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Phytohormone Supplementation for Nutrient Removal from Mariculture Wastewater by *Oocystis borgei* in Sequential Batch Operation

Yang Liu, Chengcheng Deng, Xinyue Song, Zhangxi Hu, Feng Li, Yulei Zhang, Changling Li, Xianghu Huang and Ning Zhang *

Laboratory of Algae Resource Development and Aquaculture Environment Ecological Restoration, Fisheries College, Guangdong Ocean University, Zhanjiang 524088, China; 2112101066@stu.gdou.edu.cn (Y.L.); 2112101072@stu.gdou.edu.cn (C.D.); 2112201160@stu.gdou.edu.cn (X.S.); huzx@gdou.edu.cn (Z.H.); lifeng2318@gdou.edu.cn (F.L.); zhangyl@gdou.edu.cn (Y.Z.); licl@gdou.edu.cn (C.L.); huangxh@gdou.edu.cn (X.H.)

* Correspondence: zhangn@gdou.edu.cn

Abstract: To enhance the nutrient removal efficiency of *Oocystis borgei* for mariculture wastewater (MW), the effects and processes of three phytohormones on nitrogen and phosphorus removal from synthetic mariculture wastewater (SMW) by *O. borgei* under sequential batch operation were compared. The findings revealed that the supplementation with 10^{-6} M 3-indoleacetic acid (IAA), gibberellic acid (GA3), and zeatin (ZT) resulted in the most effective elimination, while there was no appreciable difference among them. The nitrogen and phosphorus indices of the effluent dramatically reduced ($p < 0.01$) upon the supplementation of phytohormones, and the removal effects were ranked as NO_3^--N > PO_4^{3-}-P > NH_4^+-N > NO_2^--N. The removal rates for NH_4^+-N and PO_4^{3-}-P were 0.72–0.74 mg·L^{-1}·d^{-1} and 1.26–1.30 mg·L^{-1}·d^{-1}, respectively. According to physiological studies, phytohormones enhanced the levels of photosynthetic pigments and chlorophyll fluorescence parameters (Fv/Fm and φPSII), thereby improving photosynthetic activity. Additionally, they stimulated Nitrate Reductase (NR) and Glutamine Synthetase (GS) activities to promote nitrogen metabolism and increased Superoxide Dismutase (SOD), Catalase (CAT), and carotenoid contents to mitigate oxidative stress damage caused by abiotic stress. These activities contribute to the proliferation of *O. borgei*, which in turn resulted in an increase in the assimilation of nitrogen and phosphorus from SMW. In conclusion, phytohormone supplementation significantly increased nutrient removal from SMW by *O. borgei* in a sequential batch reactor, which has potential application in MW treatment.

Keywords: *Oocystis borgei*; phytohormone; mariculture wastewater; nitrogen metabolism; antioxidant system

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1. Introduction

With the rapid development of the mariculture industry, the discharge of mariculture wastewater (MW) containing large quantities of residual bait and excrement has worsened water eutrophication, endangered aquatic ecosystems, and put human health at risk [1]. The removal of nutrients from MW has become a challenge for water environmental protection due to its high salt concentration [2,3]. The removal of nitrogen (N) and phosphorus (P) from MW is considered a basic way to control water eutrophication [4]. In recent years, microalgal-based MW treatment has been recognized as one of the most promising technologies for N and P removal. This potential is mainly attributed to the high-salinity tolerance, excellent pollutant removal capacity, absence of secondary pollution, and ability to generate high-value microalgae biomass [5–9]. However, due to microalgae's low photosynthetic efficiency under natural conditions, microalgae ponds used for treating MW need to be as large as possible to allow for complete nutrient removal [10]. With

this magnitude comes an increase in the occupied land area and investment costs [11]. Thus, it is imperative to find less-land-occupying, non-polluting, and commercially feasible strategies to boost the photosynthetic efficiency of microalgae for the treatment of MW [12].

Phytohormones are a class of small-molecule plant growth regulators (PGRs) capable of regulating life activities such as proliferation, biomass synthesis, and resistance to adverse environments at low doses [13–16]. There are many kinds of plant hormones, which are mainly divided into nine categories, as reported by Waadt [17]. Indole-3-acetic acid (IAA) is the predominant auxin in plants and plays a crucial role in the growth of *Cyanobacteria* [18]. IAA had been found to exert the strongest effect on both growth and metabolite accumulation in *Chlorella vulgaris* [19–21], as well as playing an important role in enhancing resistance against N-limitation stress in *Chlorella sorokiniana* [22]. Gibberellins (GAs) are an essential plant hormone involved in many biological processes, such as plant growth and development [23,24]. Among them, GA3 is one of the most biologically active forms, produced through fungal fermentation, primarily by *Gibberella fujikuroi* during industrial production [25]. GA3 plays a pivotal role in mitigating abiotic-stress-induced perturbations in microalgae and improving its adaptation abilities to low-level-polluted aquatic environments [26]. Several studies have demonstrated that GA3 affects microalgae growth and metabolism by increasing the absorption and utilization of nutrients from the growth medium [27–29]. In addition, zeatin (ZT), a natural plant cytokinin, plays an important role in cell growth response and the embedding of lipids [30]. For instance, 0.1 $mg \cdot L^{-1}$ of ZT significantly promoted the growth and lipid production of *Acutodesmus obliquus* while simultaneously improving its photosynthetic performance under nitrogen stress [31].

In microalgae culture, the supplementation of phytohormones resulted in a higher economic viability for the production of low-cost microalgal biomass. Salama et al. [32] conducted an economic feasibility analysis of using the phytohormones for the mass production of *Scenedesmus obliquus*, and they found that the supplementation of IAA and DHA resulted in more economical biomass production compared to the control. In a study conducted by Park et al. [33], it was also found that the use of phytohormones resulted in higher economic viability when compared to normal synthetic media and acetate for *Chlamydomonas reinhardtii* cultivated for biodiesel production. In addition, the ability of microalgae to absorb nitrogen and phosphorus is proportional to biomass, so it is a safe, environmentally friendly, and economically feasible method to increase the yield of microalgae and then improve the efficiency of the microalgae purification of wastewater by supplementation with phytohormones [32–35]. Previous studies have demonstrated that supplementation with phytohormones such as IAA, ZT, and brassinosteroid (Br) can effectively promote the removal of N and P by *Tetraselmis cordiformis* from domestic wastewater [36]. Additionally, Yu et al. found that naphthalene acetic acid (NAA) and indole butyric acid (IBA) increased TN removal efficiency by 59% in *Chlorella* sp. SDEC-18 when treating anaerobically digested wastewater diluted with seawater from kitchen waste [37]. Furthermore, 2,4-dichlorophenoxyacetic acid (2,4-D) had been found to promote N and P pollutant elimination in municipal wastewater using *Chlorella* sp. as the bioremediation agent [38]. However, limited research has explored the combined application of microalgae and phytohormones for MW treatment.

