were collected by centrifugation (5000 × *g;* 5 min) and freeze-dried (Martin Christ Alpha 1-2 LDplus, Germany). Samples were analyzed

**Table 1** Primer sequences used for real-time PCR amplification of specific target primers in the study. The target gene's abbreviation and the nucleotide sequence of the forward and reverse primers used for amplification are included

| Target Gene | Forward Primer | Reverse Primer |
|---|---|---|
| *HSP70* | 5´-GTTTGATGTGTCCCTGCTG-3´ | 5´-AAGGCGGTTGTCGAAATCC-3´ |
| *HSP100* | 5´-GCTGGACATGAGCGAGTACATGG-3´ | 5´-ATGCGCCTTCTCGACCTCGTC-3' |
| 18S rRNA | 5´-GCCCGTCGCTCCTACCGATT-3' | 5´-TGGGGCGGTTTGGAGAACTT-3´ |

to assess proximal composition (protein, lipid, and ash content), pigments, and fatty acid profiles.

### Proximal composition

For protein content, elemental analysis of total nitrogen was conducted using a Vario EL III (Elementar Analysen Systeme GmbH, Germany). The resulting nitrogen percentage was multiplied by 4.78 to calculate the final protein content (Schüler et al. 2017).

The total lipid content was determined gravimetrically using a modified Bligh and Dyer method (Bligh and Dyer 1959). Lipids were extracted from the lyophilized biomass using an Ultra-Turrax T10B disperser (IKA-Werke, Germany) and a mixture of chloroform, methanol, and water (2:1:1), as detailed in Pereira et al. (2011).

Ash content was obtained by incinerating 50 mg of biomass at 515 ºC for 8h, following the method outlined by Barreira et al. (2017). The difference between the dry biomass and the resulting ash residue measured the ash content.

All analyses were performed in triplicate, and the average values were used for data interpretation.

### Pigment analysis

Pigment extraction and analysis were performed as described by Schüler et al. (2020). Pigments were extracted from lyophilized biomass with methanol assisted with glass bead-based cell disruption. The supernatant was collected after centrifugation (2500 ×*g* for 5 min), and the extraction process was repeated until the pellet and the supernatant became colorless. The extracts were then evaporated under a nitrogen flow, resuspended in methanol, and filtered.

The carotenoid profiles of the samples were analyzed by an HPLC system equipped with a LiChroCART RP-18 column (5 µm, 250 × 4 mm, LiChrospher) and a photodiode-array detector set to 450 nm. For identification and quantification, calibration curves of the pure neoxanthin, violaxanthin, lutein, zeaxanthin, and $\beta$-carotene standards (Sigma-Aldrich, Portugal) were established.

### Fatty acid methyl esters profile

The fatty acid methyl esters (FAME) profile was determined using a modified protocol described by Pereira et al. (2012), with slight modifications described by Schüler et al. (2017). Lyophilized biomass was resuspended in a solution of methanol and acetyl chloride (20:1, v/v) containing tricosanoic acid (C23:0, Sigma-Aldrich, Portugal) to assess the efficiency of the fatty acid derivatization and extraction. The samples were dispersed using an Ultra-Turrax T10B disperser, followed by the addition of *n*-hexane and derivatization of the fatty acids at 70 ºC for 60 min. FAMEs were extracted from the reaction by centrifugation (2000 ×*g* for 5 min) and residual water was removed using anhydrous sodium sulfate. The samples were then filtered (0.2 µm) and the solvent was evaporated under a nitrogen flow. The resulting pellet was resuspended in a known amount of gas chromatography-grade hexane.

FAME profiles were analyzed on a Bruker gas chromatograph coupled to a mass spectrometry system (Bruker SCION 456-GC, SCION TQ MS) equipped with a ZB-5MS capillary column (30 × 0.25 mm internal diameter with 0.25 µm film thickness; Phenomenex). Helium was used as the carrier gas at a 1 mL $min^{-1}$ flow rate. The temperature program consisted of an initial hold at 60 ºC for 1 min, followed by a ramp of 30 ºC $min^{-1}$ until 120 ºC, 4 ºC $min^{-1}$ until 250 ºC, and 20 ºC $min^{-1}$ until 300 ºC, with a final hold of 4 min. The injection temperature was set at 300 ºC in splitless mode.

To identify and quantify the FAMEs, a Supelco 37 component FAME Mix (Sigma-Aldrich, Portugal) was used as a standard to prepare calibration curves of the individual fatty acid methyl esters.

### Statistical analysis

Data were tested for normality using the Shapiro-Wilk test (XLStat software, Vers. 2016.02.27444, Addinsoft, USA). To compare the means of treatments, ANOVA and two-sided Dunnett's tests were performed with a 95% confidence interval.

## Results

### Effect of temperature and salinity shifts on cell growth

The growth of *T. striata* CTP4 cells was evaluated under different temperature and salinity conditions under batch growth mode. Figure 1 shows that cells grown at 20 °C and with a salinity of 5 ppt reached the highest biomass concentration (0.7 g $L^{-1}$) on day 12. However, by day 15, cells grown at 20 °C and 30 °C, regardless of salinity, reached biomass concentrations similar to those of the best-performing culture. The observed plateauing in biomass dry weight for the culture grown at 30 °C with a salinity of 5 ppt may be due to nutrient depletion. This often takes place in batch cultures where no further nutrient addition is made, causing the cells to enter the stationary phase.

A shift from 20 to 10 °C resulted in significantly lower growth, with biomass concentrations approximately half of those at 20 °C or after a thermal upshift to 30 °C. Interestingly, salinity had a minor impact on growth when cells were downshifted to 10 °C compared to those exposed to a

