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The Detrimental Effect of High Salinity on the Growth and Microcystins Contamination of *Microcystis aeruginosa*

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Abstract: The occurrence of cyanobacterial species, especially toxic ones, poses a great threat to coastal and estuary areas. In this study, the toxigenic *Microcystis aeruginosa* (*M. aeruginosa*) FACHB-905 was exposed to BG-11 medium with different salinities (1, 4, 7, 10 and 15 ppt) to investigate the physiological responses of this species in terms of oxidative stress, chl *a* fluorescence and microcystins (MCs) contamination. The results showed that low salinity (≤7 ppt) favored the electron transfer of photosystem II, which promoted the growth and photosynthesis of *M. aeruginosa* and induced MCs production. However, increased salinity (≥10 ppt) suppressed the growth and photosynthesis of *M. aeruginosa* and aggravated the oxidative stress of the strain. Salinity of 15 ppt reduced MCs contamination and caused irreversible damage to the photosynthetic system of *M. aeruginosa*, leading to the lysis and death of algal cells. These results indicated that changes in salinity exerted important regulations on the growth and MCs contamination of the toxic *M. aeruginosa*, which may provide a reference for the risk assessment of the harmful cyanobacterial species in the coastal and estuary areas.

Keywords: salinity; *Microcystis aeruginosa*; photosynthesis; microcystins; oxidative stress

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1. Introduction

Global climate change affects precipitation, sea level and many other factors, leading to seawater intrusion, making salinity one of the most important issues in coastal water resource management [1,2]. The continuing increase in the salinity of water bodies has affected aquatic organisms to various extents [3,4]. Previous studies have shown that some freshwater algae can migrate through rivers or artificial canals to brackish water [5]. Recently, the worldwide distributed freshwater algae *Microcystis* has been reported in many low salinity areas, such as the Swan River in Australia [6], San Francisco Estuary in North America [7], St. Johns River in Florida [3] and Río de la Plata Estuary in South America [8], indicating that the species has a certain tolerance to salinity. As reported by Li et al. [5], *Microcystis aeruginosa* (*M. aeruginosa*) could grow in environments with low to medium salinity (5~18 ppt). Additionally, low nitrogen availability in the environment might increase the salinity tolerance threshold of the genus, which is beneficial to its survival and the outbreak of water blooms in estuaries.

Elevated salt concentrations can cause the leakage of algal cells and accelerate the excretion of cellular contents. Thus, the emergence of toxic strains, such as *Microcystis*, poses a serious threat to the management of affected coastal environments. Generally, the occurrence of *Microcystis* blooms is often accompanied by highly prevalent hepatotoxic microcystins (MCs), which can cause multiple organ toxicity, genotoxicity, neurotoxicity, immunotoxicity and potential carcinogenicity. Exposure to MCs can cause liver failure in wild

animals, livestock and aquatic animals and even death [9–11]. Nowadays, although there is an increasing focus on harmful *Microcystis* blooms in brackish water, studies on the effect of salinity on toxigenic characteristics of *Microcystis* have no consistent conclusions [3,12]. Some researchers have pointed out that elevated salinity induced the production and release of MCs, leading to the increase in both intracellular MCs (IMCs) and extracellular MCs (EMCs) content [13–15]. However, other studies proved that elevated salinity reduced MCs production but promoted the release of MCs, enhancing the risk of aquatic organisms exposed to high concentrations of EMCs [16,17].

Microalgae typically adapt to environmental change through a combination of biochemical pathways, e.g., expelling ions, synthesizing penetrants and adjusting antioxidant defense systems [18]. As reported, high salinity can cause oxidative stress by the generation of reactive oxygen species (ROS), commonly including superoxide anions ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl free radicals ($\cdot OH^-$). ROS contain unpaired electrons and have high chemical reactivity that can cause serious damage to growth factors, transcription factors, proteins, nucleic acids, carbohydrates and lipids [3,19,20], which in turn causes peroxidation of membrane lipids, formation of lipid peroxidation products, such as malondialdehyde (MDA) and 4-hydroxynonenal, and changes to the fluidity and permeability of cell membranes. Ross et al. [3] found that redox homeostasis in *M. aeruginosa* cells was disrupted after salinity surpassed 7 ppt, leading to the increase in the percentage of ROS-positive *M. aeruginosa* cells and the content of H_2O_2. Microalgae protect themselves against oxidative damage by activating enzymatic or non-enzymatic antioxidant systems in vivo [21]. Superoxide dismutase (SOD), the first line of defense in the antioxidant system, can specifically eliminate superoxide radicals ($O_2^{-\cdot}$), causing disproportionation reactions to generate O_2 and H_2O_2. Glutathione peroxidase (GPx) and catalase (CAT) represent the second line of defense in antioxidant systems. They continue to decompose H_2O_2 or other hydroperoxides into nontoxic substances and prevent peroxidation from maintaining the oxygen balance of the intracellular environment. Thus, these biomarkers (e.g., SOD, GPx, CAT and MDA) are useful for describing salinity-induced oxidative damage to microalgae.

For algae, at high salinity levels, Na^+ competes with Ca^{2+} for the binding sites of cell walls, reduces K^+ levels, disturbs cellular ion homeostasis and affects protein synthesis and photosynthesis in algal cells [22]. During photosynthesis of algae, the energy absorbed by antenna chlorophyll (chl) is converted into chemical energy through the electron transfer of photosystem II (PSII) and photosystem I (PSI), and the remaining energy is dissipated in the form of heat and fluorescence [23]. After dark-adapted algae are suddenly exposed to visible light, the algae cells emit a dark red fluorescence with varying intensity. The curve describing fluorescence change with time is known as the chl *a* fluorescence rise kinetics curve (OJIP curve), also known as the Kautsky curve [23]. The response of the OJIP curve to different environments determines morphological changes, which contain a large amount of information about the original chemical reaction of PSII [24,25]. Thus, the OJIP curve can be applied as a powerful tool for studying the photosynthesis of algae under salinity stress.

As *Microcystis* presents various physiological responses to the change in salinity, we hypothesized that salinity stress has an important regulatory effect on the toxigenic and photosynthetic ability of the species. To test the hypothesis, an axenic *M. aeruginosa* strain was selected as a model organism in this study. The responses of *M. aeruginosa* to increased salinity were revealed from the aspects of growth status, chl *a* fluorescence, antioxidant activities and MCs production.