The selection of appropriate microalgae species is crucial for enhancing the efficiency of MW by microalgae. The ability of microalgae to absorb N and P determines their effectiveness in treating MW. With the advantages of good stability, euryhalinity, self-sedimentation capability, and strong resistance [39–43], the *Oocystis borgei* population has shown a good performance in nutrient regulation in aquaculture water of shrimp [44–46]. Notably, the self-sedimentation characteristics of *O. borgei* are particularly prominent during sequential batch operation for wastewater, which enables the efficient recovery of microalgae through sedimentation in large ponds, while allowing the discharge of treated supernatant water. However, there is currently no report on the utilization of *O. borgei* for nutrient removal from wastewater. In this study, the phytohormones IAA, GA3, and ZT

were supplemented into the sequential batch reactor (SBR) of *O. borgei*. Firstly, the effect of phytohormones on the removal of N and P from MW by *O. borgei* was investigated. Furthermore, changes in photosynthetic activity, nitrogen metabolism, and antioxidant capacity were studied to elucidate the mechanism of these phytohormones.

2. Materials and Methods

2.1. Microalgae, Phytohormones, and Synthetic Mariculture Wastewater

O. borgei was supplied by our laboratory and pre-cultured in culture room with Zhanshui 107–13 medium prepared with autoclaved artificial seawater (Table S1) [47]. The normal culture conditions were a light intensity of 45 μmol photons·m^{-2}·s^{-1}, a photoperiod of 12 h:12 h, and a temperature of 25 ± 2 °C. Phytohormones purchased from Hefei Bomei Biotechnology Co., Ltd. (Hefei, China) were prepared as a mother solution of IAA (10^{-2} M), GA3 (10^{-2} M), and ZT (10^{-2} M) according to the product's instructions. The mother solution was filtered and decontaminated using a 0.22 μm microporous filter membrane.

To simulate highly saline MW, the synthetic mariculture wastewater (SMW) was prepared using artificial aquaculture wastewater (Table S2) [48] and autoclaved artificial seawater (Table S1). The salinity and initial concentrations of ammonium-nitrogen (NH_4^+-N), nitrite-nitrogen (NO_2^--N), nitrate-nitrogen (NO_3^--N), and orthophosphate (PO_4^{3-}-P) in the SMW were 30, 1.21 mg·L^{-1}, 5.20 mg·L^{-1}, 3.62 mg·L^{-1}, and 2.09 mg·L^{-1}, respectively.

2.2. Determination of Optimal Concentration of Phytohormone

Five concentrations (10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M, and 10^{-8} M) of IAA, GA3, and ZT were screened to determine the optimal phytohormone concentration. The stocks of *O. borgei* were collected through centrifugation at 5000 rpm for 10 min at 25 °C and then resuspended in 200 mL of sterile SMW (OD_{680} = 0.263, cell density 1.13 × 10^6 cells·mL^{-1}) in a 250 mL triangular beaker. Different amounts of IAA, GA3, and ZT mother solution were added to triangular beakers to form various concentrations of phytohormone groups, and a control group without phytohormone was set up. Each treatment was performed in three replicates and cultured in an illumination incubator (PRX-350C, Ningbo Prant Instrument Co., Ltd., Ningbo, China) for 4 d with shaking and randomly changing positions five times a day to ensure uniform illumination. Cultivation conditions were the normal conditions as previously described. The 3 mL microalgae sample was collected every 24 h, and the biomass (as defined in Equation (1)) and specific growth rate (as defined in Equation T(2) [36]) of *O. borgei* were measured using a spectrophotometer (UV-1900i, Shimadzu, Japan) to determine the optimal phytohormone concentration.

The standard curve of cell density and OD_{680} absorbance value of *O. borgei* is as follows:

$$Y = 0.0023\ X + 0.0218\ (R^2 = 0.9997) \quad (1)$$

In this equation, Y represents the OD_{680} value and X represents the cell density (10^4 cells·mL^{-1}).

Specific growth rate:

$$\mu = \frac{(\ln N_{t_2} - \ln N_{t_1})}{t_2 - t_1} \quad (2)$$

In this equation, μ represents the specific growth rate (d^{-1}), while t_1 and t_2 represent time, and N_{t1} and N_{t2} represent the microalgae cell density (cells·mL^{-1}) measured at times t_1 and t_2, respectively.

2.3. Nitrogen and Phosphorus Determination

Following the aforementioned procedure, the centrifuged microalgae stock was re-suspended in 250 mL triangular beakers containing 200 mL of sterile SMW, and optimal concentrations of IAA (10^{-6} M), GA3 (10^{-6} M), and ZT (10^{-6} M) (see results and discussion) were supplemented into the culture flasks. A control flask without added phytohormones was established. The experiment employed sequential batch operation for 11 cycles (days)

with a hydraulic retention time (HRT) of 24 h. The supernatant from culture flasks was sampled every 24 h by centrifugation (5000 rpm, 10 min, 25 °C) and subsequently used to analyze the concentrations of N and P. Fresh SMW with different kinds of phytohormones was then used to resuspend the microalgae. All the experiments were carried out in triplicate in an illumination incubator. The concentrations of NH_4^+-N (salicylic acid-hypochlorite method), NO_2^--N (N-(1-naphthalene)-diaminoethane method), NO_3^--N (hydrazine reduction method), and PO_4^{3-}-P (molybdenum blue method) of the collected supernatant were determined using an automated discrete analyzer (SmartChem 200, AMS, Guidonia, Italy) [48] following standard spectrophotometric methods. The removal rates were calculated according to Equation (3) to investigate the effect of phytohormones on the N and P removal efficiency by *O. borgei*.

