



# Article Increasing Temperature Alters the Effects of Extracellular Copper on Thalassiosira Pseudonana Physiology and Transcription

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Abstract: Copper (Cu) is essential for many physiological processes in phytoplankton, including electron-transfer reactions and high-affinity Fe transport systems. However, at high concentrations, Cu can have a toxic effect on phytoplankton. Phytoplankton's tolerance to certain toxicants in marine ecosystems cannot always be solely attributed to the presence of compounds, as various environmental factors including temperature can also indirectly influence their effects on organisms. In this study, we investigated the effects of different Cu concentrations (0, 19.6, 160, 800, and 8000 nM) on the growth and physiological changes in the centric diatom Thalassiosira pseudonana (CCMP 1335) when simultaneously applied with temperature cultivation of 20 °C and 25 °C. At low (0 nM) and high (8000 nM) Cu concentrations, the growth rate of T. pseudonana was inhibited, though an increase in temperature lessened this inhibition. There were no significant changes in the POC:PON ratio during all of the treatments. However, increasing the temperature significantly decreased the POC: POP, PON: POP and BSi: POP ratios of T. pseudonana. The intracellular Cu content of T. pseudonana varied from 0.13 to 13.28 fg cell $^{-1}$  in response to increases in ambient Cu concentrations. Lastly, an increase in the Cu concentration decreased the transcriptional expression of CTR (copper transporter), 3Hfcp (photosynthetic protein), and Sit1 (silica shell formation) encoding genes. In conclusion, our results indicated that T. pseudonana can adapt to physiological processes and molecular mechanisms in response to varying Cu concentrations and ambient temperatures.

Keywords: T. pseudonana; copper; temperature; growth; transcription

# 1. Introduction

Trace metals are required for numerous physiological processes in phytoplankton [1–3]. In particular, copper (Cu) is critical for photosynthesis [4], respiration [5] and the quenching of reactive oxygen species via superoxide dismutase [6]. Its availability can limit or inhibit the growth of phytoplankton in natural environments [1,2,7–9]. Cu concentrations in open oceans range from 0.3–6.0 nM, and it is often a factor in limiting the growth of phytoplankton [10]. However, Cu concentration can reach up to 300 nM in coastal waters, and such high Cu concentrations have been observed to reduce growth rate of marine phytoplankton [10,11], inhibit photosynthesis [12], decrease nitrate uptake [13], hinder silicic acid uptake [14] and alter the community composition of phytoplankton [8]. Most organisms have evolved mechanisms to survive under both optimal and suboptimal conditions in order to acclimatize to dynamic environments [3]. Plasticity is particularly evident in the use of trace metals, wherein elemental substitutions can occur under limiting conditions and excess intracellular accumulation of metals can occur when external concentrations are high [15,16]. Phytoplankton can modulate their growth, elemental stoichiometry and photosynthetic activity to respond to fluctuating levels of Cu concentration [17,18]. More-



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**Copyright:** © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). over, varying Cu concentrations can alter the community compositions of phytoplankton, because diatoms have a higher tolerance than cyanobacteria and dinoflagellates [8].

Diatoms contribute roughly 20% of the total global primary production [19,20]. Consequently, diatoms were used as optimal-biological models to investigate ecotoxicological and environmental effects arising from stressors on marine ecosystems [21–23]. Cu influences phytoplankton growth [24,25] and cellular-elemental stoichiometry, as well as a range of biochemical process such as pigmentation, photosynthesis and biochemical composition [26]. Furthermore, variation in physicochemical factors including temperature, salinity and pH, can impose additional stress on marine organisms, while also influencing metal contaminants, thereby affecting their availability and toxicity to organisms. In particular, seawater temperature is a key factor that influences the toxicity of chemicals on phytoplankton [17]. Chemical toxicity generally varies with temperature [27] and a rise in temperature can promote a higher Cu tolerance in periphytic bacterial communities. These early studies assumed that an acceleration in pollution induced the acquisition of community tolerance via temperature-induced physiological enhancements [28]. In contrast, Morin et al. [28] demonstrated that increased Cu concentrations induced tolerance in diatom communities, but that tolerance decreases with increasing temperatures. Further, other studies have indicated that the toxicity of cadmium (Cd) generally increases with increasing temperature in the marine diatom Thalassiosira nordenskioeldii [29]. Thus, both the specific metal element and the diatom species should be considered when designing experiments to investigate metal toxicity in diatoms.

