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Heavy Metals Exacerbate the Effect of Temperature on the Growth of *Chlorella* sp.: Implications on Algal Blooms and Management

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Abstract: With the accelerated urbanization and rapid development of the industrial and agricultural sectors, concern about the pollution of water environments is becoming more widespread. Algal blooms of varying sizes are becoming increasingly frequent in lakes and reservoirs; temperatures, nutrients, heavy metals, and dissolved oxygen are the factors that influence algal bloom occurrence. However, knowledge of the combined effect of heavy metals and temperature on algal growth remains limited. Thus, this study investigated how specific concentrations of heavy metals affect algal growth at different temperatures; to this end, two heavy metals were used (0.01 mg/L Pb^{2+} and 0.05 mg/L Cr^{6+}) at three incubation temperatures (15, 25, and 30 °C) with the alga *Chlorella* sp. A higher incubation temperature contributed to a rise in soluble proteins, which promoted algal growth. The density of algal cells increased with temperature, and catalase (CAT) decreased with increasing temperature. *Chlorella* sp. growth and catalase activity were optimal at 30 °C (algal cell density: 1.46×10^7 cell/L; CAT activity: 29.98 gprot/L). Pb^{2+} and Cr^{6+} significantly promoted *Chlorella* sp. growth during incubation at 25 and 30 °C, respectively. At specific temperatures, 0.01 mg/L Pb^{2+} and 0.05 mg/L Cr^{6+} promoted the production of soluble proteins and, hence, the growth of *Chlorella* sp. The results provide a useful background for the mitigation and prevention of algal blooms.

Keywords: water pollution; temperature; heavy metals; *Chlorella* sp.

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1. Introduction

After the construction of the Three Gorges reservoir in China, algal blooms have become widespread in tributary backwaters [1], causing increased concern. Algae are primary producers in water ecosystems, with microalgae contributing to at least 32% of global photosynthesis [2]. However, high temperatures, excessive amount of nutrients, and suitable amounts of dissolved oxygen in water can lead to algal blooms in lakes and reservoirs. Of these three factors, water temperature is the main cause of seasonal changes to eutrophication processes [3–5].

Wang et al. [6] demonstrated the toxicological effects of heavy metals on algae, showing that Pb^{2+} has a low-promoting and high-inhibiting effect on *Microcystis aeruginosa*. Furthermore, Cr^{3+} and Cd^{2+} positively affected the growth of *Chlorella* sp. at certain concentrations, and high concentrations negatively affected its normal growth [7]. Thus, low concentrations of heavy metals in the water appear to promote algal growth. However, the combined effect of heavy metals and temperature on algal growth remain unclear. The presence of heavy metals in water ecosystems stimulates the production of reactive oxygen

species (ROS) in algal cells, inducing higher antioxidant enzyme activity (e.g., superoxide dismutase (SOD), catalase (CAT)). Soluble proteins reduce intracellular osmotic pressure to maintain normal water supply and cellular physiological functions under the presence of heavy metals. Therefore, it is necessary to establish how heavy metals impact algae [8].

Chlorella sp. is a dominant algae genus found in the Three Gorges reservoir area. It has a typical growth cycle of approximately 12 d, and survives laboratory conditions easily due to its good physiological tolerance. Therefore, *Chlorella* sp. represents a standard test organism for ecotoxicological studies [9]. Most studies on the eutrophication of water bodies focus on certain environmental factors, such as temperature, light, nutrients, and organic matter [2,10,11]. Further, these studies have explored the effects of temperature and heavy metals on algal growth as separate environmental factors, rather than combined [12–16]. For example, Staehr and Sand-Jensen [17] found that the interaction between nutrient availability, time of the year and, thus, ambient temperature was responsible for most of the observed variability in phytoplankton growth, photosynthesis, and respiration. Bestion et al. [18] developed a theoretical model to understand that the changes in temperature affect competitive interactions among phytoplankton. Carfagna et al. [19] found that both heavy metals, Pb and Cd, could alter the ultrastructure of algal cells and their physiological properties (growth, photosynthesis, respiration, and enzyme activity). Therefore, it is necessary to explore the combined effects of heavy metals and temperature on algae.

Here, we investigated how two heavy metals (Pb^{2+} and Cd^{6+}) impact the growth and physiology of *Chlorella* sp. at different incubation temperatures. For different combinations of heavy metals and temperature, we analyzed the correlation between the growth and oxidative stress of *Chlorella* sp. by quantifying the protein, malondialdehyde (MDA), SOD, and CAT enzyme activity. Our results are expected to provide a theoretical basis for preventing and controlling eutrophication and water pollution in the Three Gorges reservoir area of China, and provide a useful background for the mitigation and prevention of algal blooms.