Removal rate:

$$\eta = \frac{(\text{Cinf} - \text{Ceff})\text{Q}}{\text{V}} \quad (3)$$

In this equation, η represents the removal rate ($mg \cdot L^{-1} \cdot d^{-1}$), while Cinf and Ceff represent the concentration of NH_4^+-N (or NO_2^--N, NO_3^--N, and PO_4^{3-}-P) in the influent and effluent water, respectively ($mg \cdot L^{-1}$). Q represents the flow rate (0.20 $L \cdot d^{-1}$), and V represents the effective volume of the reactor (L).

2.4. Determination of Photosynthetic Pigments and Chlorophyll Fluorescence Parameter

Microalgae samples (3 mL) were collected every 24 h, and the chlorophyll fluorescence parameters, including the actual photochemical efficiency (φPSII) of the photosystem (PSII) and the maximum quantum yield of PSII (Fv/Fm), were measured using a pulse-modulated fluorometer (FMS-2, Hansatech Instruments, Pentney, UK) as described in the previous studies [41]. Subsequently, the 3 mL samples were extracted overnight using 95% ethanol at 4 °C [49] for photosynthetic pigment analysis. The content of photosynthetic pigments was determined according to Sözgen's method for chlorophyll determination [50] and Chazaux's method for carotenoid determination [51], respectively.

2.5. Determination of Nitrogen-Metabolism-Related Enzymes and Antioxidant Enzymes

In this study, microalgae samples were sampled after 4 cycles (4 d) of sequential batch operation under phytohormone stimulation and then assayed for activity of Nitrate Reductase (NR, microplate method), Glutamine Synthetase (GS, colorimetric method), Superoxide Dismutase (SOD, WST-1 method), and Catalase (CAT, ammonium molybdate method) using assay kits (Nanjing Jiancheng, Nanjing, China).

2.6. Economic Feasibility Analysis of Phytohormones for MW Treatment

The cost of the microalgal biomass obtained in medium supplemented with IAA, GA3, and ZT for 4 d of sequential batch operation was calculated using Equation (4), which was modified according to Park et al. [32]:

$$\text{Cost}\left(\text{CNY} \cdot 10^9\ \text{Cells}^{-1}\right) = \frac{(A \times B + C) \times D}{\text{E}} \quad (4)$$

where *A* is the supplementation amount of phytohormone to a unit of culture medium ($g \cdot m^{-3}$), *B* is the price of the phytohormone ($CNY \cdot g^{-1}$), *C* is the price of the unit SMW ($CNY \cdot m^{-3}$), *D* is the value for sequential batch operation days, and E is the cell density of *O. borgei* after sequential batch operation of *D* days (1012 cells$\cdot m^{-3}$).

2.7. Statistical Analyses

All data are expressed as mean ± standard deviation, and statistical analysis was performed using GraphPad Prism 9.0.0 (GraphPad Software, San Diego, CA, USA) through one-way analysis of variance (ANOVA) and Tukey's test to compare the effects of phytohormones on the growth and removal of N and P of *O. borgei*.

3. Results and Discussion

3.1. Evaluating the Effect of Phytohormone on Growth of O. borgei

A dose–response analysis was performed to assess the fitness effects of three phytohormones on *O. borgei*. The results showed that IAA, ZT, and GA3 exhibited a dose-dependent enhancement in the growth of *O. borgei* (Figure 1). In the case of IAA (Figure 1A) and GA3 (Figure 1C) treatments, both high concentrations (10^{-4} M) and very low concentrations (10^{-7} M and 10^{-8} M) exerted an inhibitory effect on *O. borgei* growth compared to the control group. Notably, a highly significant negative effect was observed with 10^{-4} M GA3 from the first day of SMW treatment ($p < 0.01$). Conversely, 10^{-5} M and 10^{-6} M of IAA and GA3 demonstrated a growth-promoting effect on *O. borgei*. Regarding ZT treatment, concentrations ranging from 10^{-4} M to 10^{-6} M stimulated the growth of *O. borgei*; however, a slight inhibition in its growth was observed at concentrations of 10^{-7} M and 10^{-8} M (Figure 1E).