The Cu transport system is well-understood at the molecular level in *Chlamydomonas reinhardtii* [3], *Saccharomyces cerevisiae* [30] and plants [31]. However, the mechanisms of Cu transport and homeostasis in diatoms are not well understood. Cu uptake is biphasic, wherein two types of transporters may be involved: high-affinity systems (CTR) and low-affinity systems (ZIPs and NRAMP) [32,33]. Previous studies have suggested that the two Cu-transport systems are differentially controlled by low Fe and/or Cu. Other studies have demonstrated that exposure to Cu can induce the expression of photosynthetic genes and silica-shell formation genes. Furthermore, temperature can alter the metabolism of marine organisms, altering their response to heavy metal. *Thalassiosira pseudonana* is one of the most widely distributed marine diatoms worldwide and a genome is available for this species. In this study, integrative analyses were used to explore the possible response mechanisms of the *T. pseudonana* physiology and transcription toward the combined effects of Cu exposure and temperature variation.

#### 2. Materials & Methods

In order to address the combined effects of temperature and Cu concentrations on *Thalassiosira pseudonana*, cultures of *T. pseudonana* (CCMP 1335) were exposed to two levels of different temperatures (20 and 25 °C) and five levels of Cu concentrations. The second factor was Cu concentration (0, 19.6, 160, 800 and 8000 nM). Thus, a two by five factorial design was chosen, with triplicate values of each response variable at each treatment. Details of this overview are given in the following sections.

### 2.1. Cell Culture and Experimental Design

*Thalassiosira pseudonana* (CCMP 1335) were obtained from the Xiamen University Center for Marine Phytoplankton and semi-continuously cultured at 20 °C in constant light condition in the medium Aquil (N: 300 mM) that was filter-sterilized using 0.22- $\mu$ m of polycarbonate filters (Supplemental Table S1). Cultures were acclimated to a range of Cu concentrations (0, 19.6, 160, 800 and 8000 nM) at temperatures of 20 °C and 25 °C and a light intensity of 90  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup> for more than 10 generations prior to experimentation. All the glassware used in the experiments were cleaned with 10% of nitric acid, rinsed with ultrapure water and autoclaved before use. A stock solution of 2 g Cu L<sup>-1</sup> was prepared in ultrapure water using copper sulfate pentahydrate (sigma). Cultures from the high temperature (25 °C) and the low temperature (20 °C) were in turn exposed to five Cu-concentration treatments. The algal cells were cultured in a 500 mL conical flask in a light incubator for 3 days, and the algal was mixed every 2 h. Then, cultures were collected to analyze the response variables from each of the triplicates in every treatment.

# 2.2. Growth Rates

Cell densities were obtained using a Z2 Coulter Counter. Growth rates were calculated from observed cell numbers using the equation:  $\mu = (LnN_{t2} - LnN_{t1})/(t2 - t1)$ , where  $N_{t2}$  and  $N_{t1}$  are the cell concentrations of the culture at t2 and t1. Growth rates were calculated from triplicate measurements of cell abundances for each culture.

### 2.3. Pigments and Chlorophyll Fluorescence Parameters

Absorbance spectra of cell extracts were determined at day 3 by filtering 15 mL of cultures through a Whatman GF/F glass-fiber filter (25 mm diameter). The filters were placed in 10 mL centrifuge tubes and 8 mL of 90% acetone was immediately added while avoiding light. The samples were then placed at a temperature of 4 °C overnight to extract the pigments, and then centrifuged at  $5000 \times g$  for 10 min. The supernatant was removed and measured using a UV-Vis spectrophotometer (SP-752, Shanghai spectrum instruments) at absorbances of 480, 510, 630, 664 and 750 nm. Pigment values were then calculated using the Ritchie method [34].

Chlorophyll fluorescence was determined using a pulse-amplitude modulated fluorometer (water-PAM, Walz, Germany). Two samples were taken from each flask. One sample was incubated in the dark, adapted for 15 min first to relax any photosynthetic activity. The maximal efficiency of PS II photochemistry was then determined as the ratio of variable to maximal chlorophyll fluorescence (Fv/Fm), where Fv = (Fm – F0), and Fm and F0 represent the maximal and minimal fluorescence yield of a dark-adapted suspension, respectively. The chlorophyll fluorescence of the other sample was determined at an actinic light of 96 µmol photons m<sup>-2</sup> s<sup>-1</sup>. The effective efficiency of PS II photochemistry was then determined as the ratio of variable to effective chlorophyll fluorescence (Fv'/Fm'), where Fv' = (Fm' – F0'), and Fm' and F0' are the maximal and minimal fluorescence yield of an actinic light suspension, respectively, and the non-photochemical quenching-NPQ = (Fm – Fm')/Fm'.