2. Materials and Methods

2.1. Culture Conditions and Experimental Design

M. aeruginosa FACHB-905 (hereafter *M. aeruginosa*) was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). The unicellular strain was cultured in 1 L Erlenmeyer flasks with BG-11 medium under constant conditions (25 °C, illumination of 50 μmol/(m^2·s) on 12:12 h

light/dark cycle). Cultures were shaken three times daily and rearranged randomly to reduce disturbances caused by the uneven light intensity in the incubator.

To evaluate the effects of elevated salinity on *M. aeruginosa*, pre-cultures in the exponential phase were harvested by centrifugation (D1524R, DLAB Scientific Inc., Beijing, China) (8000 rpm, 10 min, 4 °C) and then inoculated in 600 mL of BG-11 medium with different salinity for 9 days. The salinities of the treatments were adjusted to 1 (control, standard BG-11 medium), 4, 7, 10 and 15 ppt by sterilized NaCl solution. The initial cell density was 5×10^6 cells/mL, and the culture conditions were the same as described above. All experiments were conducted in triplicate. Cell density and the maximum quantum efficiency of photosystem II (F_v/F_m) were analyzed daily, and the salinity changes in culture systems were monitored at the same time. The chl *a* fluorescence transient, concentrations of EMCs and IMCs, MDA content and enzyme activities of SOD, CAT and GPx were analyzed every three days. The culture volume and sample amount for each analysis are shown in Figure 1.

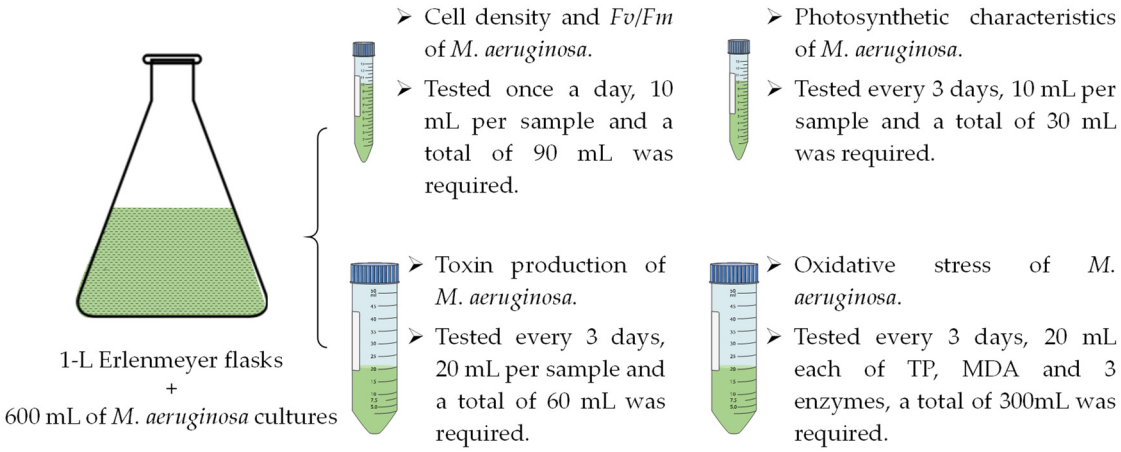

Figure 1. Culture volume for Erlenmeyer flasks and sample amount for each analysis.

2.2. Analysis Methods

2.2.1. Salinity and Cell Density

The salinity in the culture system was detected by a portable conductivity meter (DDB-303A, INESA Scientific Instrument, Co., Ltd., Shanghai, China). The cultures were analyzed by full-wavelength scanning (190–1100 nm) using an ultraviolet-visible spectrophotometer (DR 6000, HACH, Loveland, CO, USA), presenting a maximum absorption at 680 nm wavelength. Algal suspensions with different cell density gradients were prepared to detect optical density at 680 nm wavelength (OD680). Then, the calibration curve between cell density and OD680 of *M. aeruginosa* was established. The OD680 (x) of the cultures was measured daily to calculate the cell density (y) using the equation $y = 4 \times 10^7 x + 85{,}045$ ($R^2 = 0.9998$). At the end of the experiment, the inhibition rate (μ) of cells grown under elevated salinity treatments was calculated by the equation $\mu\ (\%) = (1 - y_2/y_1) \times 100\%$, where y_2 and y_1 were the cell density of the treatment group and control group, respectively.

2.2.2. F_v/F_m and the Chlorophyll a Fluorescence Transient Analysis

The F_v/F_m and chl *a* fluorescence transient were measured by a hand-held AquaPen (AP 100-C, Photon Systems Instruments, Drásov, Czech Republic). Cultures samples (5 mL) were kept in darkness for 10 min at room temperature to allow all reaction centers to open. Additionally, the samples were transferred to a cuvette and shaken thoroughly to avoid the precipitation and (or) floating of cells. Red LEDs (630 nm) were used as measuring, actinic and saturation lights. A saturation pulse (3000 μmol/m^2·s, 500 ms) was applied to determine F_v/F_m. After relaxation for 30 s in the dark, the fast fluorescence transient (OJIP curve) under actinic light (200 μmol/m^2·s, 2 s) was measured.

The OJIP curve is composed of a series of phases as follows. The minimum fluorescence signal is found at point O, corresponding to the minimum fluorescence intensity, F_o, which reflects the fluorescence yield of plants under dark adaptation conditions when they are completely photochemically quenched. The peak fluorescence signal is found at point P (400 ms), the maximum fluorescence yield when PSII is completely closed. In the electron transfer process, point J (2 ms) is generated by the rapid increase in fluorescence due to the large accumulation of primary quinone quinone-type electron acceptor (Q_A), whereas point I (30 ms) appears in the process of electron transfer from Q_A to Q_B (the secondary quinone-type electron acceptor) [26].

According to our previous study [27], the parameters V_J, M_0, φ_{Po}, ψ_o and φ_{Eo} were selected for J-test analysis. V_J refers to the relative variable fluorescence intensity in the J step, and M_0 corresponds to the initial slope of the OJIP curve. The parameters φ_{Po}, ψ_o and φ_{Eo} are involved in the quantum efficiencies of the electron transport chain (ETC), wherein φ_{Po} represents the maximum quantum yield of primary photochemistry, ψ_o represents the efficiency that a trapped exciton can move an electron into the ETC beyond the primary acceptor plastoquinones and φ_{Eo} represents the quantum yield of electron transport. Furthermore, several parameters, including ABS/RC (energy fluxes ratio), TR_o/RC (trapped energy fluxes ratio), ET_o/RC (electron transport flux) and DI_o/RC (dissipated energy flux ratio per reaction center), involved in the specific energy flux ratios in the electron transport chain, were also analyzed.