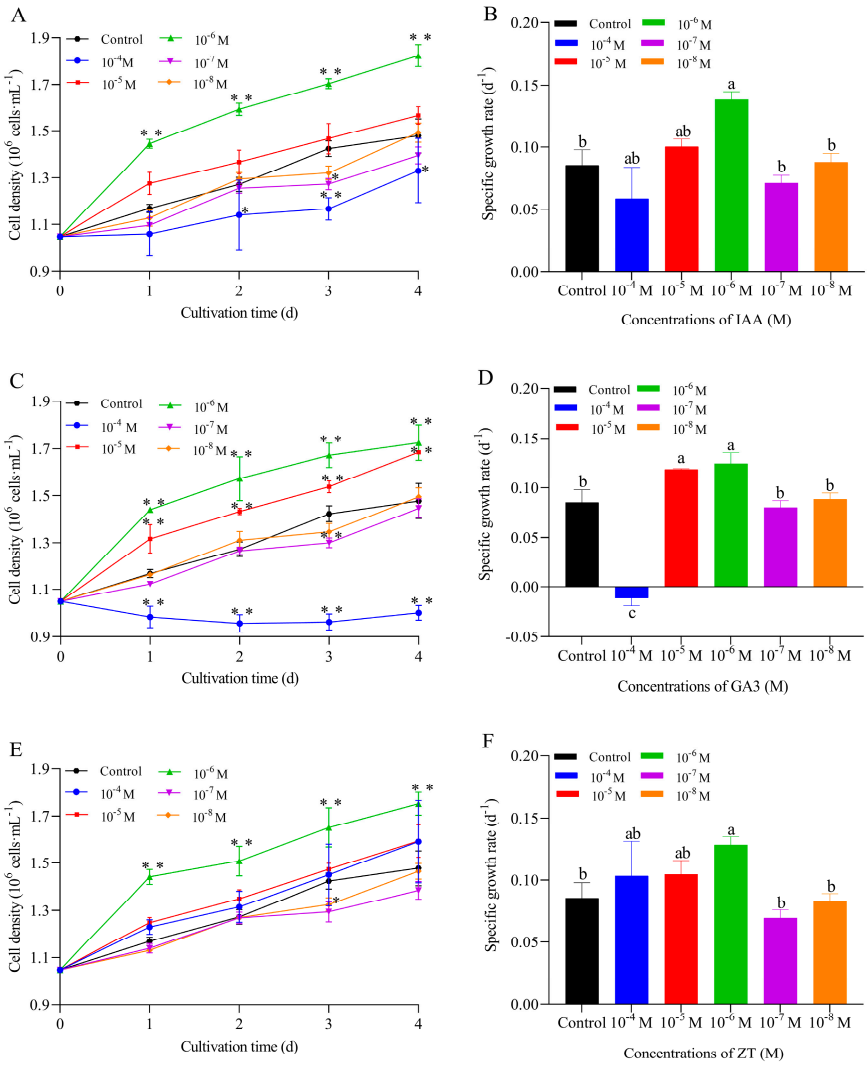

Figure 1. Effect of different concentrations of phytohormones IAA (**A**,**B**), GA3 (**C**,**D**), ZT (**E**,**F**) on the growth and specific growth rate of *O. borgei*. The differences between the control and experimental groups were statistically significant at $p < 0.05$ (*) and $p < 0.01$ (**), respectively. Different letters indicate significant differences among groups ($p < 0.05$ or $p < 0.01$).

Supplementation with 10^{-6} M of IAA, GA3, and ZT significantly promoted the growth of *O. borgei* ($p < 0.01$) throughout the cultivation. However, no significant difference was observed among the three phytohormones (Figure 1). The cell density after 4 days of

incubation was 1.23, 1.17, and 1.19 times higher than that of the control group, respectively. The maximum average specific growth rate over 4 d was achieved at a concentration of 10^{-6} M in all the three phytohormone-treated groups, namely 0.138 d^{-1} (IAA, Figure 1B), 0.124 d^{-1} (GA3, Figure 1D), and 0.128 d^{-1} (ZT, Figure 1F). These values were significantly higher than that of the control (0.085 d^{-1}) ($p < 0.01$).

The effects of phytohormones are dose-dependent, and supplementation with an optimal phytohormone concentration can dramatically boost the growth rate and biomass of a variety of microalgae [21,36,52]. Similarly, consistent effects influenced by the phytohormone were also observed in *O. borgei*. In all the cases with different phytohormone treatments, the maximum specific growth rate was obtained with the supplementation of 10^{-6} M of the phytohormone. Moreover, the effect of phytohormones on the growth of *O. borgei* showed low dosage-promotion and high dosage-inhibition effects, which has also been reported in some other microalgae. For instance, Chung et al. [53] found that a low concentration of IAA (10^{-4} M) promoted the growth of *Desmodesmus komarekii* after 5 days of culturing in the medium, but high concentrations of IAA (>3×10^{-4} M) inhibited cell growth. For *Isochrysis galbana*, 4 $mg \cdot L^{-1}$ of GA3 was the most effective in promoting growth rate, whereas 6 $mg \cdot L^{-1}$ of GA3 had an inhibitory effect on biomass [28]. In terms of the impact of ZT on *A. obliquus*, it was found that ZT supplementation above the optimum concentration (0.1 $mg \cdot L^{-1}$) resulted in decreased biomass productivity [31]. Furthermore, the inhibitory effects on *O. borgei* were observed at very low concentrations (10^{-7} M and 10^{-8} M) compared to the control in this study. Similarly, the higher GA3 concentration (10^{-5}–10^{-6} M) enhanced the growth of *Chlorella minutissima* while lower GA3 concentrations (10^{-7}–10^{-8} M) were slightly inhibitory over the 7-day inoculation, which can be attributed to the non-uptake of GA3 from the media, as indicated by the endogenous GA content after 7 days of GA3 treatment [54]. Based on these findings, it was deduced that very low concentrations (10^{-7} M and 10^{-8} M) of phytohormones were less likely to be taken up by *O. borgei* and thus lead to the results in this study.

In laboratory settings, centrifugation can be utilized for the recovery of *O. borgei;* however, this approach proves impractical for large-scale wastewater treatment. *O. borgei* exhibited self-sedimentation properties, and our previous research found that the sedimentation ratio of *O. borgei* was positively correlated with the biomass ($R^2 = 0.8243$) [43]. Supplementation with the phytohormones in this study promoted the growth of *O. borgei*, enabling efficient and cost-effective separation of the microalgae with treated wastewater, while facilitating discharge of the treated supernatant water and the recovery of *O. borgei* biomass for the production of high-value components such as fucose, linolenic acid (C18:3 ω3), and EPA (C20:5 ω3) [55].

3.2. Phytohormones Facilitate the Removal of N and P by O. borgei

The concentrations of NH_4^+-N, NO_2^--N, NO_3^--N, and PO_4^{3-}-P in the influent and effluent of SMW were quantified during sequential batch operation treatment, supplementing with 10^{-6} M of IAA, GA3, and ZT by *O. borgei*. The removal rate of nutrients from the effluent water (Figure 2 and Table S3) indicated that the supplementation with 10^{-6} M of IAA, GA3, and ZT significantly improved N and P removal by *O. borgei* ($p < 0.01$). There was no significant difference in the enhancement effect of the three phytohormones, and their nutrient removal rates were ranked as NO_3^--N > PO_4^{3-}-P > NH_4^+-N > NO_2^--N.

Supplementation with 10^{-6} M IAA, GA3, and ZT resulted in a significant increase in NH_4^+-N removal by *O. borgei* ($p < 0.01$) from the first day, and the maximum removal efficiency was achieved on day 6 ($p < 0.01$), after which it reached a steady state from days 7 to 11 ($p < 0.01$) (Figure 2A). The average removal rate of NH_4^+-N by the three phytohormones over 11 cycles was significantly higher (IAA—at a rate of 0.730 $mg \cdot L^{-1} \cdot d^{-1}$ (1.78 times higher); GA3—at a rate of 0.718 $mg \cdot L^{-1} \cdot d^{-1}$ (1.75 times higher); and ZT—at a rate of 0.738 $mg \cdot L^{-1} \cdot d^{-1}$ (1.8 times higher)) than that observed in the control group's average removal rate of NH_4^+-N (0.41 $mg \cdot L^{-1} \cdot d^{-1}$) ($p < 0.01$).