#### 2.4. Intracellular Copper

Cells were filtered with 1.2  $\mu$ m of polycarbonate filters, and the cells retained by the filters were washed with 15 mL of a 1 mM EDTA·2Na solution to remove surface-adsorbed metals and then rinsed with ultrapure water [35]. Samples were then digested for 6 h at 65 °C on a hot plate with 2 mL of ultrapure HNO<sub>3</sub>. Total and intercellular metal extracts were then filtered before diluting to 10 mL of ultrapure water in a 15 mL volumetric flask, and then analyzed using an Agilen ICP-MS (7700).

## 2.5. Intracellular POC, PON, POP and BSi

To measure particulate organic carbon (POC), nitrogen (PON), Particular organic phosphorus (POP) and biogenic silicon (BSi), each sample was taken at 25 mL to filter through pre-weighed, pre-combusted (450 °C for 6 h) GF/F filters. The filter membranes were then placed in a sealed sample box with concentrated hydrochloric acid and then dried at 65 °C. The result samples were then analyzed with an elemental analyzer (PerkinElmer 2400 II). Biogenic silicon (BSi) and particular organic phosphorus (POP) were analyzed following procedures by Passche [36] and Solórzano et al. [37], respectively.

#### 2.6. RNA Extraction and Quantitative Real-Time PCR

Cells from the cultures were harvested onto 25 mm polycarbonate filters with 1.2  $\mu$ m porosity and then flash-frozen in liquid nitrogen and stored at -80 °C until further RNA extraction. Cell lysis and total-cellular RNA isolation were performed using an RNAiso Plus kit (Takara). In the first treatment (hereafter designated as the RNAiso Plus + liquid N2 method), the homogenization step was replaced by a simultaneous flash-freezing of the cells

using liquid nitrogen in a pre-chilled mortar, followed by continuous grinding with a pestle while adding 1 mL of the RNAiso Plus reagent to the lyse cells and efficiently extracting RNA. Care was taken in order to prevent the cells from thawing before the RNAiso Plus regent was added into the Sample. As the frozen RNAiso Plus-cell mixture started to thaw, homogenization was continued until the RNAiso Plus was also pulverized, as previously described [38]. The remaining steps were as recommended in the manufacturer RNAiso Plus protocol. The purity of extracted RNA was verified by RT-qPCR to ensure the lack of genomic DNA contamination. Purified total-RNA was quantified spectrophotometrically, and 1000 ng were reverse-transcribed into first-strand cDNAs in 20  $\mu$ L reaction using the  $2 \times$  RealStar Power SYBR Mixture (GenStar). Gene-specific primers for RT-qPCR, with amplicon sizes in the range of 90–200 bp, were designed based on the available genomic sequence data for *T. pseudonana* or otherwise based on previous reports [17,39,40]. cDNA was then amplified with RT-qPCR using those gene-specific primers (Table 1) and SYBR Green Supermix. Each 20  $\mu$ L PCR reaction contained 0.2  $\mu$ mol L<sup>-1</sup> each of the forward and reverse primers, and 2 ng transcribed RNA. RT-qPCR was performed on the iQ5 real-time system with thermal cycling conditions as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Melt curve analysis began at 95 °C for 1 s, followed by 65 °C for 15 s. Transcript abundance was then determined using the  $2^{(-\Delta Ct)}$  method [41] from technical duplicates of each biological triplicate. The  $\Delta$ Ct values were calculated after normalization to the template numbers of the endogenous reference gene TpActin\_25772.

Genes (Name_ID)	Primer Sequence (5'to3')		Tm (°C)	Annotation	References
CTR_24275 -	Forward	GCATGCGTCGGCTTATTCTA	58.50	<ul> <li>Cu transporter</li> </ul>	- - - - - - - - - - -
	Reverse	CACATGACCTGCCATCATTC	56.85		
CTR_9391 -	Forward	GTCAAATGCTCTGTCCAGCA	58.47	<ul> <li>Cu transporter</li> </ul>	
	Reverse	CGTTTGATCCATTTGGAGGT	56.02		
CTP_263051 -	Forward	GTGCTGAGCAGAACAGTGAGC	61.79	Cu transporting p-type ATPase	
	Reverse	GCAGATAGACAACGGCGATT	58.43		
CTP_264357 -	Forward	TTGACAAGACGGGAACCATT	57.35	Cu transporting p-type ATPase	
	Reverse	TTCACTGCCGAGGGTAAATC	57.60		
COX17_264096 -	Forward	AAAGTGGGCATCAAATCAGG	56.27	— Cu chaperone	
	Reverse	CTCTTACAATCCGCGTCCTC	58.16		
COX11_37139 -	Forward	AGTCGCCACCTACAATGTCC	59.75	— Cu chaperone	
	Reverse	ACTGGCATATCCACCGTTTC	57.96		
ZIP_32375 -	Forward	AACGTAGGAACAGCCCTCCT	60.54	— ZIP transporter	
	Reverse	ATGACACCTGCCGACAAAC	58.37		
ZIP_268980 -	Forward	TGGTGTGTGGTCACTTCGTT	59.75	<ul> <li>ZIP transporter</li> </ul>	
	Reverse	GCCTCCAACAACAACTCCAT	58.38		
ZIP_22351 -	Forward	CAACGCAGACGAAGAAAACA	57.27	<ul> <li>ZIP transporter</li> </ul>	
	Reverse	GGGAACGCATACTTCGACAT	58.06		
ZIP_11826 -	Forward	CATTGGTTGCAGCACTATCG	57.54	— ZIP transporter	
	Reverse	CGACAATCGTTTACCAAGCA	56.76		
ZIP_263800 -	Forward	GCCATTGCTCAAACCGTATT	57.06	<ul> <li>ZIP transporter</li> </ul>	
	Reverse	CTTAGCCGCAAGACCAAATC	57.17		