2. Materials and Methods

2.1. Materials and Instruments

Chlorella sp. was purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (Wuhan, China). The algal cell culture medium was BG11 [20], which is considered suitable for freshwater algae cultures. The algae were cultured under aseptic conditions. The medium and containers used in the experiment were sterilized in an autoclave prior to use. $Pb(NO_3)_2$ and $K_2Cr_2O_7$ were added to the BG11 medium separately according to the experimental design.

The main instruments used in the experiments and analyses were: a thermostatic climate incubator (ZRX, Qianjiang Instrument Equipment Co., Ltd., Hangzhou, China), a vertical pressure steam sterilizer (LDZF-30KB-III, Shen'an Medical Instrument Factory, Shanghai, China), a pH meter (IS128S, Yimai Instrument Technology Co., Ltd., Shanghai China), a thermostatic water bath (HHW-4, Xinno Instrumentation Co., Ltd., Shanghai, China), an ultra-clean workbench (BCM-1300, Boris Purification Technology Co., Ltd., Suzhou, China), an electronic microscope (LW40, Calvin Optoelectronic Technology Co., Ltd., Shanghai, China), an ultrasonic cell grinder (JX-1A, Jingxin Industrial Development Co., Ltd., Shanghai, China), an ultraviolet–visible spectrophotometer (T6 New Century, Seiko Scientific Instrument Co., Ltd., Shanghai, China), and a high-speed refrigeration centrifuge (SF-TGL18R, Ficchal Analytical Instrument Co., Ltd., Shanghai, China).

2.2. Experimental Design

Table 1 presents information on the experimental setup. In a preliminary experiment, 0.01 mg/L Pb^{2+} and 0.05 mg/L Cr^{6+} significantly promoted the growth of Chlorella sp. cells [21]. Therefore, the concentrations of Pb^{2+} and Cr^{6+} in the *Chlorella* sp. algae solution were set at 0.01 and 0.05 mg/L, respectively, and were incubated at 15, 25, and 30 °C. The control group contained no added metals. Three replicates of each concentration were used.

The number, chlorophyll a content, and soluble protein and antioxidant enzyme activity of the algal cells were measured daily. The mixture was shaken once in the morning and once in the evening. The optimum temperature for *Chlorella* sp. growth was determined by recording the daily biomass and chlorophyll a content in the control group, and measuring the soluble protein, SOD, MDA, and CAT activity in the *Chlorella* sp. cells. The results were used to investigate the physiological effects of the dual stress of temperature and heavy metals.

Table 1. Experimental design.

Heavy Metal	pH	Heavy Metal Concentration (mg/L)	Temperature (°C)		
CK	7	0	15	25	30
Pb^{2+}	7	0.01	15	25	30
Cr^{6+}	7	0.05	15	25	30

The number of algal cells was determined using the optical density method [22]. Chlorophyll a content was determined using the hot ethanol method [23]. Soluble protein (A045-2-2), SOD (A001-1-1), MDA (A003-1-1), and CAT (A007-2-1) were determined using kits purchased from Jiancheng Biological Engineering Research Institute in Nanjing, Jiangsu Province, China.

2.3. Statistical Analysis

Origin 2021 and SPSS Statistics 25 were used for the data analysis. One-way analysis of variance (ANOVA) was used, with significance set at 0.05 and high significance at 0.01.

3. Results and Discussion

3.1. Simultaneous Effect of Temperature and Heavy Metals on the Growth of Chlorella sp.

3.1.1. Algal Cell Growth

The biomass of *Chlorella* sp. improved with increasing temperature and changed in the presence of the two heavy metals over the experimental period (Figure 1). During the first 2 d of incubation, the *Chlorella* sp. biomass did not noticeably change under the three temperatures (Figure 1A). However, from 3–7 d, while the biomass grew slowly at 15 °C, it increased at 25 °C, and was exponential at 30 °C. The algal cell density increased with temperature (15 °C group < 25 °C group < 30 °C group), with 30 °C representing the optimum temperature for cultivation. At 15 °C, *Chlorella* sp. growth was slow in both heavy metal treatment groups (Figure 1B). In contrast, at 25 and 30 °C in the presence of heavy metals, the *Chlorella* sp. biomass was consistent and exponential, respectively. For the Pb^{2+} treatment groups at 15 and 25 °C, the *Chlorella* sp. biomass was 1.7% and 10.48% higher than that of the control, respectively. The highest *Chlorella* sp. biomass was recorded for Cr^{6+} at 30 °C, and was 4.71% higher than that of the control. Thus, temperature and heavy metals appeared to have positive synergistic effects on *Chlorella* sp. growth. Of note, the synergistic effect of 0.01 mg/L of Pb^{2+} on *Chlorella* sp. growth was cut off at 25–30 °C.