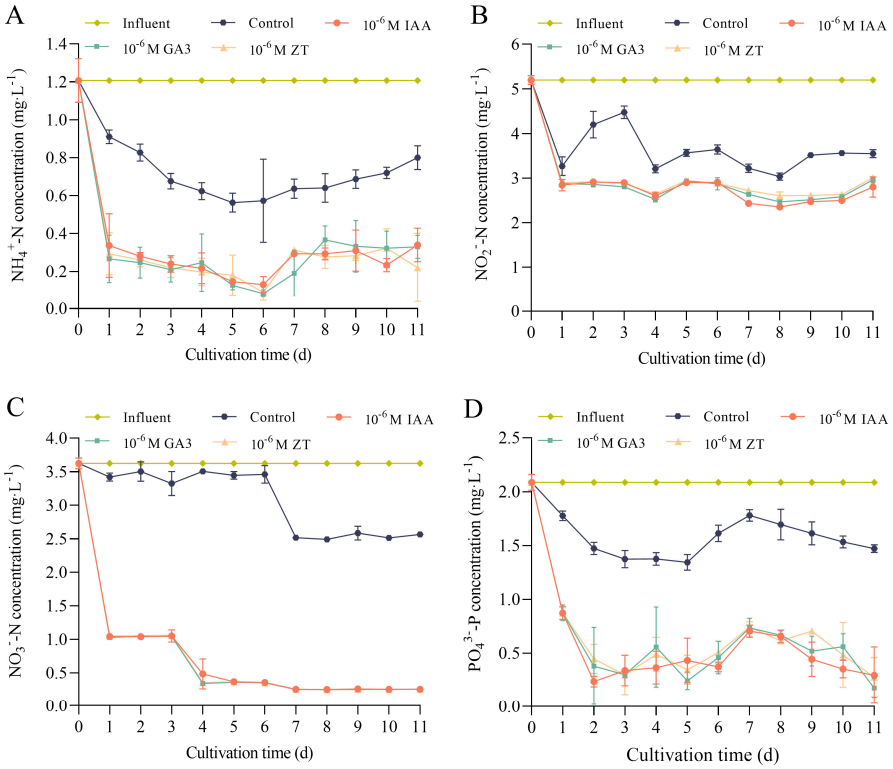

Figure 2. Assimilation curve of NH_4^+-N (**A**), NO_2^--N (**B**), NO_3^--N (**C**), and PO_4^{3-}-P (**D**) of *O. borgei* treated by IAA, ZT, and GA3.

The NO_2^--N concentration in the effluent of the IAA, GA3, and ZT groups showed a consistently stable trend from the initial day, while the control group displayed an unstable removal pattern of NO_2^--N with a delayed start-up phase (Figure 2B). After 4 days, the control group demonstrated a steady trend with an average removal rate of 1.31 $mg \cdot L^{-1} \cdot d^{-1}$ over an 11-day period. The average removal rates of NO_2^--N by IAA, GA3, and ZT were 2.01 $mg \cdot L^{-1} \cdot d^{-1}$, 1.98 $mg \cdot L^{-1} \cdot d^{-1}$, and 1.92 $mg \cdot L^{-1} \cdot d^{-1}$, respectively. These values were significantly higher than that observed in the control group ($p < 0.01$), corresponding to increases of approximately 53%, 51%, and 47%, respectively.

In terms of the removal of NO_3^--N, the IAA, GA3, and ZT groups exhibited an initial plateau period within the first three days and achieved enhanced removal after 4 d. Conversely, the control group's NO_3^--N removal commenced and stabilized on day 7 (Figure 2C). The average removal rate of NO_3^--N by phytohormones in 11 cycles was 2.49 $mg \cdot L^{-1} \cdot d^{-1}$ for IAA, 2.50 $mg \cdot L^{-1} \cdot d^{-1}$ for GA3, and 2.49 $mg \cdot L^{-1} \cdot d^{-1}$ for ZT, which were significantly higher than that of the control group (0.47 $mg \cdot L^{-1} \cdot d^{-1}$) with a fold increase of approximately 5 times ($p < 0.01$).

The removal of PO_4^{3-}-P by the supplementation of phytohormones and the control group exhibited a similar trend. Both groups reached stabilization from 2 d to 6 d, followed by a gradual decline after a slight decrease at 7 d (Figure 2D). The removal rate of IAA, GA3, and ZT on PO_4^{3-}-P was significantly higher ($p < 0.01$) compared to the control group, with values of 1.30 $mg \cdot L^{-1} \cdot d^{-1}$, 1.27 $mg \cdot L^{-1} \cdot d^{-1}$, and 1.26 $mg \cdot L^{-1} \cdot d^{-1}$, respectively. These values were also found to be approximately 3.02, 2.95, and 2.93 times higher than that of the control (0.43 $mg \cdot L^{-1} \cdot d^{-1}$) ($p < 0.01$).

There have been numerous studies indicating that microalgae possess the capability to effectively remove N and P nutrients from wastewater [9,53]. Ahmad et al. [54] conducted experiments on aquaculture wastewater treatment using *Scenedesmus obliquus*, *C. sorokiniana*, and *Ankistrodesmus falcatus*, achieving remarkable removal efficiencies of NH_4^+-N ranging from 86.45% to 98.21%, as well as NO_3^--N removal efficiencies of 75.76% to 80.85%. Additionally, *Spirulina platensis* and *S. obliquus* exhibited exceptional nutrient

removal effects in the field of wastewater treatment research [56]. Although microalgae could assimilate nutrients of wastewater, the treatment period was too long, and there was still a certain gap from practical applications; it even has no competitive advantages compared to traditional biological treatments [57,58]. The combination of microalgae and phytohormones has demonstrated excellent efficacy in aquaculture wastewater treatment. Research has demonstrated that the supplementation of 1.00 mg·L^{-1} of 2,4-D significantly enhances the N and P removal capacity of *Chlorella pyrenoidosa,* in which the total nitrogen (TN) removal rate reached 87.3%, the NH_4^+-N removal rate achieved 94.1%, and the total phosphorus (TP) removal rate attained 90.0% [38]. Furthermore, NAA could improve the efficiency of *Coccomyxa subellipsoidea* in removing TN and TP from brewery effluent [59]. By supplementation with 2 mg·L^{-1} NAA., 1 mg·L^{-1} indomethacin (INM), and 1 mg·L^{-1} 2,4-D in mariculture wastewater treatment systems, *C. pyrenoidosa* achieved a removal rate for NH_4^+-N exceeding over 95% [60]. Further, the selection of microalgae and phytohormones appears to be critical to the efficacy of wastewater treatments [6,61,62]. The present study employed a 24 h sequential batch operation to investigate the combined impact of phytohormones and *O. borgei* on the treatment efficiency of SMW. The findings demonstrated that supplementation with 10^{-6} M IAA, GA3, and ZT significantly stimulated *O. borgei* growth and enhanced N and P removal efficacy in the SMW over an 11-day treatment period.