Table 1. Genes and primers used in this study.

Genes (Name_ID)	Primer Sequence (5'to3')		Tm (°C)	Annotation	References
SCO1_30986 -	Forward	TTGGTTTCGCTCGGTGTC	57.96	— Cu chaperone	In this study
	Reverse	GTTCGCAATGCAACTTTCCT	57.58		
COPI_23273 -	Forward	ATTCGGAGTGTTGTTGCC	55.58	— Cu chaperone	
	Reverse	CATTGTCAGGGAGTTGTTCT	55.27		
FET3_5574 -	Forward	GTCGTCGAGCAAGTTTCGTT	59.15	Multi-copper oxidase	- _ [39] -
	Reverse	GGGAGCAACGTGGATAACAT	57.96		
FTR1_268009 -	Forward	GCTGGCTTGTGTTTGGAATTGT	60.48	— Fe permease	
	Reverse	CACCTTCCTCTGCCTCGTTGA	62.04		
NRAMP_9840 -	Forward	ATCGCCCAAGACGACAGAGTTGA	64.04	— NRAMP protein	
	Reverse	CACCGCTCCTAGAATCCCAGTAATG	62.75		
TpActin_25772 -	Forward	ACTGGATTGGAGATGGATGG	56.66	– Actin	
	Reverse	CAAAGCCGTAATCTCCTTCG	56.62		
3HfcpA_1778744 -	Forward	CTCCCTCCAGGTTCCTGTTG	56.66	Photosynthetic proteins	- _ [42] -
	Reverse	AGCGAGCTCAAGGAATCCAA	59.38		
3HfcpB_1778746 -	Forward	AGTTCGATGAGGAGACCAAGCT	61.16	_ Photosynthetic proteins	
	Reverse	GGCACGTCCGTTGTTCAAC	60.01		
Sil1_52355302 -	Forward	CCGTCACCCTCTCCTGAAAC	60.04	Silica shell formation	
	Reverse	ATGGGAGCAGCGGTAATGG	59.85		
Sit1_224002055 -	Forward	TTGCCGAGGATGCCTAAACTT	60.00	Silica shell	
	Reverse	TGACGAGCTACTGCAGGTTCA	61.43	formation	

#### Table 1. Cont.

## 2.7. Data analysis

All data are expressed as mean  $\pm$  1 standard deviation (SD). Each treatment was performed in triplicate. *t*-test and One-way ANOVA (LSD test) were used to compare the different parameters (e.g., growth rate, Chla, Chlc, Carotenoid, Fv/Fm, Yield, NPQ, Cu content, POC: Chla) among the 10 different treatments. We checked the normality, equal variances and independence were met before we conducted the one-way ANOVA. Multiple-way analysis of variance (MANOVA) was used to determine whether there were significant differences among the two factors (temperature and Cu content). All statistical analyses were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). The significant level was set at *p* < 0.05.

## 3. Results

## 3.1. Growth Rates

Copper addition significantly increased the growth rates of *Thalassiosira pseudonana* at 20 °C and 25 °C when Cu concentrations were less than 160 nM (Figure 1). However, increasing copper concentration significantly reduced the growth rates of *T. pseudonana* at 20 °C when Cu concentrations were more than 160 nM (Figure 1). In contrast, increased Cu did not significantly alter the growth rates at 25 °C (Figure 1). In addition, the 5 °C temperature increase also influenced the growth rates of *T. pseudonana* (Figure 1). The growth rates of *T. pseudonana* at 25 °C significantly increased relative to *T. pseudonana* at 20 °C by 41.8%, 32.1%, 26.1%, 39.7% and 49.3% at the five different Cu concentrations (p < 0.05) (Figure 1, Supplemental Table S2).