2.2.3. Microcystins Quantification

Culture samples (5–10 mL) were harvested by centrifugation at 8000 rpm for 10 min. The supernatants and sediments were used to determine the concentrations of EMCs and IMCs, respectively. The total MCs (TMCs) were the sum of IMCs and EMCs. In order to extract IMCs, the sediments were re-suspended with 5 mL of double-distilled water. The cell suspension was firstly lysed by the freeze–thaw method combined with ultrasonication and then centrifuged at 10,000 rpm for 10 min to obtain the supernatants [14]. Both EMCs and IMCs were detected using an enzyme-linked immunosorbent assay (ELISA) kit (Beacon Analytical Systems Inc., Saco, ME, USA) according to the manufacturer's instructions, tested on a microplate reader at 450 nm (Multiskan FC, Thermo Scientific, Waltham, MA, USA). The coefficient of the calibration curve was higher than 0.99, and the lower detection limit was set to 0.1 μg/L. The recovery of the spiked samples was 93.2 ± 3.5% (n = 5).

2.2.4. Antioxidant Enzyme Activity and Malondialdehyde Content

To evaluate oxidative damage of *M. aeruginosa* under elevated salinity, the enzyme activity of SOD, CAT and GPx, coupled with MDA content, were estimated. The detection of every biomarker needed to harvest 20 mL culture samples by centrifugation at 8000 rpm for 10 min, at 4 °C. Then, SOD (U/mg protein) and MDA (nmol/g) were assayed using corresponding bioassay kits provided by Nanjing Jiancheng Bioengineering Institute, China. CAT (U/mg protein) and GPx (U/mg protein) were assayed using corresponding bioassay kits provided by Beyotime Institute of Biotechnology, China. All the operations and data processing were strictly in accordance with the manufacturer's instructions.

2.3. Statistical Analysis

Statistical analysis was performed using SPSS 16.0 data for Windows (SPSS Inc., Chicago, IL, USA). The cell density, F_v/F_m, chl *a* fluorescence parameter, concentrations of IMCs and EMCs, coupled with the contents of the biomarkers among different salinities treatments, were tested by one-way analysis of variance (ANOVA) with the LSD test. The significance level was set at $p < 0.05$.

3. Results

3.1. Cell Density and F_v/F_m of M. aeruginosa under Salinity Stress

During the study, the salinities of every treatment remained stable (Figure A1). The cell density and F_v/F_m of *M. aeruginosa* under different salinity gradients were monitored, and the inhibition rate of salinity on cell growth was calculated using 1 ppt as the control (Figure 2). In terms of cell density, the exponential growth phase of *M. aeruginosa* at 1, 4 and 7 ppt started from day 3, while that of *M. aeruginosa* at 10 ppt was delayed until day 7. Compared to the control (1 ppt), *M. aeruginosa* under both 4 and 7 ppt treatments presented significantly higher cell densities from day 3 (ANOVA, $p < 0.05$), with increasing rates of 36.4% and 63.9%, respectively, at the end of cultivation. In the 10 ppt treatment, cell density was significantly lower than those of the control from day 8 (ANOVA, $p < 0.05$), presenting an inhibition rate of 36.0% at the end of cultivation. F_v/F_m at 10 ppt showed an upward trend after the first 2 days and was significantly lower than that at 1, 4 and 7 ppt from day 2 (ANOVA, $p < 0.05$). In contrast, cell density at 15 ppt began to decrease from day 2, and F_v/F_m decreased rapidly below 0.2. At the end of cultivation, a salinity of 15 ppt inhibited cell growth by 76.5% compared to the control.

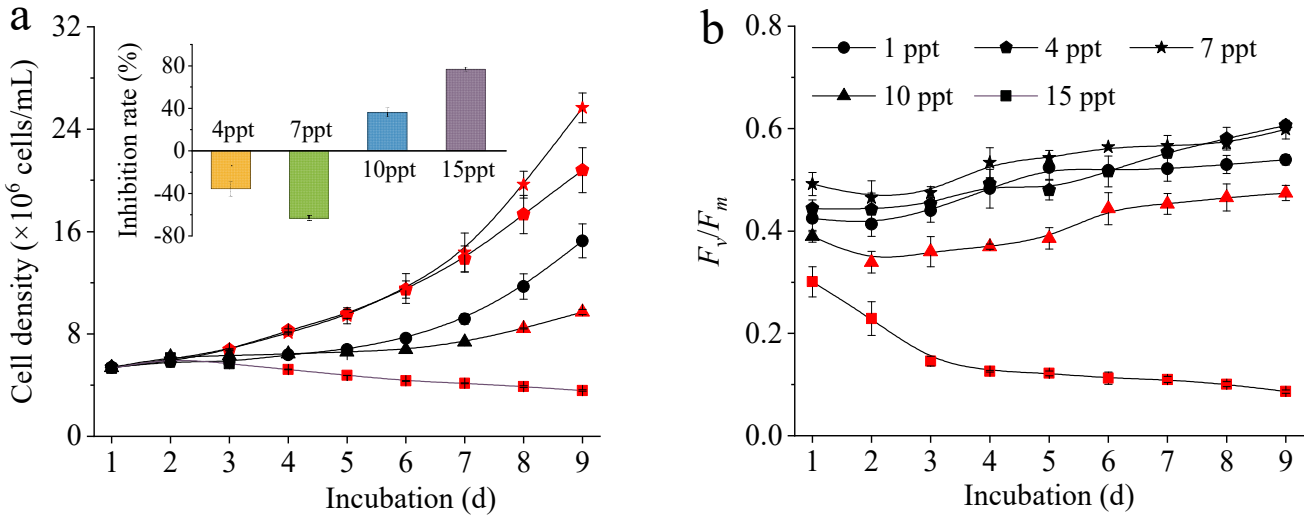

Figure 2. Changes in *M. aeruginosa* cell density and the inhibition rate of cell growth (**a**) and F_v/F_m (**b**) during the experiment under different salinity stresses. Red-filled legends indicate significant differences compared to the control (ANOVA, $p < 0.05$).