3.3. Mechanism of Phytohormones to Promote N and P Removal by O. borgei

3.3.1. Promotion of Photosynthetic Metabolism of *O. borgei*

The content of photosynthetic pigments is closely correlated with photosynthetic activity. Therefore, the levels of chlorophyll (total Chl, Chla, and Chlb) and carotenoids were quantified in *O. borgei* (Figure 3A–D). The results showed that all three phytohormones exhibited positive effects on the chlorophyll and carotenoid content of *O. borgei* in the given concentration (10^{-6} M). However, this stimulatory effect was time-dependent as significant differences in pigment content compared to the control group were only observed after 4 days of stimulation ($p < 0.05$). Among the three phytohormones tested, IAA exhibited the most potent stimulating effect followed by GA3 and ZT. Notably, the total Chl content of *O. borgei* under IAA treatment was significantly higher than that of ZT treatment ($p < 0.05$) (Figure 3A). After 4 days of stimulation with IAA, GA3, and ZT, the total chl content in *O. borgei* exhibited a significant increase compared to the control group (4.087 ± 0.077 ng·$cell^{-1}$), showing a fold change of 1.31 times ($p < 0.01$) for IAA treatment, 1.23 times ($p < 0.01$) for GA3 treatment, and 1.17 times ($p < 0.05$) for ZT treatment (Table S4). The impact of phytohormones on carotenoid content was analogous to that of total Chl (Figure 3B). Following a 4-day stimulation, all three phytohormones significantly elevated the levels of carotenoid contents in *O. borgei*, which was 1.30 ($p < 0.01$), 1.21 ($p < 0.01$), and 1.18 ($p < 0.05$) times higher than the control group's (1.033 ± 0.020 ng·$cell^{-1}$), respectively (Table S4).

Zhao et al. [36] also reported a time-dependent effect, demonstrating that IAA and ZT significantly increased the content of Chla and Chlb in *T. cordiformis* at their optimal concentrations. Similarly, Fierli et al. [63] observed a significant enhancement in the cell content of Chla in *Phaeodactylum tricornutum* when treated with GA3 at its optimal concentration (200 mg·L^{-1}). However, it is worth noting that the effects of phytohormones on chlorophyll content in microalgae are not always positive. For instance, the exposure of *I. galbana* to a culture medium containing the optimal concentration of GA3 (4 mg·L^{-1}) resulted in lower Chla contents compared to the control group, though the difference was not statistically significant [28]. Chla, Chlb, and carotenoids play crucial roles in the absorption of light energy and its transfer to microalgal cells, with certain Chla variants even exhibiting light conversion capabilities [64]. In this study, the observed increase in levels of chlorophyll and carotenoids following stimulation by phytohormones signified an augmentation in the light harvesting efficiency and energy transfer potential of *O. borgei*.