**Figure 1.** Growth rate of diatom *T. pseudonana* under different temperature and copper concentration at light:dark cycles of 12:12 under 90 µmol photons  $m^{-2} s^{-1}$  growth light. Data are mean  $\pm$  standard deviation (n = 3). Different letters at the top of each bar indicate significant differences between different Cu concentration treatments and \* indicate significant differences between different temperatures (p < 0.05).

### 3.2. Pigments and Chlorophyll Fluorescence Parameters

At the higher temperature (25 °C), the concentrations of pigments including Chla, Chlc and carotenoid increased in *T. pseudonana* (Figure 2A). In addition, Cu concentration and temperature had no effect on Chla content, and Chlc had significantly increased under high temperature at 160 nM and nM Cu concentrations (Figure 2A). After 25 °C, carotenoid content of five copper-concentration groups increased, and there were significant differences, except for 800 nM (Figure 2A). For *T. pseudonana* grown at 20 °C and 25 °C, a significant difference was observed between Fv/Fm and Fv'/Fm' with increased Cu concentration. The results indicate that elevated temperature can improve the Fv/Fm and Fv'/Fm of *T. pseudonana* (Figure 2B). Additionally, the NPQ also increased significantly in the treatment of 8000 nM Cu concentration at 20 °C and 25 °C.

## 3.3. Intracellular Elemental Contents

The intracellular Cu content in *T. pseudonana* ranged from 0.13 to 13.28 fg cell<sup>-1</sup> and increased with increasing Cu-concentration treatments (Figure 3). Increased growth temperature significantly reduced the intracellular Cu content of *T. pseudonana*, with the exception of the highest Cu concentration. There were no significant changes in the POC:PON ratio throughout all of the treatments (Figure 4A). The POC:POP, PON:POP and BSi:POP ratios of *T. pseudonana* grown at 25 °C decreased relative to that grown at 20 °C in all of the treatments (Figure 4). There were no significant changes in POC:POP and PON:POP ratios when Cu was increased at 20 °C. However, increasing Cu significantly decreased the POC:POP and PON:POP ratios at 25 °C (Figure 4B,C). Increasing Cu decreased the BSi:POP ratio of *T. pseudonana* in all of the treatments, except that grown at 0 nM Cu (Figure 4D). The POC:Chla ratio of *T. pseudonana* grown at 25 °C decreased, relative to that grown at 20 °C, by 8.7%, 12.7%, 5.1%, 15.4% and 1.9% when grown with the five different Cu concentration treatments, respectively (Figure 5). The POC:Chla ratio decreased when Cu concentrations increased from 19.6 to 8000 nM (Figure 5).



**Figure 2.** Pigment content (chlorophyll a, Chlc and Caro) (**A**) and chlorophyll fluorescence parameters ( $F_v/F_M$ , yield and NPQ) (**B**) of *T. pseudonana* after culture for three days in five different copper concentrations, at temperatures of 20 °C and 25 °C. Data are mean  $\pm$  standard deviation (n = 3) Different letters at the top of each bar indicate significant differences between different Cu concentration treatments and \* indicates significant differences between different temperatures (p < 0.05).



**Figure 3.** Intracellular Cu content of *T. pseudonana* cell after being cultured for three days in five different copper concentrations at temperatures of 20 °C and 25 °C, Data are mean  $\pm$  standard deviation (n = 3). Different letters at the top of each bar indicate significant differences between different Cu concentration treatments and \* indicates significant differences between different temperatures (p < 0.05).



**Figure 4.** The POC:PON, POC:POP, PON:POP and BSi:POP ratios (**A**–**D**) in *T. pseudonana* after being cultured for four days in five different copper concentrations at 20 °C and 25 °C. Data are mean  $\pm$  standard deviation (n = 3). Different letters at the top of each bar indicate significant differences between different Cu concentration treatments and \* indicates significant differences between different cu concentration treatments and \* indicates significant differences between different cu concentration treatments and \* indicates significant differences between different cu concentration treatments and \* indicates significant differences between different cu concentration treatments and \* indicates significant differences between different cu concentration treatments and \* indicates significant differences between different cu concentration treatments and \* indicates significant differences between different cu concentration treatments and \* indicates significant differences between different cu concentration treatments and \* indicates significant differences between different cu concentration treatments and \* indicates significant differences between different cu concentration treatments and \* indicates significant differences between different cu concentration treatments and \* indicates significant differences between differences between



**Figure 5.** The response of POC:Chla ratio to five different copper concentrations at 20 °C and 25 °C, Data are mean  $\pm$  standard deviation (n = 3). Different letters at the top of each bar indicate significant differences between different Cu concentration treatments and \* indicates significant differences between different temperatures (p < 0.05).