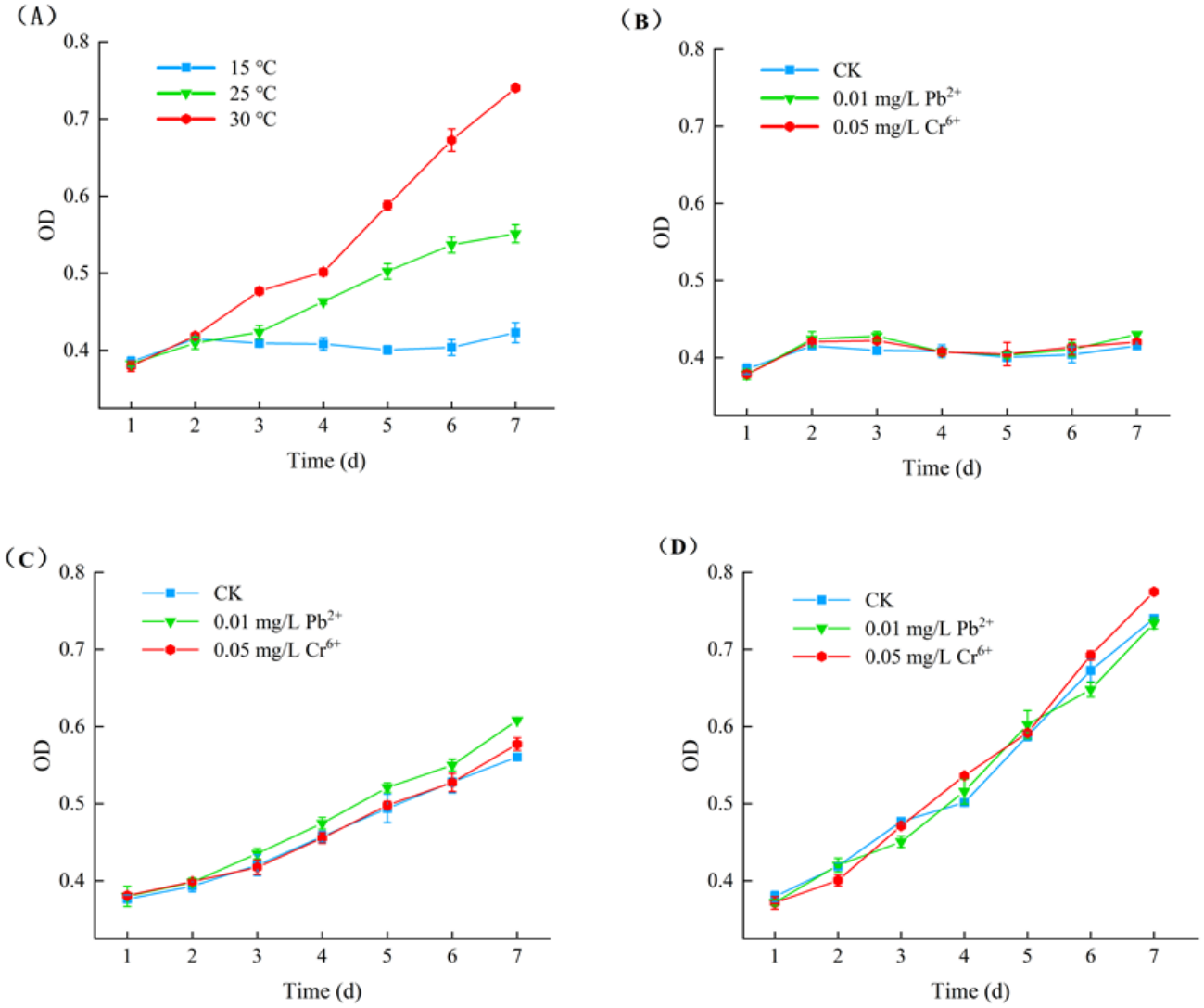

Figure 1. *Chlorella* sp. growth curve. (**A**) Change to algal cell biomass at 15 °C, 25 °C, and 30 °C over the 7-day incubation period in the absence of heavy metals. (**B**–**D**) Effect of the two heavy metals on algal cell biomass at 15 °C, 25 °C, and 30 °C over the 7-day incubation period. Error bars represent the three parallel relative standard deviations for each group of data.

3.1.2. *Chlorella* sp. Chlorophyll a

The chlorophyll a content in the *Chlorella* sp. cells generally increased under the dual influence of heavy metals and temperature (Figure 2). The chlorophyll a synthesis in *Chlorella* sp. differed significantly among the three temperatures ($p < 0.05$). The chlorophyll a content in the *Chlorella* sp. cells increased with increasing incubation temperature, with improved *Chlorella* sp. growth. The chlorophyll synthesis was at its lowest at 15 °C, demonstrating that this temperature was not optimal for *Chlorella* sp. growth. The highest chlorophyll a synthesis and best algal cell growth were recorded in the Pb^{2+} treated group at 25 °C. At 30 °C, the chlorophyll synthesis was significantly inhibited ($p < 0.05$) in the Pb^{2+}-treated group compared to that in the control group (inhibition rate: 26.7%). Thus, temperature and Pb^{2+} antagonistically affected chlorophyll synthesis in *Chlorella* sp. at 30 °C and negatively affected its growth. In contrast, the chlorophyll a was significantly promoted ($p < 0.05$) under the Cr^{6+} treatment, with concentrations being 1.42 times higher than those in the control group. This scenario generated the best *Chlorella* sp. growth among all of the treatment groups. The chlorophyll synthesis in *Chlorella* sp. was promoted

at 25 °C for 0.01 mg/L Pb^{2+} and at 30 °C for 0.05 mg/L Cr^{6+}, showing that temperature and heavy metals synergistically affect the growth of *Chlorella* sp.