3.2. Photosynthetic Characteristics of M. aeruginosa under Salinity Stress

The OJIP curves of *M. aeruginosa* under different salinity gradients recorded every 3 days are shown in Figure 3. In general, the curve at 15 ppt was in lower positions than those at the 1, 4, 7 and 10 ppt salinity gradients. On days 3, 6 and 9, the curve at 15 ppt decreased continuously with the J-I phase leveling off, and no fluorescence increase was observed in the J-P phase. Furthermore, the OJIP curves at 1, 4, 7 and 10 ppt changed in the same pattern with time. The highest chl *a* fluorescence was found in the treatment of 7 ppt, followed by order of 4, 1 and 10 ppt.

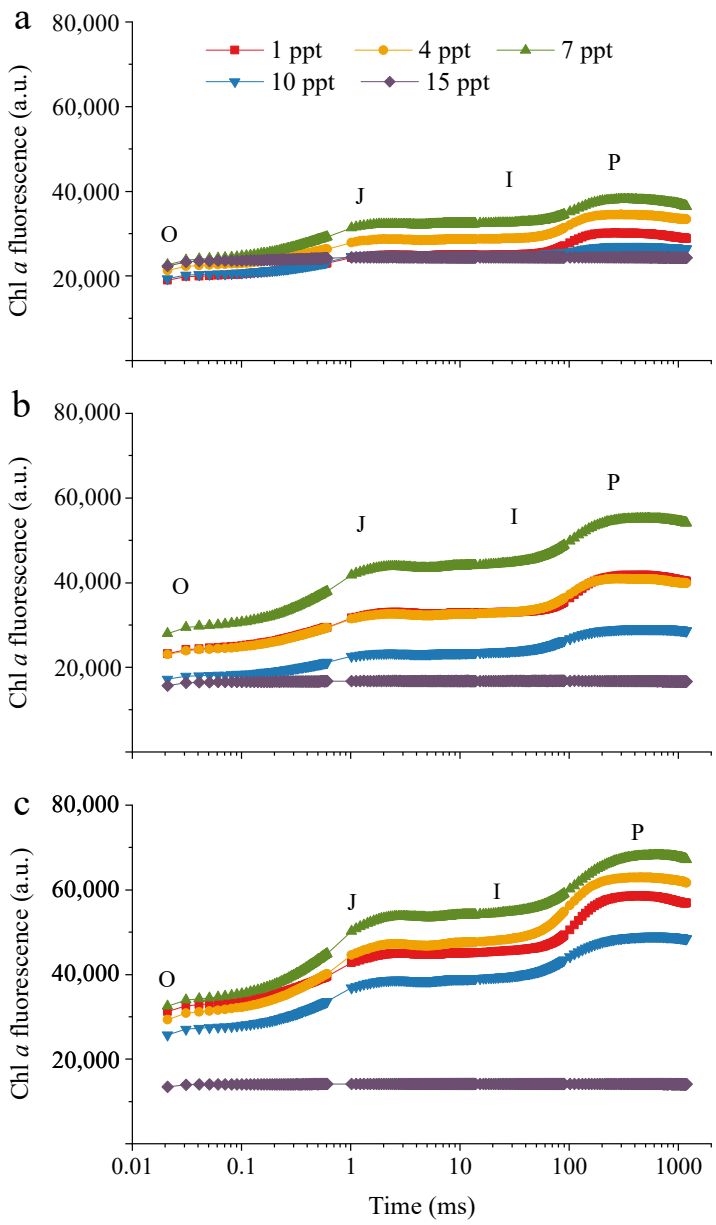

Figure 3. The OJIP curves of *M. aeruginosa* under different salinity stress on days 3 (**a**), 6 (**b**) and 9 (**c**).

Changes in the chl *a* fluorescence parameters of *M. aeruginosa* recorded every 3 days are shown in Table 1. A relatively high salinity of 15 ppt seriously damaged the PSII reaction center of *M. aeruginosa*, resulting in irregular, abnormal values of the chl *a* fluorescence parameters compared to the control (ANOVA, $p < 0.05$) and other treatments. Under the 1, 4, 7 and 10 ppt salinity, the V_J and M_0 decreased gradually as the experiment progressed, except that the V_J at 7 and 10 ppt presented higher values on day 9 than those on day 6. In contrast, the parameters φ_{Po} and φ_{Eo} increased gradually over time, and both presented the minimum and maximum values on days 3 and 9, respectively, at 10 ppt treatments. Additionally, other than the 10 ppt salinity, the parameter ψ_0 increased gradually with the prolongation of incubation, while it declined as salinity increased at the same time point. The ABS/RC, TR_0/RC, ET_0/RC and DI_0/RC in the 1, 4, 7 and 10 ppt salinity groups showed declining tendencies with fluctuation over time and as the salinity gradient increased. The indicators at 15 ppt showed no obvious trend and included outliers. Indeed, measurements could not be taken in the middle and late stages of the experiment.

Table 1. Chlorophyll *a* fluorescence parameters of *M. aeruginosa* under different salinity stresses on days 3, 6 and 9. Bold letters marked with '*' indicated significant differences compared to the control (ANOVA, $p < 0.05$); "N.D." indicated not detected.