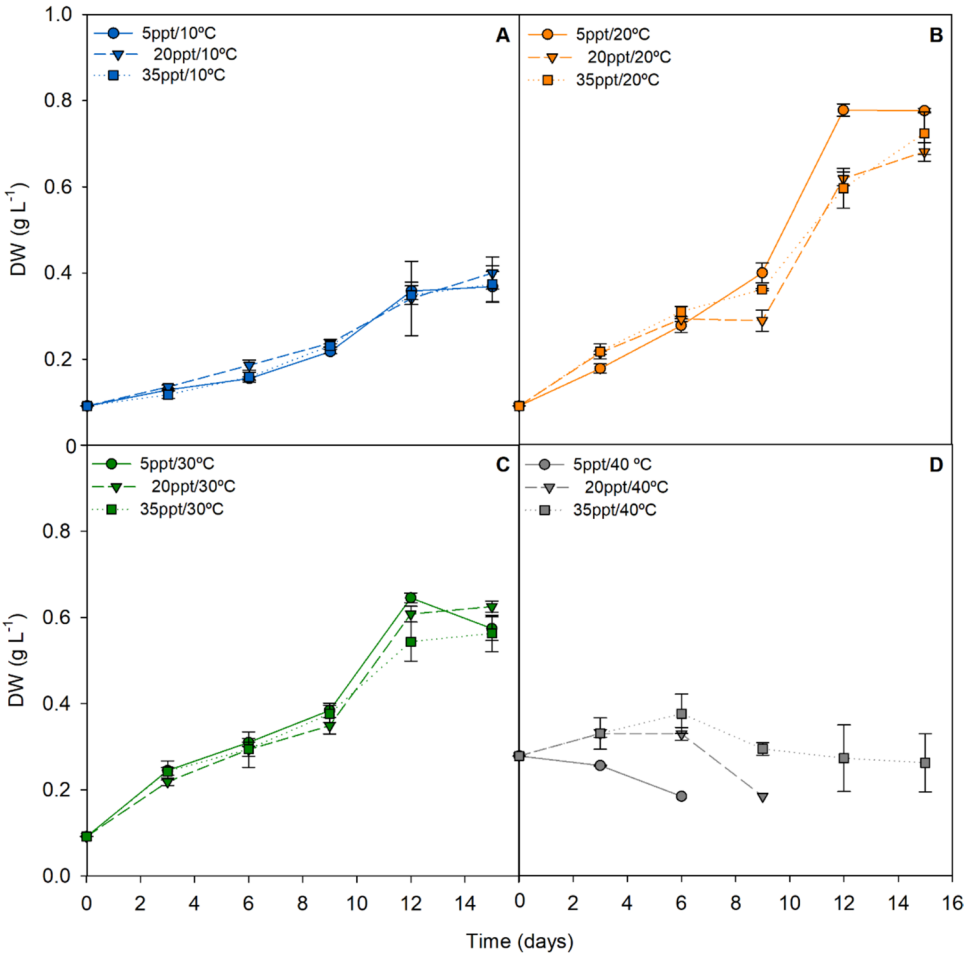


**Fig. 1** Growth in terms of dry biomass weight (DW g $L^{-1}$) of *Tetraselmis striata* CTP4 when shifted from 20 °C to different temperatures, namely 10 (blue), 30 (green), and 40 ºC (grey), using 20 °C (orange) as a control where the thermal shift was omitted. Microalgae under each thermal condition were shifted from a salinity of 20 ppt to salinities of 5 (circles) and 35 ppt (squares). Cultures at a salinity of 20 ppt (triangles) were used as an unshifted control for this abiotic factor. Data is presented as mean ± standard deviation of three biological replicates ($n$=3)

thermal upshift to 30 °C or when cells were maintained at 20 °C. Additionally, cultures that underwent a heat shock from 20 to 40 °C rapidly decreased in terms of biomass DW by day 6 and day 9 when subjected to a salinity downshift to 5 ppt or were maintained at the initial salinity of the inoculum (20 ppt), respectively (Fig. 1D).

However, the lethal condition caused by the heat shock was partially rescued when cells were exposed to a salinity upshift to 35 ppt (Fig. 1). This result strongly suggests that increased salinity toward seawater levels induces a cross-protective response toward an otherwise lethal heat shock. Indeed, unlike cells cultivated at lower salinity, cultures incubated at 40 °C and a salinity of 35 ppt remained viable until the end of the experiment, although they were unable to grow. To further validate these findings, a second assay was conducted with higher initial biomass concentrations (≈0.4 g $L^{-1}$) (Fig. 2). Cultures that underwent a temperature upshift from 20 to 30 °C displayed similar growth to those maintained at 20 °C, regardless of the salinity. Lower performance was observed, once again, when cells were exposed to a temperature downshift from 20 to 10 °C. Notably, in the more concentrated culture, complete loss of cell viability occurred within 24 hours after a heat shock from 20 to 40 °C when cells were downshifted from 20 to 5 ppt salinity, suggesting that the observed lethal effect is more acute under these growth conditions. This acceleration of cell viability loss was also observed when cells were kept at 20 ppt salinity and underwent a heat shock from 20 to 40 °C. However, the rescue of this lethal phenotype was again observed in cells upshifted to a salinity of 35 ppt. These results confirm that exposure to higher salinity protects *T. striata* cells from an otherwise lethal thermal upshift from 20 to 40 °C. Figure 3 depicts the appearance of the culture after having lost its typical green color.

Cell viability was scored using SYTOX Green (Fig. 4) to confirm the observed lethal effect. Cells grown at 20 °C and shifted to 30 °C at all tested salinities displayed high viability (Fig. 4B and C). Moderate loss of viability (15-30%) was observed upon a temperature downshift from 20 to 10 °C after 48 hours, where salinity did not significantly affect survival at lower temperatures (Fig. 4A). However, a heat shock from 20 to 40 °C resulted in rapid loss of cell viability, particularly at a salinity of 5 ppt (Fig. 4D). This

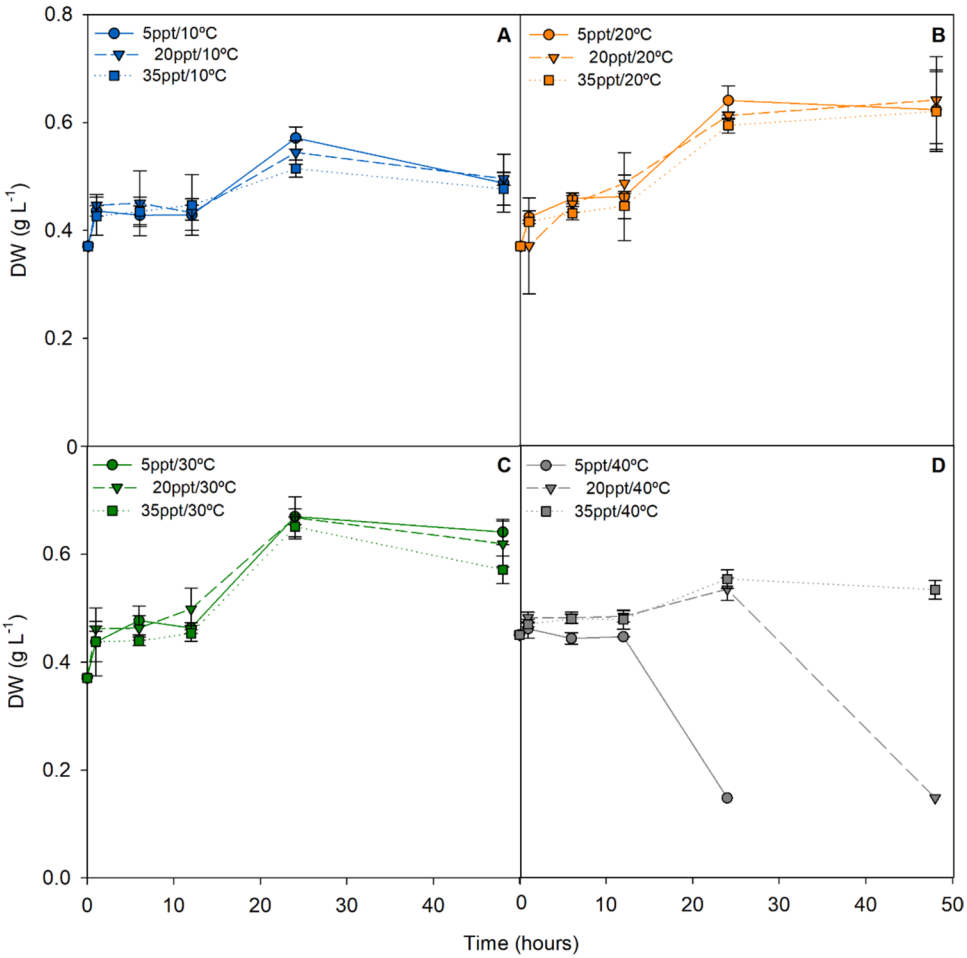


**Fig. 2** Growth in terms of dry biomass weight (DW g $L^{-1}$) of *Tetraselmis striata* CTP4 when shifted from 20 °C to different temperatures, namely 10 (blue), 30 (green) and 40 ºC (grey), using 20 °C (orange) as a control where the thermal shift was omitted. Microalgae under each thermal condition were shifted from a salinity of 20 ppt to salinities of 5 (circles) and 35 ppt (squares). Cultures kept at a salinity of 20 ppt (triangles) were used as an unshifted control for this abiotic factor. All cultures were grown in 1-L flasks for 48 hours. Data are presented as mean ± standard deviation of three biological replicates ($n$=3)

loss of viability was reduced at an intermediate salinity (20 ppt) and they almost abrogated within the first 24 hours when cells experienced a salinity upshift to 35 ppt. Some loss of viability was observed after 48 hours, thus corroborating that exposure to high salt induces cross-tolerance in *T. striata* to an otherwise lethal heat shock.