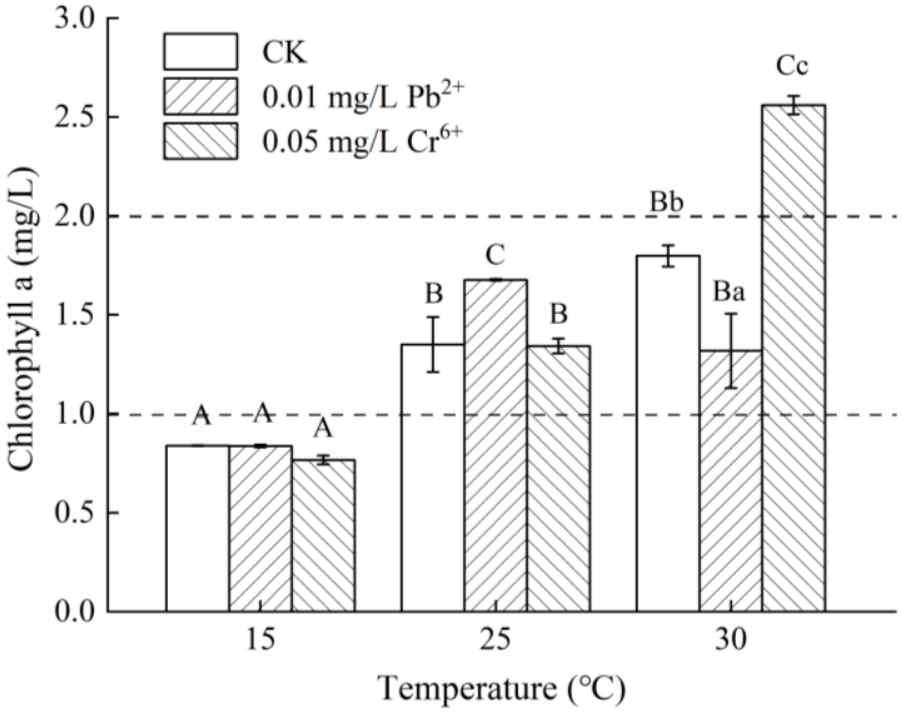

Figure 2. Chlorophyll a content of *Chlorella* sp. under dual-phase stress at the three temperatures and with the two heavy metals. Error bars represent the three parallel relative standard deviations for each group of data. Upper– and lower–case letters (e.g., A, a) at the top of columns represent significant differences within all groups and between groups, respectively, using Duncan's Multiple Test for Extreme Differences ($p < 0.05$).

3.2. Growth Mechanism of Chlorella sp.

3.2.1. Soluble Protein Content of *Chlorella* sp.

The soluble protein content in *Chlorella* sp. generally increased under the three temperatures and in the presence of the two heavy metals (Figure 3). The soluble protein content was the highest at 30 °C in the Cr^{6+} group (0.215 g/L), with soluble protein synthesis being promoted at higher incubation temperatures. The effect of temperature and heavy metals on the soluble protein content in *Chlorella* sp. was consistent with the observed effect on biomass and chlorophyll synthesis. This phenomenon was attributed to the protein content being closely correlated to photosynthesis. The increase in incubation temperature (Figure 2) promoted the synthesis of chlorophyll a, and increased the photosynthetic rate of the algal cells, which led to a significant increase in the amount of protein produced by photosynthesis in the algal cells. At 25 °C, 0.01 mg/L of Pb^{2+} significantly promoted the synthesis of soluble proteins in the algal cells ($p < 0.05$). At 30 °C, 0.05 mg/L of Cr^{6+} promoted the synthesis of soluble proteins in the algal cells. Low concentrations of heavy metals inhibited the synthesis of soluble proteins, and reduced the growth of *Chlorella* sp. at a certain temperature. Specifically, 0.01 mg/L Pb^{2+} significantly inhibited the synthesis of soluble proteins in algal cells at 30 °C ($p < 0.05$). This phenomenon might be attributed to the increased toxicity of Pb^{2+} to algal cells at 30 °C, and causing acute oxidative stress that impairs chlorophyll a synthesis and inhibits photosynthesis in *Chlorella* sp.