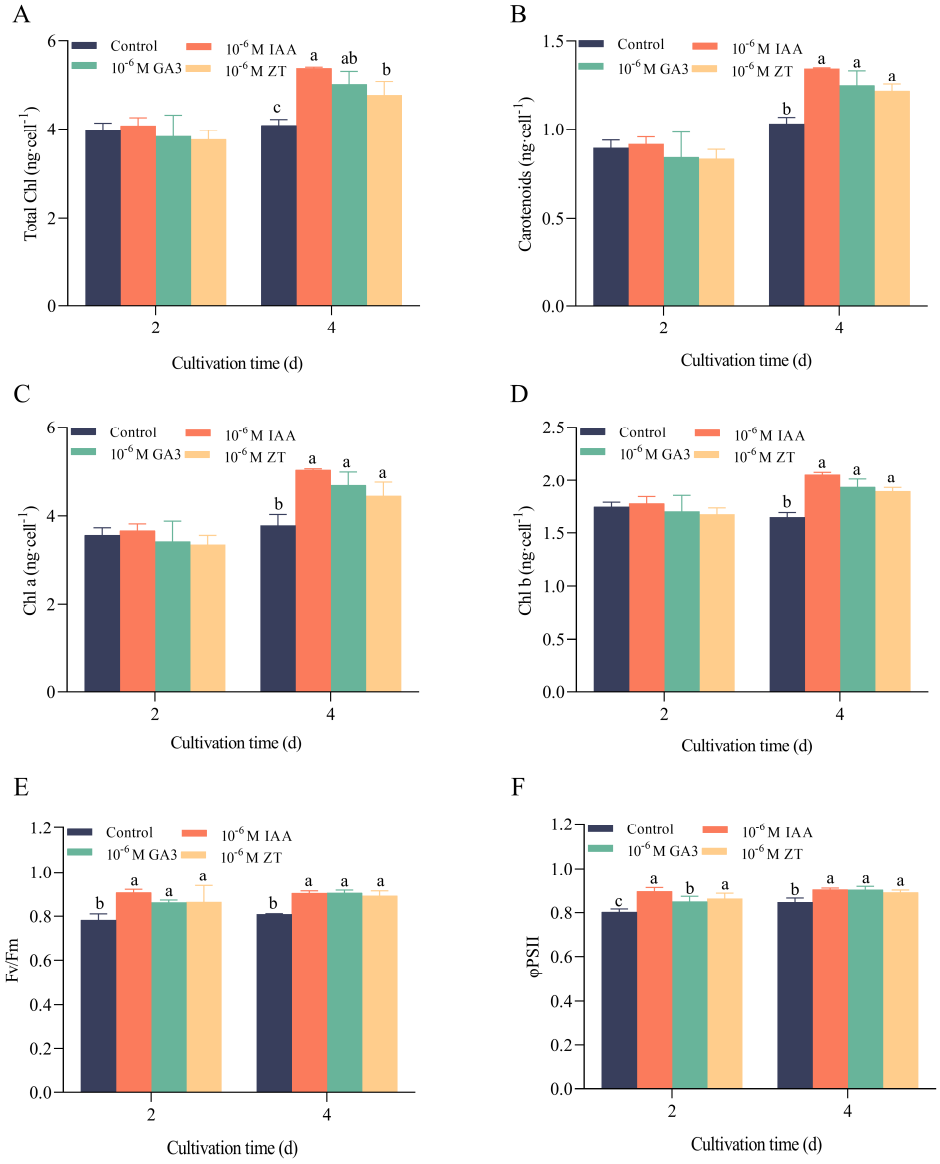

Figure 3. Effect of phytohormones on photosynthetic pigment content (total Chl, (**A**); carotenoids, (**B**); Chl a, (**C**); Chl b, (**D**)) and photosynthetic activity (Fv/Fm, (**E**); φPSII (**F**)) of *O. borgei*. Different letters indicate significant differences among groups ($p < 0.05$ or $p < 0.01$).

Two chlorophyll fluorescence parameters, Fv/Fm and φPSII, were further selected to assess the effects of phytohormones on the photosynthetic activity of *O. borgei* during the 4-day stimulation period (Figure 3 and Table S4). Treatment with 10^{-6} M IAA, GA3, and ZT significantly increased Fv/Fm (Figure 3E) and φPSII (Figure 3F) values in *O. borgei* from day 2 onwards ($p < 0.05$). Chlorophyll fluorescence parameters provide valuable information about photochemical and thermal quantum efficiency, which are crucial for plant photosynthesis and productivity enhancement [65]. Chlorophyll fluorescence parameters have become important tools in microalgal physiology research as well as biotechnological applications [66]. The dark-adapted value of Fv/Fm is an indicator of PSII energy capture efficiency in plants, and the higher the value, the higher the potential for light energy utilization [65,67]. φPSII, the most commonly used parameter for characterizing light adaptation, reflects the proportion of absorbed light actually utilized in PSII photochemistry [65,67]. In this study, supplementation with 10^{-6} M IAA, GA3, and ZT resulted in a significant increase in Fv/Fm and φPSII, indicating an enhancement in the efficiency of light energy conversion and the activity of the photoreaction centers of PSII.

In conclusion, supplementation with 10^{-6} M IAA, GA3, and ZT has been found to enhance the photosynthetic activity of *O. borgei* by increasing the content of photosynthetic pigments, which played an important role in promoting the growth of microalgae [20,31,68], and is one contributing factor for improving its N and P removal efficiency.

3.3.2. Improving the Nitrogen Metabolism of *O. borgei*

The process of nutrient removal by microalgae can be regarded as a utilization process. Therefore, the impact of phytohormones on nitrogen-metabolism-related enzymes (NR, GS) was investigated to further elucidate the mechanism underlying the phytohormone-induced enhancement in N and P removal from SMW by *O. borgei*. The corresponding results are presented in Figure 4 and Table S5. After 4 days of treatment with 10^{-6} M IAA, GA3, and ZT, the average activities of NR and GS exhibited a significant elevation compared to those in the control group ($p < 0.01$). However, there were no notable differences among the three hormone treatments. The activity of NR after phytohormone treatment was amplified by a factor of 3.61 (IAA), 3.73 (GA3), and 2.66 (ZT) relative to that of the control group ($p < 0.01$) (Figure 4A). The activity of GS after phytohormone treatment was increased by a factor of 3.90 (IAA), 3.71 (GA3), and 3.67 (ZT) compared to that of the control group ($p < 0.01$) (Figure 4B).

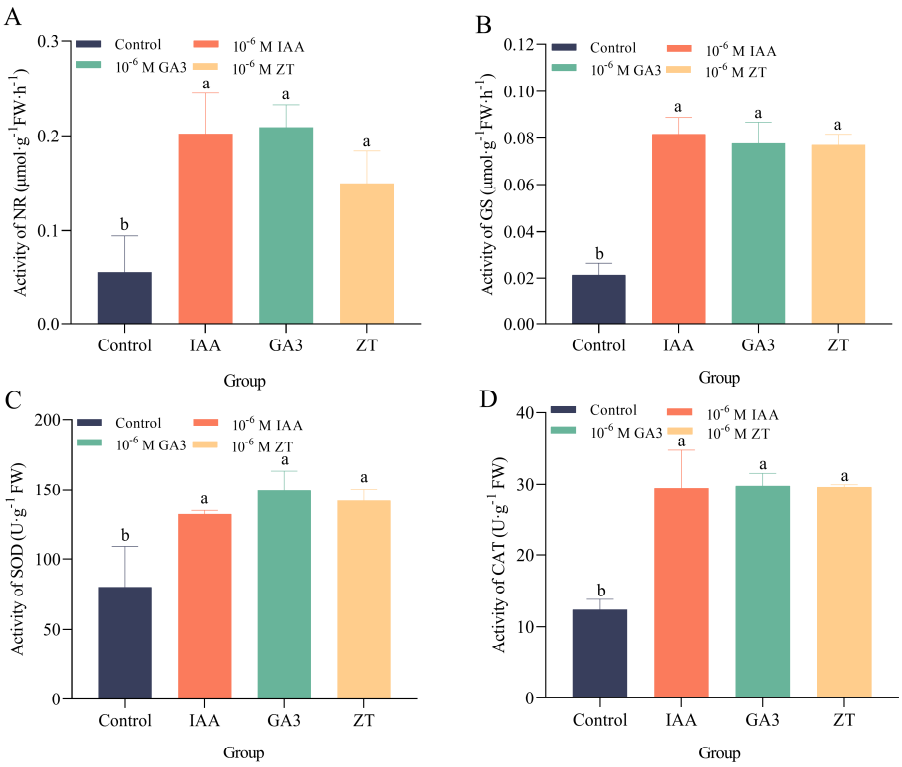

Figure 4. Effect of phytohormones on NR (**A**), GS (**B**), SOD (**C**), and CAT (**D**) activities of *O. borgei*. Different letters indicate significant differences among groups ($p < 0.05$ or $p < 0.01$).