## Effect of temperature and salinity shifts on heat shock protein gene expression

To investigate the cross-tolerance mechanisms between higher salinity and temperature in this microalgal industrial strain and to understand the conservation across different organisms, we analyzed the expression of genes encoding the heat-inducible proteins HSP70 and HSP100 in *T. striata*. These proteins play a critical role in the cellular response to a wide variety of abiotic stress conditions, such as heat, high light intensity and oxidative stress, by facilitating protein refolding to re-establish protein homeostasis (Chaudhary et al. 2019; Zhou et al. 2023). Furthermore, they are essential for the development of thermotolerance across various organisms, ranging from bacteria and yeast to animals and flowering plants (Welte et al. 1993; Vogel et al. 1995; Keeler et al. 2000; Su and Li 2008; Kobiyama et al. 2010; Tukaj and Tukaj 2010; Miot et al. 2011; Liang et al. 2020; Xu et al. 2020).

Indeed, *HSP70* and *HSP100* gene expression analysis showed no significant increased transcript levels in control cells (20 °C) or cells downshifted to 10 °C, regardless of the salinity (Fig. 5). Under these conditions the expression of these genes was maintained at constitutive levels. However, upregulation of *HSP70* and *HSP100* mRNA levels becomes quite apparent in cultures upshifted to higher temperatures (30 and 40 °C) compared to control cells grown at 20 °C and a salinity of 20 ppt. Within 6 h of growth, cultures subjected to a thermal upshift from 20 to 40 °C and a salinity upshift from 20 to 35 ppt exhibited a 5.4-fold increase in *HSP70* mRNA levels and a 3.2-fold increase in *HSP100* gene expression compared to the start of the experiment.

Furthermore, at a salinity of 20 ppt, *HSP70* gene expression was upregulated 3-fold in the cultures exposed to 40 °C. However, these cells failed to induce *HSP100* gene expression and started to lose viability (Figs. 2 and 3), leading to complete degradation of any available RNA and preventing

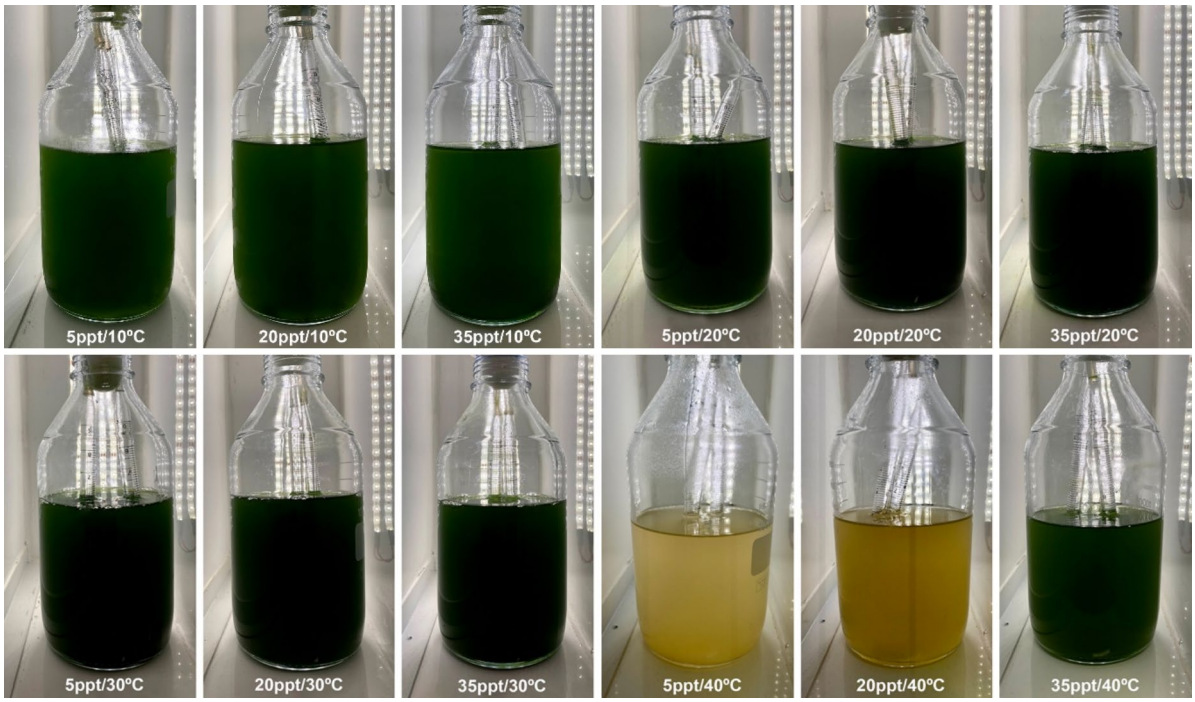


**Fig. 3** Growth of *Tetraselmis striata* CTP4 in 1-L flasks at 48 hours. Cultures were shifted from 20 °C and 20 ppt salinity (control) to different temperatures (10, 30, and 40 ºC), and salinities (5 and 35 ppt)

further gene expression analysis in these cultures. On the other hand, cultures incubated at 30 °C and a salinity of 30 ppt showed a 3.2-fold increase in *HSP100* transcript levels compared to those of the control. After 12 h of growth, the transcript levels of *HSP70* remained high in the culture growing at 40 °C shifted to the salinity of 35 ppt; however, at this time, *HSP100* gene expression returned to control levels. In contrast, cultures grown at 30 °C and at the same salinity (35 ppt) displayed a 2-fold upregulation of *HSP70* gene expression and a 4.7-fold increase in the *HSP100* transcript levels compared to those of the control.

## Effect of temperature and salinity shifts on the biochemical composition of *T. striata*

To assess the impact of salinity and temperature on the metabolism of *T. striata* CTP4 cultures, the composition of protein, lipids, and ash among the biomass grown under different conditions (Table 2) was compared. After 48 h, the ash content varied between 16.6 and 34.8% of dry weight, with significant increases observed at higher salinity (35 ppt). In comparison, lower salinity (5 ppt) resulted in significantly lower ash content at all temperatures assayed. The total lipid content ranged from 10.3 to 17.5% and increased significantly under all tested conditions compared to the initial inoculum associated with cells that underwent a salinity upshift from 20 to 35 ppt and were downshifted from 20 to 10 °C.

Upon 48 h, the protein content varied between 17.7 and 30.1%. A comparison among treatments revealed a significant increase in protein content for cells cultivated at 20 °C and subjected to a salinity downshift from 20 to 5 ppt. Conversely, a significant decrease in protein levels occurred when the salinity increased from 20 to 35 ppt, regardless of whether accompanied by a temperature shift to 10, 30, or 40 °C.