Parameter	Time (d)	1 ppt	4 ppt	7 ppt	10 ppt	15 ppt
V_J	3	0.495 ± 0.010	0.519 ± 0.017	**0.589 ± 0.011** *	**0.735 ± 0.035** *	**1.025 ± 0.122** *
	6	0.489 ± 0.007	0.507 ± 0.000	**0.552 ± 0.011** *	0.496 ± 0.028	**0.988 ± 0.016** *
	9	0.460 ± 0.008	**0.498 ± 0.009** *	**0.563 ± 0.009** *	**0.518 ± 0.010** *	**−0.061 ± 0.892** *
M_o	3	0.696 ± 0.045	0.725 ± 0.038	**0.792 ± 0.008** *	**0.940 ± 0.090** *	**2.111 ± 0.343** *
	6	0.659 ± 0.015	0.711 ± 0.018	0.672 ± 0.005	0.621 ± 0.012	**2.939 ± 0.876** *
	9	0.576 ± 0.013	0.605 ± 0.009	**0.655 ± 0.019** *	0.619 ± 0.034	**−0.463 ± 1.916** *
φ_{Po}	3	0.340 ± 0.022	0.352 ± 0.013	0.374 ± 0.013	0.306 ± 0.005	**0.046 ± 0.036** *
	6	0.418 ± 0.006	0.401 ± 0.014	**0.464 ± 0.003** *	0.443 ± 0.050	**0.012 ± 0.005** *
	9	0.4390.010	**0.506 ± 0.008** *	**0.498 ± 0.018** *	**0.520 ± 0.004** *	**0.011 ± 0.003** *
ψ_o	3	0.521 ± 0.034	**0.481 ± 0.017** *	**0.411 ± 0.011** *	**0.331 ± 0.013** *	**0.023 ± 0.002** *
	6	0.511 ± 0.007	0.493 ± 0.000	**0.448 ± 0.011** *	0.504 ± 0.028	**0.009 ± 0.002** *
	9	0.540 ± 0.008	**0.502 ± 0.009** *	**0.437 ± 0.009** *	**0.482 ± 0.010** *	**1.061 ± 0.892** *
φ_{Eo}	3	0.177 ± 0.019	0.169 ± 0.012	0.154 ± 0.003	**0.104 ± 0.002** *	**0.033 ± 0.024** *
	6	0.214 ± 0.003	0.198 ± 0.007	0.208 ± 0.004	**0.245 ± 0.008** *	**0.001 ± 0.000** *
	9	0.238 ± 0.009	0.254 ± 0.005	0.218 ± 0.010	**0.258 ± 0.009** *	**0.012 ± 0.012** *
ABS/RC	3	4.313 ± 0.425	3.968 ± 0.231	**3.604 ± 0.215** *	**4.742 ± 0.313** *	**16.721 ± 2.611** *
	6	3.216 ± 0.013	3.245 ± 0.165	**2.627 ± 0.077** *	**2.721 ± 0.050** *	N.D.
	9	2.856 ± 0.082	**2.397 ± 0.084** *	**2.335 ± 0.116** *	**2.164 ± 0.068** *	N.D.
TR_0/RC	3	1.456 ± 0.057	1.395 ± 0.029	**1.345 ± 0.039** *	**1.341 ± 0.021** *	**2.050 ± 0.097** *
	6	1.346 ± 0.016	1.408 ± 0.027	**1.219 ± 0.029** *	**1.279 ± 0.009** *	**1.974 ± 0.036** *
	9	1.254 ± 0.016	1.214 ± 0.024	**1.162 ± 0.017** *	1.195 ± 0.55	**0.006 ± 0.006** *
ET_0/RC	3	0.760 ± 0.068	**0.670 ± 0.015** *	**0.553 ± 0.031** *	**0.435 ± 0.023** *	**0.203 ± 0.162** *
	6	0.687 ± 0.008	0.709 ± 0.012	**0.547 ± 0.027** *	0.692 ± 0.026	N.D.
	9	0.678 ± 0.012	**0.609 ± 0.022** *	**0.507 ± 0.008** *	**0.576 ± 0.026** *	**0.448 ± 0.633** *
DI_0/RC	3	2.857 ± 0.379	2.573 ± 0.204	**2.259 ± 0.179** *	3.034 ± 0.117	**64.672 ± 5.485** *
	6	1.870 ± 0.025	1.866 ± 0.158	**1.408 ± 0.049** *	**1.342 ± 0.086** *	N.D.
	9	1.602 ± 0.074	**1.184 ± 0.060** *	**1.173 ± 0.101** *	1.302 ± 0.351	N.D.

3.3. Toxin Production of M. aeruginosa under Salinity Stress

The total IMCs at 1, 4 and 7 ppt increased significantly as the experiment progressed (ANOVA, $p < 0.05$, Figure 4a), whereas they remained stable at 10 ppt and presented a statistically decreasing tendency at 15 ppt (ANOVA, $p < 0.05$). Additionally, there were no significant differences in total IMCs concentrations between the treatments of 7 and 10 ppt on days 3 and 6 (ANOVA, $p < 0.05$). However, on day 9, the total IMCs at 7 ppt peaked at 649.59 μg/L and were significantly higher than that of the 10 ppt (ANOVA, $p < 0.05$). As shown in Figure 4b, the IMCs per cell only increased significantly over time at 1 ppt (ANOVA, $p < 0.05$), while they remained stable at 4 and 7 ppt. For the treatments of 10 and 15 ppt, the IMCs per cell decreased significantly from day 6 and day 3, respectively (ANOVA, $p < 0.05$). The total EMCs at 1, 4 and 7 ppt exhibited similar variations with the total IMCs over time, peaking at 19.51, 18.17 and 18.03 μg/L, respectively, on day 9 (Figure 4c). However, the total EMCs at 10 and 15 ppt sharply decreased from 5.85 and 8.65 μg/L to 0.43 and 0.05 μg/L, respectively, from day 3 to day 6, and maintained stability in the later experimental stages. Due to the low concentration of total EMCs, the TMCs were dominated by total IMCs. Thus, the changes and statistical differences of the TMCs under different salinities were consistent with the total IMCs (Figure 4d).