#### 3.4. Gene Expression

Temperature alters the transcription and expression of many genes, including Cu transport, ZIP, photosynthetic, and silica shell formation-related genes. Under low Cu concentrations, CTR\_24275 and CTR\_9391 expression increased at both 20 °C and 25 °C (Figure 6A). However, the expression of these two genes differed with respect to temperature, wherein the higher-growth temperature induced the expression of CTR\_9391 (Figure 6A). Increased Cu concentrations inhibited the expression of CTP\_263051 and CTP\_264357 (Figure 6A). In particular, the expression of CTP\_264357 was significantly inhibited at 25 °C (Figure 6A). At high-Cu concentrations, the expression of COX\_37139 and COX17\_264096 were reduced at both 20 °C and 25 °C (Figure 6A). Likewise, the expression of SCO1\_30986 was reduced at both 20 °C and 25 °C under high-Cu concentration treatments (Figure 6A). Increased temperature will promote the expression of these two genes. Higher-growth temperature induced the expression of FET3\_5574, FRE1\_11375, and FTR1\_268009 during most of the Cu concentration treatments (Figure 6B). In addition, increased-growth temperature and Cu concentrations induced the expression of NRAMP\_9840 (Figure 6B). The expression of five ZIP genes differed based on temperature and Cu concentrations. For example, the expression levels of ZIP\_11826 and ZIP\_268980 were inhibited at 25 °C (Figure 6B). Under high-Cu concentrations, the expression of ZIP\_26890 was inhibited when cultures were grown at 20 °C, but induced with growth at 25 °C (Figure 6B). 3HfcpA and 3HfcpB also exhibited lower expression levels at 25 °C, but the transcription expression of 3HfcpA\_1778744 and 3HfcpB\_1778746 decreased with increasing Cu concentrations (Figure 6C). Lastly, higher Cu concentrations inhibited the expression of Sil1\_52355302 and Sit1\_224002055 (Figure 6C).



**Figure 6.** Relative mRNA expression of genes upon combined effect of copper exposure and temperature treatment with the control (mean + SD, n = 3, Note: n = 1 for sample at 160 nM Cu under 25 °C). (**A**) Cu transporters (CTR\_24257, CTR\_9391), Cu-transporting P-type AT-Pase (CTP\_263051, CTP\_264357), Cu chaperone (COX11\_37139, COX17\_264096, SCO1\_30986, COPI\_23273); (**B**) ZIP transporters (ZIP\_11826, ZIP\_22351, ZIP\_263800, ZIP\_268980, ZIP\_32375), NRAMP protein (NRAMP\_9840), Multi-copper oxidase (FET3\_5574), Fe reductase (FRE1\_11375), Fe permease (FTR1\_268009), Heavy metal transporter (HAM\_261657); (**C**) photosynthetic proteins (3HscpA\_1778744, 3HscpB\_1778746), silicon synthesis proteins (Sil1\_52355302, Sit1\_224002055). Data are mean  $\pm$  standard deviation (n = 3). Different letters at the top of each bar indicate significant differences between different Cu concentration treatments and \* indicates significant differences between different temperatures (p < 0.05).

# 4. Discussion

# 4.1. Growth Rates

Here, we investigate the role of Cu and ambient temperatures on the growth and physiological responses of the diatom T. pseudonana. Cu is essential for many physiological processes in phytoplankton, including electron-transfer reactions and high-affinity Fe transport systems, therefore low-Cu concentrations can also limit phytoplankton growth, as observed in the coccolithophore and *T. oceanic* [7]. Therefore, slight increases in ambient Cu concentrations have been shown to trigger the growth of *P. tricornutum* [25]. We found that increased Cu concentrations appeared to stimulate the growth of T. pseudonana at lower concentrations, which is consistent with previous studies [43,44]. Taken together, the results of this study and others suggest that Cu is an essential element for phytoplankton. However, Cu can also be potentially toxic at high concentrations. Increased copper concentration significantly reduced the growth rates of *T. pseudonana* at 20 °C (Figure 1). Jordi, Basterretxea et al. have shown that Cu aerosols can inhibit phytoplankton growth in the Mediterranean sea [45]. Further, high-Cu concentrations can inhibit *Phaeocystis cordata* growth, both in batch and semi-continuous cultures [46]. T. pseudonana exhibited a reduced growth at 20 °C under high-Cu concentrations, but little inhibition at 25 °C (Figure 1). Overall, the high-Cu concentration treatments decreased the growth of *T. pseudonana*.