Total carotenoid contents decreased significantly by 33 and 69% in cultures under a salinity upshift from 20 to 35 ppt or severe heat shock from 20 to 40 °C compared to the content of the initial inoculum (6.69 mg $g^{-1}$; Fig. 6). Remarkably, while the xanthophylls neoxanthin, violaxanthin, and $\beta$-carotene decreased, lutein and zeaxanthin did not change significantly under these extreme conditions (Fig. 6). Furthermore, compared to the initial culture (0 h), violaxanthin and $\beta$-carotene contents decreased in cultures downshifted from 20 to 10 °C or when upshifted from salinity of 20 to 35 ppt, even when maintaining the initial salinity (20 ppt) or temperature (20 °C), respectively. The highest lutein contents were found in cultures exposed to a cold shock from 20 to 10 °C and a salinity downshift from 20 to 5 ppt (1.81 mg $g^{-1}$). The second-highest lutein content was observed in cultures subjected to a temperature upshifted from 20 to 30 °C, but a salinity downshifted from 20 to 5 ppt (1.57 mg $g^{-1}$), resulting in a 1.5-fold increase compared to the levels recorded at the beginning of the experiment.

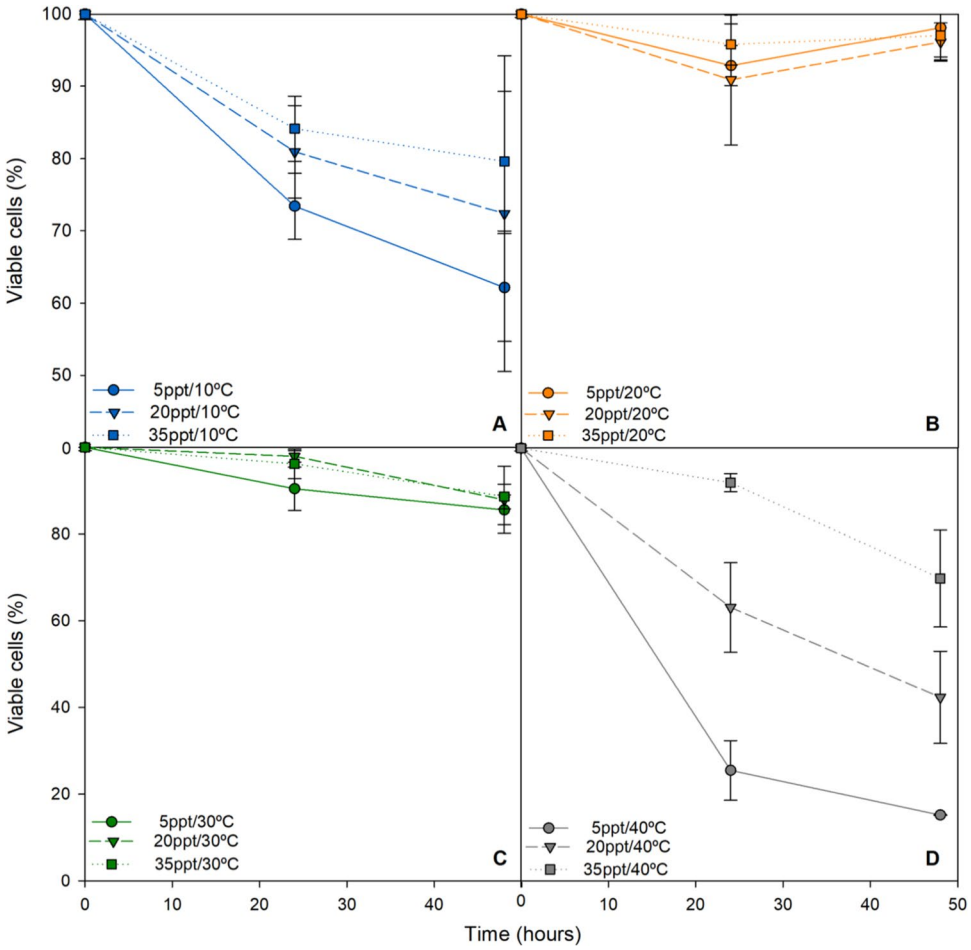


**Fig. 4** Cell viability (%) of *Tetraselmis striata* CTP4 grown at 20 °C and then shifted to different temperatures, namely 10 (blue), 30 (green) and 40 ºC (grey), using 20 °C (orange) as a control where the thermal shift was omitted. Microalgae initially grown at a salinity of 20 ppt and subjected to each thermal condition were shifted to salinities of 5 (circles) and 35 ppt (squares). Cultures kept at a salinity of 20 ppt (triangles) were used as an unshifted control for this abiotic factor. All cultures were grown in 1-L flasks for 48 hours. Data is presented as mean ± standard deviation of three biological replicates ($n$=3)

The fatty acid profile of *T. striata* CTP4 grown under different abiotic conditions was mainly composed of C16:0, C16:1, C16:3, C16:4, C18:1, C18:2, C18:4 and C20:5, which accounted for over 90% of total fatty acids. However, differences were observed in several fatty acids, except for C16:1, C18:0, and C18:1, which did not change significantly (Table 3). The concentration of C16:2*n*-6 significantly decreased in *T. striata* CTP4 cells under all conditions compared to that of the initial culture (0 h). The lowest C16:3, C18:2, C20:3, and C20:4 contents were observed in cells downshifted to 10°C or when the temperature was maintained at 20 °C or exposed to severe heat shock at 40 °C. However, their concentration remained at 30 °C, regardless of salinity. On the other hand, C20:5 had a significant drop, by 51%, when cells were shifted to the highest temperature assayed (40 °C). As a result, the *n*-6 PUFA content decreased in cells under all conditions except in cells shifted from 20 to 30 °C. Conversely, the sum of *n*-3 PUFA only became significantly lower in cells that underwent a shift to the highest temperature (40 °C). Overall, PUFA contents significantly decreased upon a salinity increase to 35 ppt, dropping by 39% when cells were exposed to the most severe heat shock (20 to 40 °C).

## Discussion

Tolerance to a heat shock from 20 to 40 °C by *T. striata* CTP4 cells was observed in two independent experiments at different initial cell concentrations. Under all conditions tested, tolerance to a high temperature was only observed upon simultaneous exposure to an increase in salinity from 20 to 35 ppt. This conclusion was further confirmed by cell viability, as previously shown for the alga *Tetradesmus obliquus* and the yeast *Rhodothorula toruloides* (Dias et al. 2020; Elisabeth et al. 2021). Overall, cell viability was also high at temperatures between 10 and 30 ºC, ranging between 62.15 ± 7.45 % and 96.82 ± 7.45 % after 48h exposure. At these milder temperatures, salinity did not influence cell viability significantly. Thus, these results corroborate a previous study where the robustness of *T. striata* CTP4 was shown at an industrial scale (Pereira et al. 2016). Compared to other species, *Chlamydomonas reinhardtii* is one of the