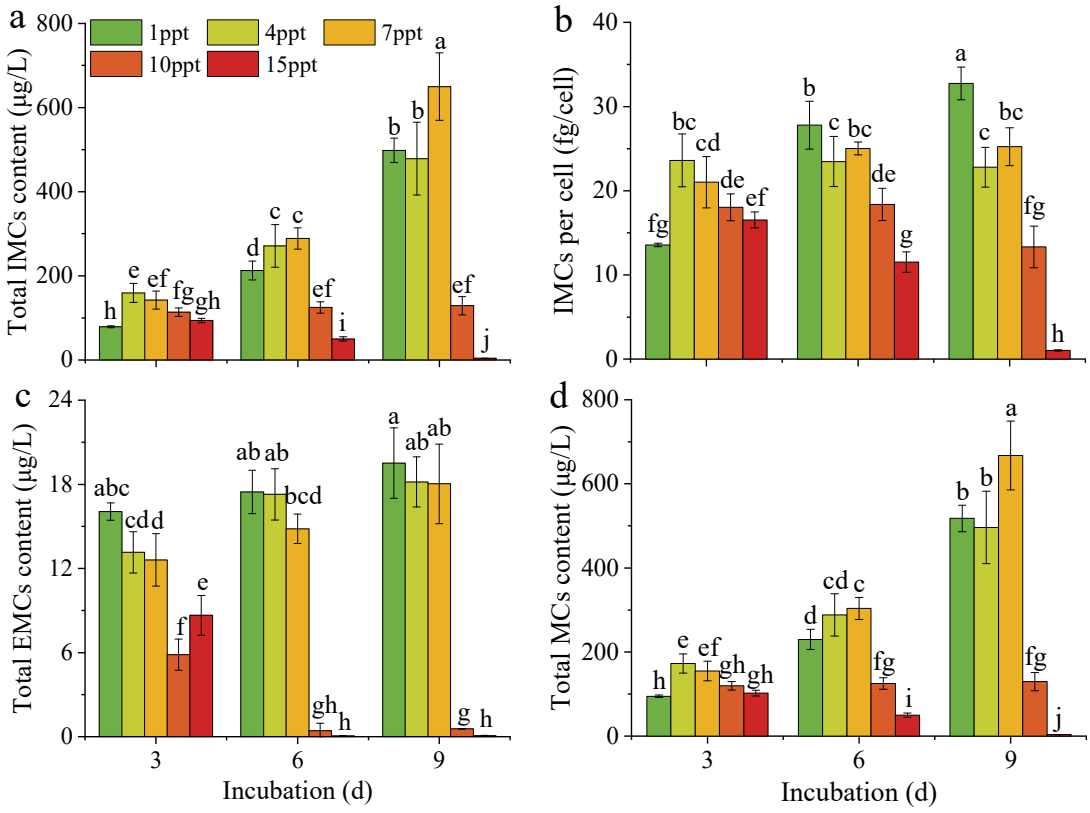

Figure 4. Contents of total intracellular MCs (**a**), intracellular MCs per cell (**b**), total extracellular MCs (**c**) and total MCs (**d**) under different salinity stresses on days 3, 6 and 9. Different letters a–j in the histogram indicate significant differences (ANOVA, $p < 0.05$).

3.4. Oxidative Stress of M. aeruginosa under Salinity Stress

The MDA contents of *M. aeruginosa* at 1, 4 and 10 ppt peaked on day 6, while that of the 7 ppt attained its maximum on day 9 (Figure 5a). While the MDA content at 15 ppt was significantly higher than the control group in the first 6 days (ANOVA, $p < 0.05$), it sharply decreased to the minimum of 0.18 nmol/g on day 9. The SOD activity of *M. aeruginosa* at the 1, 4, 7 and 10 ppt salinity gradients increased and then decreased, unlike that at 15 ppt, which peaked at 290.11 U/mg protein on day 6 (Figure 5b). SOD activity at 4 ppt was significantly lower than that at 1 ppt, whereas the SOD activities at 10 and 15 ppt were significantly higher than those at 1, 4 and 7 ppt in the late stage of the experiment (ANOVA, $p < 0.05$).

The CAT activities of *M. aeruginosa* at the higher salinity gradients were significantly higher than those at 1 ppt on day 3 (Figure 5c). Additionally, CAT activities at 4 and 7 ppt remained higher than those at 1 ppt on days 6 and 9, although the differences were not significant (ANOVA, $p > 0.05$). CAT activity at 10 ppt salinity was maintained at a certain level as the experiment progressed, whereas that at 15 ppt salinity was much higher than that at the other salinity groups. The GPx activities of *M. aeruginosa* at the 1, 4 and 7 ppt salinity gradients were largely flat during the experiment, with no significant differences detected among the groups (ANOVA, $p > 0.05$). Contrastingly, the 10 and 15 ppt salinity gradients had much higher GPx activities than the other three salinity gradients, especially in the late stage of the experiment (Figure 5d).

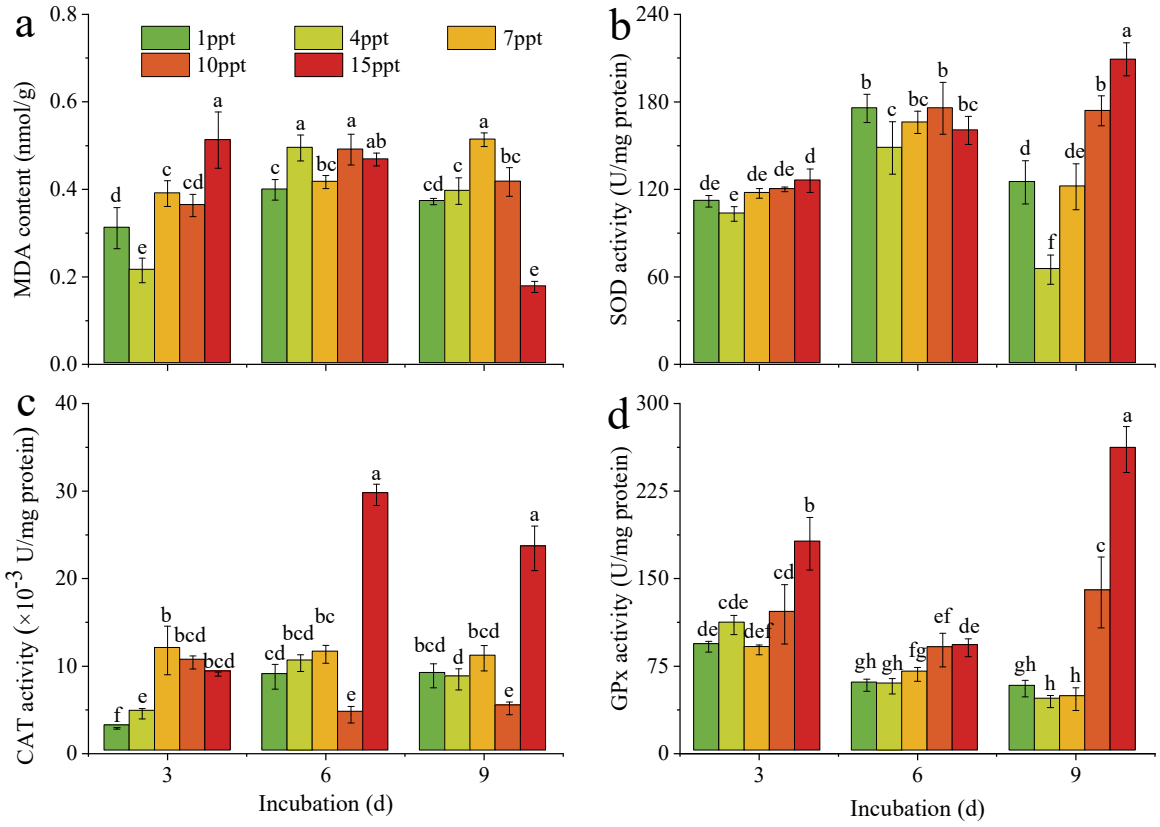

Figure 5. MDA content (**a**), and enzyme activity of SOD (**b**), CAT (**c**) and GPx (**d**) of *M. aeruginosa* under different salinity stresses on days 3, 6 and 9. Different letters a–h in the histogram indicate significant differences (ANOVA, $p < 0.05$).