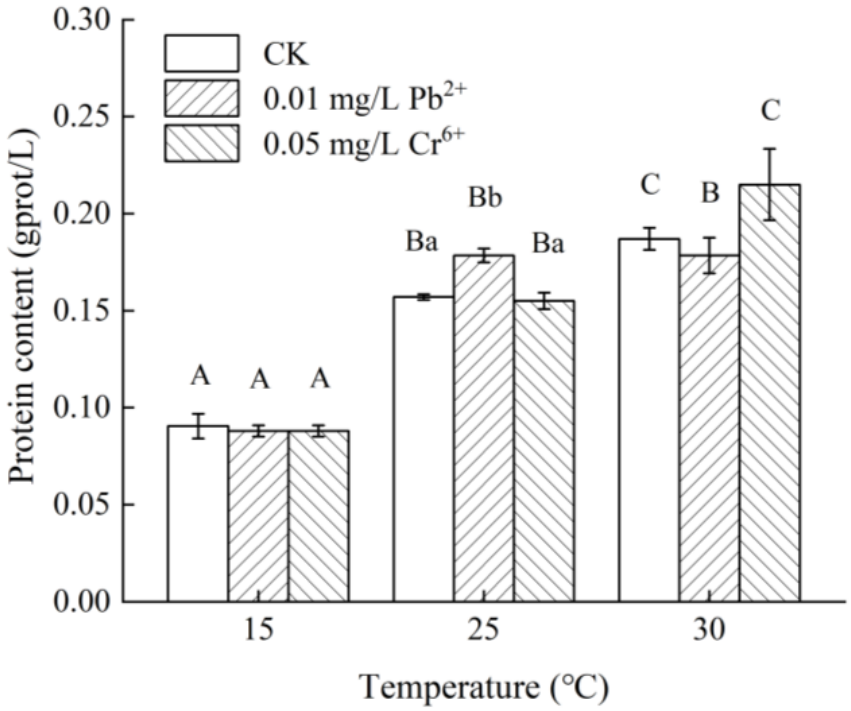

Figure 3. Soluble protein content in *Chlorella* sp. under the dual stress of temperature and heavy metals. See Figure 2 for details on statistics. Error bars represent the three parallel relative standard deviations for each group of data. Upper– and lower–case letters (e.g., A, a) at the top of columns represent significant differences within all groups and between groups, respectively, using Duncan's Multiple Test for Extreme Differences ($p < 0.05$).

3.2.2. *Chlorella* sp. MDA Content

The MDA content of *Chlorella* sp. generally increased under the dual stress of temperature and heavy metals (Figure 4). The oxidative damage to the algal cells was significant at 30 °C ($p < 0.05$) in the Pb^{2+}- and Cr^{6+} groups compared to that in the control. The degree of oxidative damage to the algal cells was highest in the Cr^{6+}- group at 30 °C, which also exhibited the highest soluble protein content (Figure 3). The faster growth of *Chlorella* sp. in this group might be attributed to the higher soluble protein content, which is required to repair oxidative damage to algal cells. Furthermore, Cr^{6+} produced less membrane lipid peroxidation damage than did Pb^{2+}, thus improving *Chlorella* sp. growth.

3.2.3. *Chlorella* sp. Antioxidant Enzymes

While the enzyme activity of the SOD of *Chlorella* sp. was variable for the two heavy metals at three temperatures, that of CAT clearly declined with increasing temperature (Figure 5). The SOD activity was significantly different at 30 °C ($p < 0.05$) compared to that at the other two incubation temperatures. This indicates that the raised temperature enhanced SOD activity in *Chlorella* sp. At 25 °C, the heavy metals did not stimulate the algal cells to produce large amounts of ROS, resulting in lower SOD activity compared to the control. Thus, temperature and heavy metals antagonistically affected damage to *Chlorella* sp. cells. The CAT activity in the control and heavy metal-treated groups clearly declined with increasing temperature. The CAT activity was the highest (91.08, 96.49, and 84.59 U/mgprot) at 15 °C. The CAT significantly differed ($p < 0.05$) between the two heavy metals at the same temperature, similar to that recorded for the SOD activity (Figure 5). For example, at 15 and 30 °C, Pb^{2+} caused the activity of both antioxidant enzymes to noticeably increase compared to that of the other experimental groups. Thus, the algal cells were likely more severely damaged in this group, leading to lower chlorophyll a content (Figure 2) and poorer growth.