Earth-system models predict that global warming will result in significant a decline in net-primary production by marine phytoplankton throughout the 21st century; this will be driven by rising temperatures which exceed the limits of thermal tolerances of phytoplankton, in addition to increasing grazing, nutrient limitations and more stratified ocean waters [47]. However, some species grow faster under increased cultivation temperatures [48,49]. Accordingly, our results also show that increased cultivation temperature significantly increased the growth rate of *T. pseudonana* (Figure 1). In addition to the direct effects of temperature on physiology, it can indirectly alter the effects of Cu concentrations on phytoplankton growth. Our results indicated that declines in growth rates were a result of higher Cu concentrations that were affected by increased temperatures. Consistent with this result, spring lacustrine phototrophic periphyton growing at 18 °C were more sensitive to a herbicide mixture than those growing at higher temperatures [50]. Further, a recent study indicated a decrease in Cu tolerance of winter phototrophic periphyton coincided with temperature changes, where higher temperatures decreased the diversity of diatom species and also exacerbated the community sensitivity towards Cu [28].

## 4.2. Chlorophyll Fluorescence Parameters

Quantum yield analysis indicated that *T. pseudonana* were characterized by relatively high Fv/Fm, suggesting that *T. pseudonana* possessed relatively high activity PS II complexes. A slight decrease in Fv/Fm values was observed in *T. pseudonana* grown with increased Cu concentration and at 20 °C (Figure 2B). In contrast, no significant differences in Fv/Fm values were observed when grown under different Cu concentrations and at 25 °C, although the Fv/Fm values were generally higher than those for *T. pseudonana* grown at 20 °C (Figure 2B). Taken together, these results suggest that the *T. pseudonana* PS II complex was relatively sensitive to Cu stress, but increased temperatures mitigated these deleterious effects. Thus, increased growth temperatures may help improve PS II recovery of *T. pseudonana* treat with high-Cu concentration stress.

#### 4.3. Intracellular Elemental Contents

*Diatom* (e.g., T. pseudonana, *P. tricornutum* and T. weissflogii) exhibit a higher tolerance to increased Cu levels compared to chrysophyceae (e.g., *Gephyrocapsa oceanica*) dinoflagellates (e.g., *Peridinium* sp.) and Cyanobacteria (e.g., *Synechococcus* spp.) [8]. It is possible that copper binds more strongly to the mucopolysaccharide cell wall of the cyanobacteria than it does to the fundamentally different types of cell walls of eukaryotic algae (predominantly cellulose) [8]. The Cu content in *P. tricornutum* has been shown to range from 1.3 to 12.8 fg cell<sup>-1</sup> [25], which coincides with the range reported here for *T*. *psuedonana*. *T. pseudonana* appeared to particularly adapt to higher Cu concentrations and grew well in the presence of 8000 nM Cu (Figure 1). Concomitantly, their intracellular Cu was far lower than that observed in other species that exhibit Cu toxicity symptoms [51,52].

Only a few studies have explored the phenotypic flexibility in C:N:P of microalgae during exponential growth; therefore, more studies are needed to better understand the effect of micro/macro-nutrient and temperature [53]. The POC:PON ratio is a key parameter in algal growth and is dependent on the variability in the availability of nutrients as well as growth temperature. Reay et al. [54] found that phytoplankton-specific affinity for nitrate is temperature-dependent below optimal temperatures, and diatom nitrate reductase activity declines rapidly as temperatures exceed optimal growth levels [55]. The higher POC:PON ratios of T. australis at low temperature, compared to high temperature (optimal temperature), suggest that suboptimal temperatures could have limited nitrate uptake relative to carbon fixation [56]. No significant differences in the POC:PON ratio were observed among the cultures in this study, although the POC:PON ratio was decreased with an increasing growth temperature (Figure 4). However, previous studies have suggested that warming can reduce intracellular POC:PON, POC:POP, PON:POP and BSi: POP ratios [57–59] due to higher temperatures promoting cell metabolism and increased carbon consumption [47,60]. Hillebrand et al. [61] observed a trend of decreasing N:P ratio and variability with increasing growth rate in phytoplankton, suggesting that fast-growing phytoplankton in general require more P and also have a relatively low PON:POP ratio compared with slow-growing phytoplankton. Another key parameter that characterizes algal growth is the POC: Chla ratio. The POC: Chla ratios increased at low and high Cu levels or low temperature, which suggested that the cell needed more energy to maintain Cu homeostasis when they are in extreme environments. Geider [62] demonstrated that the relationship between the POC: Chla ratio and growth can be described by a linear model. He also parameterized the dependence of the POC: Chla ratio on temperature and demonstrated that the POC: Chla ratio decreases with increasing growth temperature, similar to the results observed in this study (Figure 5). POC: Chla could be a useful indicator for ecological and physiological conditions of phytoplankton communities and, additionally, it is a photoacclimation index, which can help us to understand primary productivity, and is highly variable over space and time and among different species, ranging from about 20 to over 200 g  $g^{-1}$  in the ocean. Variation in POC: Chla ratios have been established as a sensitive indicator that quantifies the adjustment of phytoplankton cellular pigment levels to the new demands for photosynthesis, caused by changes in ambient temperature, nutrients, and light conditions [63].