4. Discussion

4.1. Effects of Salinity Stress on the Growth and Photosynthetic Characteristics of M. aeruginosa

Salinity is known to affect the growth of algae mainly by altering the osmotic pressure of the environment in which they are exposed, resulting in the contraction of the inner membrane structure, which changes the membrane transport process and solubility of intracellular CO_2 and O_2 and thereby reduces the metabolic rate of algal cells and slows growth [19,21]. In previous studies, the salinity tolerance of *Microcystis* was shown to differ substantially (2–35 ppt) [28,29]. The salinity tolerance threshold of *M. aeruginosa* LB2385 was 7–10 ppt [3], whereas that of *M. aeruginosa* PCC7806 was ≤10 ppt with 14 ppt, leading to death [16]. Such differences may be due to the different environments in which the algae are found and the adaptability of algal cells to salinity exposure.

In the present study, the cell density and F_v/F_m of *M. aeruginosa* at 4 and 7 ppt were higher than those of the control, indicating that these two salinities promoted the growth of *M. aeruginosa* during the early stage of culture and facilitated photosynthesis. At low NaCl concentrations, algae tended to (i) synthesize polar lipids, such as glycolipids and phospholipids, (ii) increase chloroplast and membrane lipid content, (iii) increase chl levels and (iv) show stimulated growth and photosynthesis [30,31]. In the current study, the cell density and F_v/F_m at 10 ppt increased after the initial adjustment in culture, indicating that algal cells were tolerant to this salinity and could adapt to the salinity change via self-regulation. Studies have shown that the salt tolerance of *Microcystis* is related to the synthesis of carbohydrates [32–34], which can reduce osmotic stress, help retain cell morphology and maintain intracellular physiological and metabolic processes because of their small molecular weight, high solubility and protective effect on macromolecules [35,36]. Furthermore, when photosynthetic mechanism changes, microalgae store carbohydrates in

the form of liposomes in cells, which improves their tolerance to extreme environments [37]. At 15 ppt in our study, the decrease in cell density and F_v/F_m indicated that algal cells did not exhibit photosynthetic activity and were lysed.

Additionally, Tanabe et al. [38] found that salt-tolerant genotypes of *M. aeruginosa* contain sucrose synthesis genes that are the products of horizontal gene transfer and have a short evolutionary history. Although it was not clear whether *M. aeruginosa* FACHB-905 possessed such a gene, the present results indicate that the salinity tolerance threshold of this species was between 10 and 15 ppt. Therefore, an increase in salinity was beneficial to the growth of algal cells within a specific range, and these cells also had a tolerance to high salinity conditions. However, salinity at levels higher than the tolerable range caused irreversible damage to the organism.

The photosynthetic characteristics of *M. aeruginosa* under the different salinity gradients tested here were similar to the results for cell density and F_v/F_m, i.e., the characteristics at 4 and 7 ppt were improved relative to those at 1 ppt, indicating that an appropriate increase in salinity stimulated the donor side of the *M. aeruginosa* photosynthetic system, which was conducive to the use of light absorbed by the reaction center for electron transport. The openness of the active reaction center and the increase in the reduction rate of Q_A were also conducive to photosynthesis. A large adjustment in the photosynthetic characteristics was observed at 10 ppt, and ψ_0 decreased as salinity increased, indicating that an increase in salinity reduced the openness of the active reaction centers, which was reflected in the tolerance of algal cells to salinity at these levels. Lu et al. [39] demonstrated that salinity stress inhibited electron transfer between the donor and acceptor sides of PSII, resulting in sharp declines in the fluorescence yields for the J, I and P phases of the OJIP curve and leading to phycobilisome damage as well as significantly decreased phycocyanin content, whereas salinity-adapted algae cells were shown to maintain a higher excitation energy conversion efficiency through down-regulation of PSII. In the present study, the continuous decrease of the OJIP curve and chl fluorescence parameters at 15 ppt indicated that the donor side of the algal cell photosynthetic system had been irreversibly damaged at this salinity level, which affected the reaction centers of *M. aeruginosa*, e.g., by reducing the electron transport flux of each reaction center. However, within a certain salinity range, *M. aeruginosa* could self-adjust to salinity changes, e.g., by reducing the dissipation energy flux of each reaction center. Salinity stress is known to reduce the chl content of algal cells [40,41], inhibit electron transfer between the donor and acceptor sides of the photosynthetic system PSII [42] and stimulate the activity of PSI [43,44], thereby affecting the photosynthesis of algae. Overall, an appropriate increase in salinity (1–7 ppt) was beneficial to the PSII of the photosynthetic system of *M. aeruginosa*, while salinity could inhibit electron transfer at sufficiently high levels (>10 ppt).

4.2. Effect of Salinity Stress on the Toxicity of M. aeruginosa

A variety of nonbiological factors, such as light and temperature, can affect the MCs content in the water column by influencing the abundance of toxin-producing *M. aeruginosa* or by adjusting concentrations of IMCs and EMCs. Moreover, chemical elements, such as nitrogen and phosphorus, in agricultural runoff can alter MC production in *M. aeruginosa* [45,46]. For salinity stress, it has been reported to reduce the production of MCs in *M. aeruginosa* cells in some studies [47,48], whereas no relationship between salinity and toxin concentration was found in another study [49].

Here, excessive salinity (15 ppt) decreased both the total IMCs content and the IMCs per cell during the experiment, while the control group exhibited opposite results. Thus, the changes in the total IMCs contents at 15 ppt and the control group might result from the variations in MCs production of the single algal cell. Additionally, for 4 and 7 ppt treatments, as the IMCs per cell were kept stable during the experiment, the increase in total IMCs might be related to the increased cell densities (Figure 2a). The 10 and 15 ppt salinity gradients presented significantly lower total EMCs concentrations than the other treatments (Figure 4c). This may be due to the degradation effect of high salinity on

MCs [15], which was consistent with the results of previous studies [15,16,50]. As a kind of secondary metabolite of *Microcystis*, MCs are involved in several life processes, such as intracellular signal transduction and gene regulation [51]. When encountering mechanical treatment or stress conditions, MCs released by *Microcystis* stimulate the transcription of *mcyB gene* of the rest *Microcystis* cells; thus, the algal cells could produce more MCs to adapt to the stress environment [52]. Chen et al. [14] also found that NaCl increased MCs content in *M. aeruginosa* and promoted MCs release to the extracellular environment. However, Luna et al. [53] reported that salinity stress resulted in the reduced transcription of the *mcyD* gene, which in turn reduced the content of intracellular MC-LR. Meanwhile, the MCs family currently includes at least 279 hepatotoxins containing cyclic heptapeptides [54]. The MCs detection method used in this study can obtain the MCs contents but cannot distinguish MC-LR and other variants [55]. Therefore, further study is needed to clarify the effects of salinity stress on the production and degradation of different MCs variants.