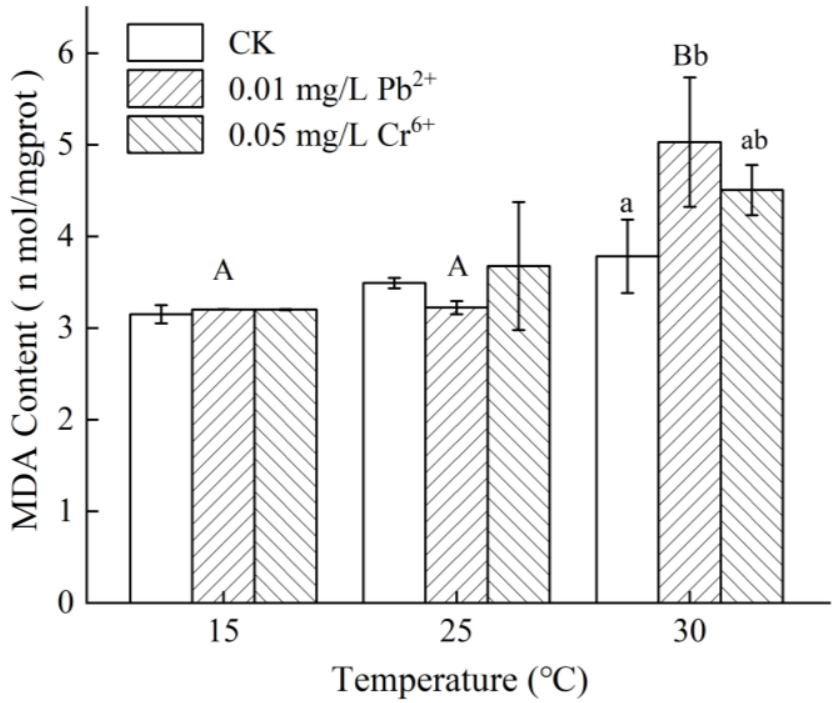

Figure 4. MDA content in *Chlorella* sp. under the dual stress of temperature and heavy metals. Error bars represent the three parallel relative standard deviations for each group of data. Upper– and lower–case letters (e.g., A, a) at the top of columns represent significant differences within all groups and between groups, respectively, using Duncan's Multiple Test for Extreme Differences ($p < 0.05$).

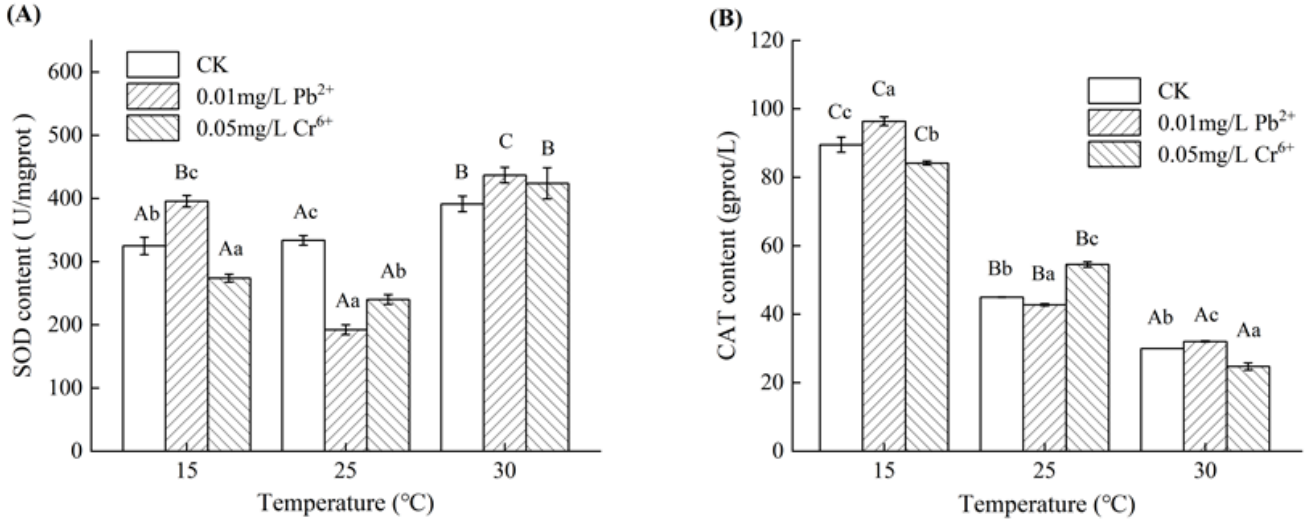

Figure 5. (**A**) SOD and (**B**) CAT content in *Chlorella* sp. under the dual stress of temperature and heavy metals. Error bars represent the three parallel relative standard deviations for each group of data. Upper– and lowercase-letters (e.g., A, a) at the top of columns represent significant differences within all groups and between groups, respectively, using Duncan's Multiple Test for Extreme Differences ($p < 0.05$).

4. Discussion

4.1. Effects of Temperature and Heavy Metals on Chlorella sp. Growth

Chlorophyll a is crucial for photosynthesis in *Chlorella* sp. algal cells, and is an important indicator of algal growth [24,25]. This study showed that *Chlorella* sp. grows slowly at 15 °C, with a low chlorophyll content. However, the number of *Chlorella* sp. cells increased with increasing temperature, demonstrating that temperature enhances the growth of *Chlorella* sp. cells. The density of the algal cells was organized as: 30 °C group > 25 °C group > 15 °C group, with 30 °C representing the optimum temperature for *Chlorella* sp. growth (Figure 1). Our results were generally consistent with those of previous studies. For instance, Zeng et al. also reported that *Chlorella* sp. growth was the fastest and chlorophyll a content was the highest at 30–40 °C, after incubating *Chlorella* sp. at 15, 20, 25, 30, 35, and 40 °C, with the slowest growth occurring at 15–20 °C. Zhang et al. also reported that *Chlorella* sp. growth increased from 15 to 35 °C, but that it was inhibited at 40 °C.