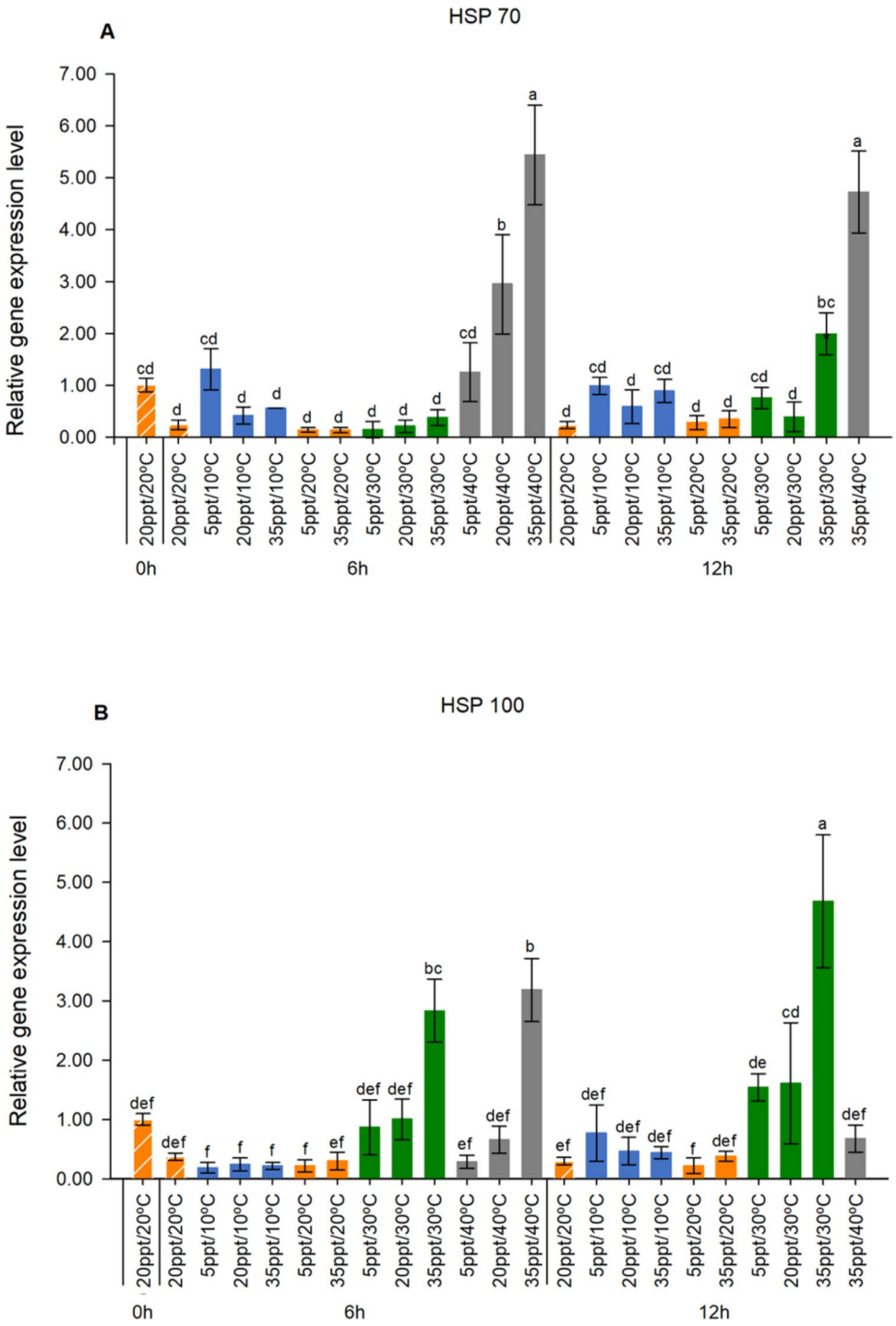


**Fig. 5** Relative expression of genes encoding stress-inducible heat shock proteins HSP70 (A) and HSP100 (B) in *Tetraselmis striata* CTP4 under mesophilic and stress conditions. RNA was extracted at 0h from the control condition (20 ppt, 20 ºC) and at 6 and 12 h after temperature and salinities shifts. Vertical bars represent the mean value ± standard deviation ($n$=3). Statistical analysis was conducted using a one-way analysis of variance (ANOVA) followed by the Tukey (HSD) test for multiple comparisons, with a significance level set at $p < 0.05$. Significant differences between treatments are indicated by different letters, when compared with the control condition (20 ppt, 20 ºC) at 0h

few species that may resist temperatures of 40 ± 5 ºC, but even so for periods of 48 h (Zhang et al. 2022b). As the latter is a freshwater species belonging to the "core chlorophytes," this suggests that at least *C. reinhardtii* can show thermotolerance even in the absence of significant salinity. However, the euryhaline *T. striata* CTP4 cells still need simultaneous exposure to high salinity to become thermotolerant to severe heat stress. To the best of the authors' knowledge, this has been demonstrated in microalgae for the first time in the present report, suggesting that similar cross-protective mechanisms might exist in marine and brackish water microalgal species. In general, other marine species such as the diatoms *Nitzschia closterium* and *Nitzschia paleacea,* have optimum growth temperatures around 20 °C, but when subjected to temperatures above 30 °C, their cell counts can drop 15-fold (Renaud et al. 1995).

**Table 2** Proximal composition of *Tetraselmis striata* CTP4 under various temperature and salinity conditions. The composition includes protein, lipid, and ash content, measured at different time points (0 h and 48 h) and under different temperature and salinity treatments. The treatments are denoted by their salinity (ppt) and temperature values (ºC). Data are presented as mean ± standard deviation of three biological replicates ($n$=3). Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by the Tukey (HSD) test for multiple comparisons, with significance level set at $p < 0.05$. Significant differences between treatments are indicated by different letters

| | Treatments | Proteins | Lipids | Ashes |
|---|---|---|---|---|
| 0 h | 20ppt/20°C | $23.7 \pm 0.3^{bc}$ | $8.3 \pm 0.1^{g}$ | $25 \pm 2.5b$ |
| 48 h | 20ppt/20°C | $27.0 \pm 2.1^{ab}$ | $12.4 \pm 0.4^{d}$ | $25.9 \pm 1.8^{b}$ |
| 48 h | 5ppt/10°C | $24.2 \pm 0.5^{bc}$ | $13.6 \pm 0.3^{c}$ | $17.7 \pm 0.6^{c}$ |
| | 20ppt/10°C | $21.9 \pm 0.9^{cde}$ | $11.9 \pm 0.5^{de}$ | $26.0 \pm 1.8^{b}$ |
| | 35ppt/10°C | $19.7 \pm 1.0d^{ef}$ | $10.3 \pm 0.3^{f}$ | $32.0 \pm 1.5^{a}$ |
| | 5ppt/20°C | $30.1 \pm 1.3^{a}$ | $17.5 \pm 0.3^{a}$ | $16.6 \pm 1.2^{c}$ |
| | 35ppt/20°C | $23.9 \pm 1.4^{bc}$ | $16.5 \pm 0.5^{a}$ | $33.0 \pm 1.8^{a}$ |
| | 5ppt/30°C | $26.3 \pm 0.7^{b}$ | $12.3 \pm 0.5^{d}$ | $18.9 \pm 0.4^{c}$ |
| | 20ppt/30°C | $22.6 \pm 1.4^{cd}$ | $13.8 \pm 0.2^{c}$ | $25.9 \pm 1.9^{b}$ |
| | 35ppt/30°C | $19.1 \pm 0.9^{e}f$ | $11.0 \pm 0.5^{ef}$ | $32.2 \pm 1.3^{a}$ |
| | 35ppt/40°C | $17.7 \pm 0.9^{f}$ | $15.2 \pm 0.6^{b}$ | $34.8 \pm 3.2^{a}$ |