#### 4.4. Gene Expression

Gene transcription represents a rapid physiological response to environmental change. Previous research has demonstrated that Cu addition provokes a decreased relative expression of two CTR-like genes (CTR\_24275 and CTR\_9391), two putative Cu-transporting P1B-type ATPases (CTP\_263051 and CTP\_264357) and two Cu-chaperones (COX11\_37139 and COX17\_264096) over short time scales [40]. In the aforementioned study, CTR\_24275 and COX\_264096 mRNA had rebounded to almost to the initial expression levels after 12 h of incubation with Cu addition. These results suggest that the expression of the two genes CTR\_24275 and COX\_264096 can acclimate to environmental changes. In this study, the relative expression of COX\_264096 was only inhibited by increasing copper additions (Figure 6A). This difference may be explained by coastal diatoms exhibiting a lower sensitivity to environmental conditions than oceanic diatoms. In addition, the genes ToCTR1 and ToCTR2 were down-regulated in *T. oceanica* in response to increasing Cu concentrations [10]. ZIPs and NARMP are the low affinity Cu transport system that also exhibited altered expression with varying Cu concentrations and temperature. In addition, the expression of respiration-related genes can be altered when acclimating to varying Cu concentrations and temperatures. COX11 is described as an inner mitochondrial membrane integral protein that is essential for cytochrome c oxidase assembly [64,65]. Likewise, COX17 is involved in the assembly of the cytochrome c oxidase protein and is involved in copper donation to both SCO1 and COX11 [66], in the intermembrane space [16,67,68]. Our results indicated that *T. pseudonana* reduced the absorption of Cu by reducing the expression of COX17\_264096, COX11\_37139 and SCO1\_30986 in order to protect against Cu toxicity to the respiration system. Numerous studies have indicated that warming accelerates the respiratory activity of organisms [47,60]. Consistent with these observations, our results indicated that the expression of COX17\_264096, COX11\_37139 and SCO1\_30986 increased with increased growth temperatures (Figure 6A). The increased expression of photosynthetic proteins 3HfcpA\_1778744 and 3HfcpB\_1778746 in *T. pseudonana* likely occurred to maintain photosynthetic performance in response to environmental stress, which is consistent with other studies [17,67]. Our results indicate that *T. pseudonana* can grow fast by inducing the expression of 3HfcpA\_1778744 and 3HfcpB\_1778746 under low Cu concentration conditions (Figure 6C).

#### 5. Conclusions

Cu is a micronutrient that is involved in many of the metabolic processes in diatoms including photosynthesis, respiration, antioxidant activities, cell well metabolism, and hormone perception. Consequently, the growth of diatoms is inhibited when they experience Cu-limitation in open oceans. However, excessive Cu concentrations can also physiologically damage diatoms growing in coastal waters. In this study, increased growth temperature altered the effect of excess Cu concentrations on T. pseudonana growth. There is no interactive effects of temperature and Cu concentrations on T. pseudonana pigment content, POC, PON, POP, BSi (or POC: PON, POC: POP, PON: POP and BSi: POP ratios), although each was responsive to individual factors. Nevertheless, the combination of increased temperature and Cu concentrations altered the transcription of genes, including photosynthetic and silicon synthesis genes under different Cu concentration and temperature conditions. Heavy metal accumulation is an intensive contemporary problem for marine environments [69]. Consequently, understanding metal homeostasis is critical to ascertain the effects of heavy metals on marine ecosystem taxa and processes. Cu is a particularly integral trace metal for phytoplankton, and thus, exploring Cu homeostasis, including g intracellular location [70] and Cu-sensing and regulation-processing [71], is necessary to evaluate the combined effect of Cu and environmental stressors on marine taxa and ecosystems. The results reported here provide a framework for better understanding the adaptability of important marine diatoms to increase metal stress and ambient temperatures.

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