Of the common heavy metals, Cu and Zn are essential for the growth of many organisms, while Cr and Pb are not [22,24]. However, this study demonstrated that the growth pattern of *Chlorella* sp. was affected by the addition of Cr^{6+} and Pb^{2+}, which synergistically promoted *Chlorella* sp. growth at certain incubation temperatures. This phenomenon was attributed to the production of soluble proteases by algal cells under certain conditions. In particular, *Chlorella* sp. growth was enhanced by Pb^{2+} at 30 °C, being significantly higher compared to that in the control (Figure 1). This difference was attributed to chlorophyll organelles in the algal cells being damaged through the severe peroxidation of membrane lipids, inhibiting photosynthesis. Consequently, the produced protein enzymes were insufficient to counteract the damage caused by Pb^{2+} to algal cells, negatively impacting cell growth [26].

4.2. Physiological Characteristics of Chlorella sp. under Dual Stress of Temperature and Heavy Metals

Soluble proteins are crucial for maintaining normal physiological functions. Most soluble proteins in algal cells are enzymes involved in various types of algal metabolism. This process ensures normal water supply when plants are subjected to heavy metal stress, allowing normal cellular physiological functions to be maintained [27]. The soluble protein content in algal cells increased with increasing incubation temperature in our study, reflecting the growth trend of algal cells. Therefore, the content of soluble proteins is a good indicator of the physiological and biochemical responses of algae or plants [28,29]. However, both the growth and soluble protein content in *Chlorella* sp. of the Pb^{2+}-treated group at 25 °C and the Cr^{6+}-treated group at 30 °C was the highest compared to that in the other experimental groups (Figure 4). Du et al. [30] reported that *Nitzschia hantzschia* adapted to stressful environments by using its own soluble proteins and other organic matter; thus, soluble proteins likely enhanced the growth of *Chlorella* sp.

The current study showed that the MDA content and SOD activity increased with increasing temperature, whereas CAT activity decreased. Thus, damage to algal cell membranes likely increases with increasing temperature, with membrane lipid peroxidation in algal cells being weaker at 15 °C. This is likely because 15 °C is within the temperature range for the growth of *Chlorella* sp., with soluble sugars in algal cells exhibiting a protective role in low-temperature stress [31]. The CAT activity decreased significantly ($p < 0.05$) with increasing temperature, possibly because it accelerated the decomposition of H_2O_2; however, further confirmation is required on this aspect. The ROS produced by plants exposed to heavy metal stress trigger or exacerbate lipid peroxidation in membranes. MDA is a product of membrane lipid peroxidation in plants, and is often used as an indicator of oxidative damage, degree of membrane lipid peroxidation in cells, and the strength of the plant's response to adverse conditions [32]. A higher MDA content indicates a higher degree of oxidation in *Chlorella* sp. cell membranes, and poorer growth. Plants exposed to heavy metal stress develop several physiological defense mechanisms to mitigate damage (Figure 6). Antioxidant enzymes are crucial for the scavenging of ROS produced in response

to the presence of heavy metals. SOD catalyzes the decomposition of superoxide anions to produce H_2O and H_2O_2, whereas CAT synergistically scavenges H_2O_2 and other peroxides [33,34]. To counteract this effect, plants produce antioxidants (e.g., SOD, CAT) and non-enzymatic antioxidants. When *Chlorella* sp. was incubated at 25 °C, the Pb^{2+}-treated group exhibited a lower MDA content and, therefore, lower antioxidant enzyme activity in algal cells than that in the control and Cr^{6+}-treated groups. Thus, this temperature and Pb^{2+} likely had an antagonistic effect on the MDA synthesis of *Chlorella* sp., thus promoting growth. Our study showed that the Pb^{2+}-treated group exhibited the best growth at 25 °C (Figure 1). When *Chlorella* sp. was incubated at 30 °C, the MDA content significantly increased in the Pb^{2+} and Cr^{6+} treatments compared to that in the control. Thus, temperature and heavy metals appear to synergistically affect the MDA synthesis of *Chlorella* sp. under this condition, with a certain degree of membrane lipid peroxidation occurring in cells, which compromised *Chlorella* sp. growth. In contrast, Cr^{6+} promoted the production of a large amount of soluble protein, which alleviated the damage to the algal cells, promoting the growth of *Chlorella* sp. at 30 °C (Figure 3). Therefore, *Chlorella* sp. growth was optimal in the presence of Cr^{6+} at 30 °C.