The upregulation of heat shock proteins (HSPs) under stress conditions (heat, salinity, osmotic, light) is a conserved response of all organisms as a process of acclimation to new environmental conditions (Lindquist and Craig 1988; Al-Whaibi 2011). These proteins are classified into five groups according to their molecular weight, namely HSP100/ClpB, HSP90, HSP70, HSP60, and small HSPs (Wang et al. 2004). Among these, the HSP70 assists in refolding and preventing irreversible aggregation of proteins, while the HSP100 is important for protein disaggregation and unfolding, becoming essential to develop tolerance to severe thermal stress. Here, we found upregulation of both the *HSP70* and *HSP100* genes in *T. striata* CTP4 cells exposed for 6 h to a temperature upshift from 20 to 40 °C and a salinity increase from 20 to 35 ppt (Figure 5). This has been shown in other microalgal species such as *Chlamydomonas reinhardtii* (Tanaka et al. 2000; Kobayashi et al. 2014), *Chlorella vulgaris* (Chankova et al. 2013), *Auxenochlorella protothecoides* (Xing et al. 2022), *Desmodesmus subspicatus* (Tukaj and Tukaj 2010) and *Dunaliella bardawil* (Liang et al. 2020) that they upregulate the expression of heat shock proteins when challenged with thermal stress. Moreover, in *C. vulgaris* and *C. reinhardtii,* upregulation of *HSP70* gene expression occurred when cells were under salt stress (Abdellaoui et al. 2019). However, the results suggest that cells can only survive under extreme temperatures when both *HSP70* and *HSP100* are induced. Although in the cultures grown at a salinity of 20 ppt and upshifted from 20 to 40 °C *HSP70* was induced after 6 h, the upregulation of *HSP100* was not observed (Figure 5), which eventually led to cell death upon 48 h (Fig. 2). Since *HSP100* is crucial for the resolubilization of denatured proteins, failure to rescue stress-induced protein aggregates under extreme heat stress is thus a likely reason as to why cells incubated at 40 °C at a salinity of 20 ppt did not survive. The importance of HSP100 Clp proteases for cells to obtain induced thermotolerance has been shown in the study on the yeast HSP104 analog and the cyanobacterium *Synechococcus* sp., demonstrating that cells with a deletion in the *HSP100* gene are not able to survive heat stress (Sanchez and Lindquist 1990; Eriksson and Clarke 1996).

However, other factors might be at play. Recently, Chankova et al. (2009) clearly demonstrated that HSPs are not the only protective mechanism against stress in the cell, being unable, for example, to protect the cells against radiation-induced damage. In addition, mannitol, the primary osmoregulatory solute in the closely related species *Tetraselmis subcordiformis* helps protect proteins from structural disruption under high salinity and temperature, aiding cellular survival and potentially supporting the synthesis of HSP (Kirst 1977).

Therefore, it is important to determine what happens to the cells regarding their overall metabolism. In fact, the effect of temperature and salinity was noticeable in the proximal composition of *T. striata* CTP4, especially in the protein, lipids, and ash contents. Protein content was negatively affected by high salinity. This decrease could be due to a regulatory response to repress housekeeping gene expression and release amino acids for the synthesis of stress-responsive proteins and/or due to more general metabolic changes. For example, the extrusion of intracellular $Na^+$ from microalgal cells can be carried out by NADH-dependent plasma membrane electron (redox) transport systems and $Na^+$ P-type ATPases

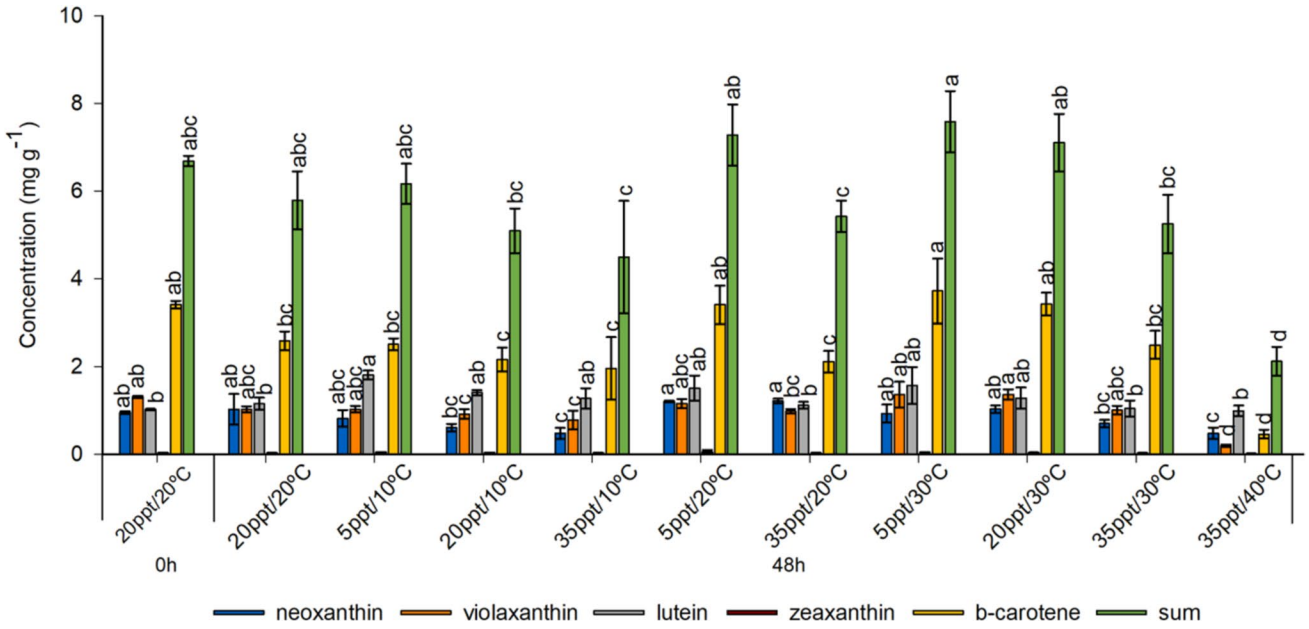


**Fig. 6** Carotenoid contents (mg $g^{-1}$) of *Tetraselmis striata* CTP4 initially grown at 20 °C and at a salinity of 20 ppt and then exposed to different temperature and salinity shifts. Neoxanthin, violaxanthin, lutein, zeaxanthin, *β*-carotene were measured at different time points (0 h and 48 h) and under different treatments of temperature and salinity The salinity (ppt) and temperature (ºC) values denote the treatments. Data are presented as mean ± standard deviation of three biological replicates ($n$=3). Statistical analysis was conducted using a one-way analysis of variance (ANOVA) followed by the Tukey (HSD) test, with significance level set at $p < 0.05$. Significant differences between treatments are indicated by different letters, when compared with the control condition (20 ppt, 20ºC) at 0 h

(Katz and Pick 2001; Matalin et al. 2021). Thus, it is possible that cells diverted ATP and reduced equivalents away from other ATP/NADH-consuming processes, such as amino acid and protein biosynthesis. Lipid content was significantly higher at 20 ºC when exposed to the highest and lowest (35 and 5 ppt) salinities. This diversion of metabolites might have been exacerbated by the accumulation of lipids as a mechanism for energy storage when *T. striata* is under stressful growth conditions, e.g., low/high temperature or salinity (Klok et al. 2013; Schüler et al. 2017; Monteiro et al. 2023). This could be seen as a potential strategy to improve the lipid contents in this microalga, if that is the desired end result of the operator.