Certain environmental factors, such as phosphate deficiency and iron stress, have been shown to affect MCs synthesis without affecting cell growth [56,57], whereas other factors, such as darkness, excess phosphate or nitrate deficiency, affect cell growth without affecting MCs synthesis [56,58,59]. Our results suggested that an excessive increase in salinity not only disadvantages the growth and photosynthesis of *M. aeruginosa* but also reduces MCs contamination.

4.3. Effects of Salinity Stress on the Oxidative Stress of M. aeruginosa

Salinity stress could affect the photosynthetic electron transport and CO_2 reduction in the Calvin cycle of *M. aeruginosa*, resulting in the accumulation of ROS in algal cells, which in turn destroys redox homeostasis in *M. aeruginosa* [60,61]. Oxidative stress caused by excessive ROS concentration negatively affects the structure of the cell membrane and other parts. Given the tiny cellular structure of cyanobacteria, its energy metabolism, i.e., photosynthesis and respiration, are closely linked, with both processes being located in the thylakoid membrane close to the cell membrane [62]. Therefore, any damage to the membrane by ROS may affect the normal function of these two processes [63].

MDA is a product of lipid peroxidation and reflects the degree of membrane lipid peroxidation [34]. Salinity stress is known to alter the redox status by inhibiting algal electron transport and producing excessive ROS resulting in increased MDA content and membrane permeability [64]. In this study, there were some differences in the influence of elevated salinities on the MDA content of *M. aeruginosa*, but generally showed a promoting effect and roughly strengthened with the increased salinity, indicating that salinity stress induced the oxidative stress of *M. aeruginosa*. Among them, the MDA content at 15 ppt was significantly higher than the control group in the first 6 days but decreased sharply after that, which may be caused by the massive fragmentation and decomposition of algal cells under high salinity. Additionally, salinity stress can increase CAT activity in many autotrophic organisms, including cyanobacteria [65,66], and the enzyme has been shown to protect photosynthesis under osmotic stress [67] as well as prevent programmed cell death caused by ROS in phytoplankton [68]. In the present study, compared to the control, SOD activities at 4 and 7 ppt were reduced, and GPx activities of the two treatments also presented lower values during the later stage of the experiment. This might indicate that *M. aeruginosa* at 4 and 7 ppt salinity levels produced ROS at the early experimental stage, resulting in activation of the antioxidant enzyme system; however, as the experiment progressed, algal cells gradually adapted to higher salinity conditions via self-regulation, thereby reducing the activity of antioxidant enzymes. The SOD or GPx activities of algal cells at 10 and 15 ppt were significantly higher than those at 1 ppt in general, indicating that the algal cells under these two salinity conditions produced more ROS and exhibited more oxidative damage. As reported by Chen et al. [14], high levels of NaCl increased the content and accumulation of ROS in *M. aeruginosa*, and they not only changed the redox state of algal cells but also inhibited the electron transport of photosynthesis. Notably, the redox state regulates the expression of the photosynthesis-related genes *psbA* and *cpcB* [69].

Zhang et al. [70] also found that an increase in ROS induced chlorosis, photoreduction and triplet chl formation in algal cells, damaging the PSI, PSII and chl of algae. Thus, increased salinity affected the redox state in algal cells and produced destructive ROS, which caused oxidative stress and affected antioxidant enzyme activity in *M. aeruginosa*.

5. Conclusions

The results of this study suggest that an appropriate salinity of ≤7 ppt was beneficial for the growth and photosynthesis of the strain *M. aeruginosa* FACHB-905 and promoted MCs synthesis. Contrastingly, excessive salinity (≥10 ppt) inhibited the growth of the strain and accelerated cell disruption, leading to the release and degradation of MCs. A salinity of 15 ppt served as a fatal environmental condition for *M. aeruginosa*, which caused severe oxidative damage and declined MCs contamination, combined with irreversible damage to the photosynthetic system of the strain. This work underscores the important role salinity plays in regulating the growth and toxin production of *M. aeruginosa*, which is beneficial to assess the risk of harmful *Microcystis* blooms in low-salinity areas such as estuaries and coastal areas. Future studies should pay attention to the physiological effects of different salinities on regulating the growth and toxicity of *Microcystis* from molecular aspects, especially its regulations for the synthesis and degradation of the main derivatives of MCs.

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Appendix A

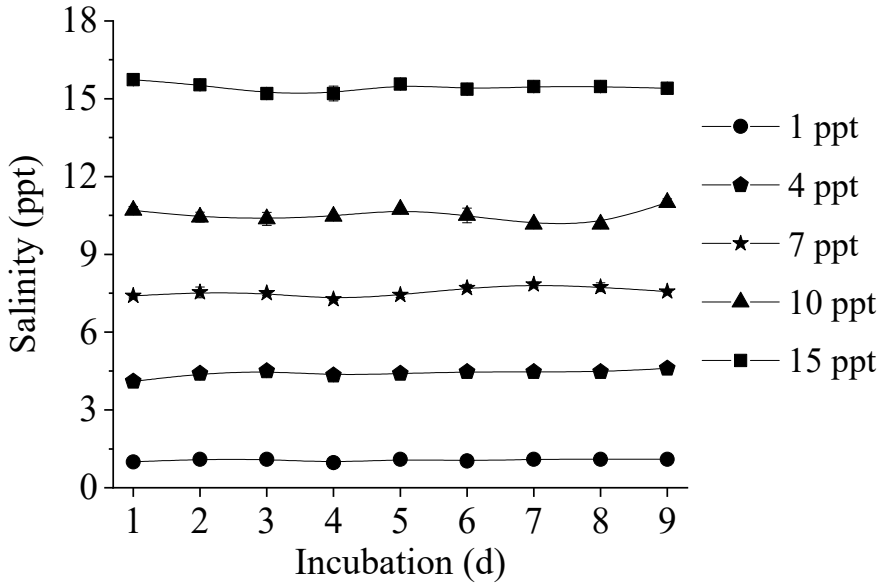

Figure A1. Variation in salinity during the study.

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