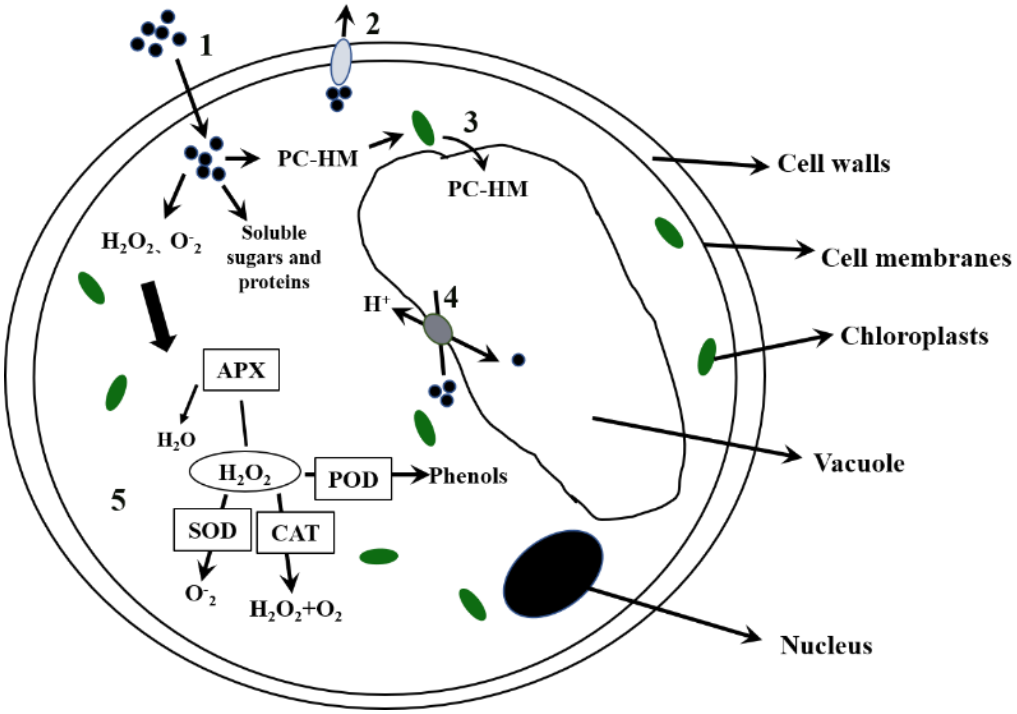

Figure 6. Map of heavy metal tolerance mechanisms in *Chlorella* sp. e.g., 1: Reduction of metal influx across the plasma membrane. 2: Metal chelation in the cytosol by ligands such as phytochelatins, metallothionein, organic acids, and amino acids. 3: Transport of metal−ligand complexes through the tonoplast and accumulation in the vacuole. 4: Sequestration in the vacuole by tonoplast transporters. 5: ROS defense mechanisms. Black dots: metal ions.

5. Conclusions

This study demonstrated the combined effects of three incubation temperatures (15, 25, and 30 °C) and two heavy metals at low concentrations (0.01 mg/L Pb^{2+} and 0.05 mg/L Cr^{6+}) on the growth of *Chlorella* sp. The results indicated 30 °C to be the optimum temperature for *Chlorella* sp. growth. Further, *Chlorella* sp. growth significantly improved with increasing temperature ($p < 0.05$). This correlation was attributed to the increased synthesis of soluble proteins at high temperatures, which protected *Chlorella* sp. and promoted the growth. The higher temperature might have also caused the decomposition of H_2O_2 and other hydroperoxides, reducing the risk of oxidation in *Chlorella* sp. cells; however, this suggestion needs further evaluation for confirmation. Both the heavy metals synergistically

affected the growth of *Chlorella* sp. at certain incubation temperatures (25 °C for 0.01 mg/L Pb^{2+} and 30 °C for 0.05 mg/L Cr^{6+}), with 25–30 and 30 °C being optimal temperatures for *Chlorella* sp. growth in the presence of Pb^{2+} and Cr^{6+}, respectively. This relationship was attributed to the antagonistic effect of temperature and Pb on MDA synthesis in *Chlorella* sp., and the effect of Cr at 30 °C. Specifically, Cr stimulated algal cells to produce large amounts of soluble protein to protect the organism, thus promoting growth. Thus, temperature and heavy metals have the potential to aggravate the eutrophication of water bodies. This study provides an evidence base towards mitigating and preventing algal bloom. It also provides a theoretical basis for preventing and controlling eutrophication and water pollution in the Three Gorges reservoir area, and other similar regions globally.

Author Contributions: H.Z.: conceptualization. L.H.: methodology. Q.L.: formal analysis. M.Y.: methodology, formal analysis. X.Y.: methodology, formal analysis. Z.Z.: methodology. J.W.: writing—original draft. A.Z.: conceptualization, project administration. B.Y.: supervision, writing—review and editing, project administration. H.W.: writing—review and editing. C.F.: resources, funding acquisition, project administration. Y.W.: supervision, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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