Concerning pigment content, an increase in the lutein content of *T. striata* CTP4 under both high (30 °C) and low (10 °C) temperature shifts was observed (Fig. 6). Lutein has been previously identified as the most important protective carotenoid in this species due to its structural and scavenging roles (Schüler et al. 2020). Increased temperatures have also led to higher carotenoid contents in other microalgal species, e.g., *H. pluvialis* (Tjahjono et al. 1994), *C. sorokiniana* (Cordero et al. 2011), and *Scenedesmus almeriensis* (Sánchez et al. 2008). However, when the temperature was upshifted to 40 °C, carotenoid production in *T. striata* CTP4 was inhibited, which is another indication that this extreme thermal stress has a dramatic effect on the overall cell metabolism, including the stability of the photosynthetic apparatus and the capacity of this microalga to maintain the levels of photoprotective pigments present in cells grown under mesophilic conditions. Decreased cell growth and carotenoid levels in microalgal cells under extreme temperatures have also been reported for *D. bardawil* and *H. pluvialis* (Hong et al. 2015; Liang et al. 2020).

The obtained fatty acids profiles are previously reported profiles for *T. striata* CTP4 (Schulze et al. 2017; Pereira et al. 2019; Cardoso et al. 2020; Monteiro et al. 2023). *T. striata* is known for its robustness and ability to tolerate a wide range of salinity conditions with no significant impact on growth (Pereira et al. 2016; Trovão et al. 2019). However, the experimental conditions applied in this study, specifically variations in temperature and salinity, did lead to significant differences in the fatty acid composition (Conde et al. 2023). Temperatures of 10, 20, and 40 ºC led to a significant decrease in C16:2*n*-6 and C18:2*n*-6, which negatively impacted the biosynthesis of other *n*-6 PUFA, while the pathway to *n*-3 PUFA was not affected (Fernandes et al. 2016). Yet, these differences did not alter the sum of PUFA, which only changed significantly when cells were exposed to extreme temperatures upon a cold or heat shock.

**Table 3** Fatty acids profile (mg $g^{-1}$) of *Tetraselmis striata* CTP4, exposed to different temperatures and salinity. The composition includes fatty acid profile measured at various time points (0 h and 48 h) and under different temperature and salinity treatments. The treatments are denoted by their salinity (ppt) and temperature values (ºC). Data are presented as mean ± standard deviation of three biological replicates ($n$=3). Statistical analysis was conducted using a one-way analysis of variance (ANOVA) followed by the Tukey (HSD) test with significance level set at $p < 0.05$. Significant differences between treatments are indicated by different letters. SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids

| | 0h | 48h | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| mg $g^{-1}$ | 20ppt/20ºC | | 5ppt/10ºC | 20ppt/10ºC | 35ppt/10ºC | 5ppt/20ºC | 35ppt/20ºC | 5ppt/30ºC | 20ppt/30ºC | 35ppt/30ºC | 35ppt/40ºC |
| C14:0 | 0.45 ± 0.15$^{ab}$ | 0.28 ± 0.01$^{bc}$ | 0.25 ± 0.06$^{c}$ | 0.29 ± 0.04$^{abc}$ | 0.27 ± 0.04$^{bc}$ | 0.30 ± 0.05$^{abc}$ | 0.25 ± 0.04$^{c}$ | 0.33 ± 0.05$^{abc}$ | 0.29 ± 0.03$^{abc}$ | 0.27 ± 0.05$^{bc}$ | 0.45 ± 0.09$^{a}$ |
| C16:0 | 15.61 ± 1.27$^{ab}$ | 11.70 ± 1.52$^{bc}$ | 12.06 ± 3.20$^{ab}$ | 13.66 ± 2.69$^{ab}$ | 10.15 ± 0.42$^{b}$ | 11.45 ± 1.37$^{ab}$ | 11.22 ± 1.46$^{ab}$ | 13.09 ± 2.23$^{ab}$ | 12.30 ± 1.00$^{ab}$ | 11.91 ± 1.43$^{ab}$ | 16.37 ± 2.34$^{a}$ |
| C18:0 | 0.57 ± 0.51 | 0.39 ± 0.32 | 0.16 ± 0.02 | 0.28 ± 0.12 | 0.88 ± 0.47 | 0.48 ± 0.50 | 0.45 ± 0.47 | 0.24 ± 0.08 | 0.17 ± 0.02 | 0.34 ± 0.27 | 0.62 ± 0.06 |
| **∑ SFA** | **16.63 ± 1.93$^{ab}$** | **12.36 ± 1.85$^{ab}$** | **12.46 ± 3.28$^{ab}$** | **14.23 ± 2.86$^{ab}$** | **11.30 ± 0.94$^{b}$** | **12.22 ± 1.92$^{ab}$** | **11.92 ± 1.97$^{ab}$** | **13.65 ± 2.37$^{ab}$** | **12.75 ± 1.05$^{ab}$** | **12.52 ± 1.75$^{ab}$** | **17.43 ± 2.50$^{a}$** |
| C16:1 | 3.82 ± 0.70 | 2.49 ± 0.54 | 2.65 ± 0.70 | 3.08 ± 0.15 | 2.55 ± 0.25 | 2.53 ± 0.34 | 2.61 ± 0.66 | 2.64 ± 0.45 | 3.00 ± 0.39 | 2.85 ± 0.41 | 3.69 ± 0.32 |
| C18:1 | 13.32 ± 0.89 | 12.45 ± 1.15 | 15.26 ± 3.60 | 15.49 ± 1.32 | 12.48 ± 0.93 | 12.63 ± 1.36 | 11.80 ± 1.46 | 12.68 ± 2.02 | 12.28 ± 1.16 | 11.94 ± 1.34 | 12.84 ± 1.90 |
| **∑ MUFA** | **17.14 ± 1.59** | **14.94 ± 1.68** | **17.92 ± 4.30** | **18.57 ± 1.47** | **15.03 ± 1.18** | **15.16 ± 1.70** | **14.41 ± 2.13** | **15.32 ± 2.48** | **15.28 ± 1.54** | **14.79 ± 1.74** | **16.53 ± 2.22** |
| 16:2*n-6* | 1.63 ± 0.02$^{a}$ | 0.74 ± 0.09$^{cd}$ | 0.84 ± 0.23$^{bcd}$ | 0.91 ± 0.10$^{bc}$ | 0.71 ± 0.07$^{cd}$ | 0.51 ± 0.19$^{d}$ | 0.69 ± 0.13$^{cd}$ | 0.95 ± 0.23$^{bc}$ | 1.09 ± 0.15$^{bc}$ | 1.21 ± 0.11$^{ab}$ | 0.89 ± 0.00$^{bcd}$ |
| 16:3*n* -3 | 4.19 ± 0.03$^{a}$ | 2.33 ± 0.62$^{bc}$ | 0.82 ± 0.24$^{de}$ | 0.89 ± 0.15$^{de}$ | 0.50 ± 0.02$^{e}$ | 1.90 ± 0.26$^{cd}$ | 1.67 ± 0.34$^{cde}$ | 4.01 ± 0.84$^{a}$ | 4.24 ± 0.55$^{a}$ | 3.47 ± 0.36$^{ab}$ | 2.47 ± 0.21$^{bc}$ |
| 16:4*n* -3 | 9.98 ± 0.22$^{ab}$ | 13.00 ± 1.97$^{a}$ | 12.89 ± 3.37$^{a}$ | 14.03 ± 0.93$^{a}$ | 10.95 ± 1.35$^{ab}$ | 13.18 ± 1.46$^{a}$ | 12.11 ± 2.15$^{a}$ | 10.53 ± 1.63$^{ab}$ | 10.11 ± 0.94$^{ab}$ | 9.26 ± 0.97$^{ab}$ | 6.15 ± 0.59$^{b}$ |
| 18:2*n* -6 | 12.17 ± 0.39$^{a}$ | 7.72 ± 1.73$^{bc}$ | 4.81 ± 1.24$^{cd}$ | 4.91 ± 0.52$^{cd}$ | 3.37 ± 0.15$^{d}$ | 6.61 ± 0.70 | 6.29 ± 1.19$^{cd}$ | 11.15 ± 2.39$^{ab}$ | 11.61 ± 1.40$^{a}$ | 10.23 ± 0.89$^{ab}$ | 8.12 ± 0.20$^{bc}$ |
| 18:3*n* -3 | 0.79 ± 0.03$^{ab}$ | 0.63 ± 0.19$^{ab}$ | 0.85 ± 0.27$^{ab}$ | 0.76 ± 0.08$^{ab}$ | 0.69 ± 0.05$^{ab}$ | 0.73 ± 0.12$^{ab}$ | 0.62 ± 0.11$^{ab}$ | 0.87 ± 0.18$^{ab}$ | 0.93 ± 0.10$^{a}$ | 0.85 ± 0.11$^{ab}$ | 0.48 ± 0.04$^{b}$ |
| 18:4*n* -3 | 3.97 ± 0.03$^{abc}$ | 6.10 ± 1.56$^{a}$ | 4.70 ± 1.19$^{ab}$ | 5.63 ± 0.15$^{ab}$ | 4.54 ± 0.94$^{ab}$ | 5.45 ± 0.64$^{ab}$ | 5.72 ± 1.39$^{ab}$ | 3.54 ± 0.74$^{bc}$ | 3.53 ± 0.31$^{bc}$ | 3.33 ± 0.31$^{bc}$ | 2.12 ± 0.22$^{c}$ |
| 20:3*n* -6 | 0.90 ± 0.16$^{a}$ | 0.61 ± 0.13$^{bcde}$ | 0.41 ± 0.10$^{ef}$ | 0.46 ± 0.04$^{def}$ | 0.37 ± 0.04$^{f}$ | 0.51 ± 0.04$^{cdef}$ | 0.56 ± 0.11$^{cdef}$ | 0.80 ± 0.12$^{ab}$ | 0.71 ± 0.07$^{abc}$ | 0.67 ± 0.09$^{abcd}$ | 0.57 ± 0.01$^{bcdef}$ |
| 20:4*n* -6 | 1.64 ± 0.07$^{ab}$ | 1.04 ± 0.28$^{cde}$ | 1.05 ± 0.25$^{cde}$ | 1.13 ± 0.06$^{bcde}$ | 0.89 ± 0.07$^{e}$ | 1.55 ± 0.16$^{abc}$ | 1.13 ± 0.13$^{bcde}$ | 1.83 ± 0.25$^{a}$ | 1.55 ± 0.14$^{abc}$ | 1.43 ± 0.20$^{abcd}$ | 0.96 ± 0.07$^{de}$ |
| 20:5*n* -3 | 1.99 ± 0.50$^{ab}$ | 1.73 ± 0.22$^{abc}$ | 1.93 ± 0.50$^{ab}$ | 1.98 ± 0.20$^{ab}$ | 1.45 ± 0.10$^{bc}$ | 2.41 ± 0.41$^{a}$ | 1.47 ± 0.16$^{bc}$ | 2.17 ± 0.30$^{b}$ | 1.67 ± 0.12$^{abc}$ | 1.46 ± 0.18$^{bc}$ | 0.97 ± 0.18$^{c}$ |
| **∑ PUFA** | **37.26 ± 1.45$^{a}$** | **33.90 ± 6.79$^{ab}$** | **28.31 ± 7.39$^{ab}$** | **30.69 ± 2.22$^{ab}$** | **23.48 ± 2.80$^{ab}$** | **32.85 ± 3.98$^{ab}$** | **30.25 ± 5.71$^{ab}$** | **35.85 ± 6.69$^{a}$** | **35.43 ± 3.80a** | **31.91 ± 3.23$^{ab}$** | **22.74 ± 1.53$^{b}$** |
| **∑ *n* -3** | **20.91 ± 0.82$^{ab}$** | **23.79 ± 4.56$^{a}$** | **21.19 ± 5.57$^{ab}$** | **23.29 ± 1.51$^{a}$** | **18.13 ± 2.46$^{ab}$** | **23.66 ± 2.89$^{a}$** | **21.57 ± 4.15$^{a}$** | **21.12 ± 3.70$^{a}$** | **20.47 ± 2.03$^{ab}$** | **18.37 ± 1.94$^{ab}$** | **12.19 ± 1.24$^{b}$** |
| **∑ *n* -6** | **16.35 ± 0.64$^{a}$** | **10.11 ± 2.23$^{cd}$** | **7.12 ± 1.82$^{de}$** | **7.41 ± 0.72$^{de}$** | **5.35 ± 0.33$^{e}$** | **9.19 ± 1.09$^{de}$** | **8.67 ± 1.56$^{de}$** | **14.73 ± 2.99$^{ab}$** | **14.96 ± 1.77$^{a}$** | **13.54 ± 1.29$^{abc}$** | **10.55 ± 0.28$^{bcd}$** |

## Conclusions

The study highlights the impact of temperature and salinity shifts on *T. striata* CTP4 cells, including growth, viability, gene expression, and biochemical composition. The cross-protective response induced by increased salinity suggests a mechanism for developing thermotolerance in this species that belongs to an evolutionary branch, Chlorodendrophyceae, that diverged early from other core chlorophytes. The observation that this microalga can only fully tolerate a thermal shift to 40 °C in the presence of salt might be a remnant of its marine evolutionary history. Future research should explore how conserved this requirement is in other microalgae, especially freshwater species, as this adaptation may have been crucial for their terrestrial colonization, which then gave way to the embryophytes, including flowering plants.

These findings offer important insights into how microalgae acclimate to changing environmental conditions, which could be applied to prevent heat-induced collapse in outdoor cultures used for industrial microalgal biomass production. The ability of microalgae strains to withstand high temperatures is critical to sustainable production, as large amounts of freshwater are often used to cool outdoor cultivation systems. The ability of *T. striata* to thrive under fluctuating temperatures highlights its potential for large-scale biomass production, especially in industrial settings with variable temperature conditions.

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**Author contribution** T.F.S. contributed to Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization. H.P.: Conceptualization, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition. L.S.: Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. I.B.M.: Formal Analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. R.J.: Validation, Investigation, Writing – Review & Editing. G.B.: Investigation, Writing – Review & Editing. F. P.: Investigation, Writing – Review & Editing. L. B.: Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition. J.V.: Conceptualization, Methodology, Validation, Resources, Writing – Original Draft, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition.

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**Data availability** All raw data are available on request.

## Declarations

**Competing interests** The authors declare no competing interests.



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