Nitrogen metabolism is a fundamental physiological process crucial for the growth and development of microalgae, as well as an essential pathway for nitrogen removal from wastewater. The primary nitrogen source for microalgae was NH_4^+-N, while NO_3^--N and NO_2^--N must be converted to NH_4^+-N before they can be assimilated by microalgae [69–71]. Liu et al. [72] also discovered that *O. borgei* exhibited a preference for utilizing different forms of soluble nitrogen in the following order: NH_4^+-N > NO_2^--N > urea-N > NO_3^--N. The conversion of NO_3^--N to NO_2^--N, the first step in nitrogen metabolism, catalyzed by NR is considered the rate-limiting step in nitrogen reduction [73,74]. To equalize the decrease in substrate for the reduction process, the microalgae must absorb

more NO_3^--N from the surrounding environment [75,76]. The present study demonstrated that supplementation with phytohormones significantly enhances NR activity in *O. borgei*, suggesting a promotion of NO_3^--N reduction. This finding also elucidated the higher removal rate of NO_3^--N by *O. borgei*. Additionally, GS, the first key enzyme identified to be involved in plant nitrogen metabolic pathways, facilitates the direct conversion of NH_4^+-N from diverse sources into intermediate metabolites such as glutamine and glutamate, which are indispensable for protein synthesis [77–80]. The upregulation of GS activity observed in this study indicated that supplementation with phytohormones stimulates the conversion of NH_4^+-N into organic nitrogen compounds, facilitating growth and enhancing nitrogen absorption by *O. borgei*.

3.3.3. Mitigation of Oxidative Stress Damage Caused by Abiotic Stresses

It has been demonstrated that centrifugal collection potentially compromises microalgae cellular integrity due to high gravity and shear forces in the process of wastewater treatment [81,82]. In this study, *O. borgei* was subjected to centrifugation for sequential batch operation every 24 h. In order to verify whether phytohormones would mitigate these negative effects, the effect of phytohormones on the activities of SOD (Figure 4C) and CAT (Figure 4D) in *O. borgei* was investigated (for more details, see Table S5). The results showed that the activities of the SOD and CAT in *O. borgei* were significantly enhanced by the three phytohormones. Among them, supplementation with 10^{-6} M GA3 exhibited the greatest enhancement, leading to a 2.4-fold increase in CAT activity ($p < 0.01$) and a 1.87-fold increase in SOD activity ($p < 0.01$), compared to the control after 4 days of stimulation.

Microalgae produce a large amount of reactive oxygen species (ROS) when subjected to abiotic stress, and excessive ROS can damage various cellular components of microalgae, thereby affecting their physiological functions [83,84]. Microalgae have an antioxidant defense system to overcome oxidative damage. There are two types of antioxidant defense mechanisms, namely enzymatic and non-enzymatic antioxidants [85]. SOD and CAT are pivotal antioxidant enzymes in microalgal cells, with SOD acting as the first defense against oxidative stress. Moreover, carotenoids have antioxidant characteristics and maintain cells against free radicals, inhibit lipid peroxidation, increase the stability of the photosynthetic apparatus, and protect integrity membranes [86]. In this study, 10^{-6} M IAA, GA3, and ZT significantly increased the levels of antioxidant enzymes SOD and CAT as well as carotenoids (Figure 3B), indicating that *O. borgei* enhanced antioxidant capacity in response to abiotic stress. This also serves as a way to increase its biomass. Similarly, Piotrowska-Niczyporu et al. [87] demonstrated that the supplementation of auxin and cytokinin effectively triggered the activation of the antioxidant defense system, thereby enhancing the resistance of microalgae *A. obliquus* to Pb toxicity. Moreover, the supplementation of phytohormones has been reported as a feasible method to mitigate oxidative stress damage induced by abiotic stress and increase microalgae biomass production [88].

3.4. Economic Evaluation of Phytohormones for MW Treatment

It is worth noting that only a very low dose of phytohormones is required to effectively improve the biomass of microalgae and extract valuable substances [13–16,31]. However, the economics of phytohormone application for the cultivation of microalgae or for MW treatment must be considered before large-scale implementation. Table 1 summarizes the economic estimation of supplementation with phytohormones for SMW treatment by *O. borgei* in sequential batch operation. The total cost of SMW treatment mainly depends on the cost of the SMW media, amount of cell density produced (sequential batch operation time), and the cost of phytohormones as described in Equation (4). The total cost of the SMW treatment with supplementation with IAA and GA3 was more economically feasible than in SMW treatment without phytohormones (control), due to the high biomass productivity under the influence of phytohormones. However, the economic evaluation with ZT was worse than that of the control, due to the excessively high cost (2170 CNY·g^{-1}). The observations are in good agreement with previous studies showing the economic

analysis of microalgal biomass production using the phytohormones for *Chlamydomonas reinhardtii* [32] and *S. obliquus* [33].

Table 1. Economic feasibility analysis of phytohormone supplementation for MW treatment by *O. borgei* in sequential batch operation.

Bulk	Amount of Phytohormone ($g \cdot m^{-3}$)	Price		Value for Cultivation Day	Cell Density (10^{12} Cells$\cdot m^{-3}$)	Cost (CNY$\cdot 10^{9}$ Cells^{-1})
		Phytohormone (CNY$\cdot g^{-1}$)	SMW (CNY$\cdot m^{-3}$)			
Control	0	0	1970	4	1.48	5.32
IAA	0.18	3.01	1970		1.82	4.33
GA3	0.35	5.04	1970		1.73	4.56
ZT	0.22	2170	1970		1.75	5.60

Notes: The price of the three phytohormones from the Hefei Bomei Biotechnology Co., Ltd. (Hefei, China). The price of SMW was calculated based on prices of Sinopharm Chemical reagent.

According to the economic feasibility analysis, the synthetic SMW added significantly contributes to the total cost. Thus, the findings of this study re-emphasize the importance of replacing synthetic media with various natural wastewaters (sources of nitrogen, phosphorus, and carbon) for the large-scale cultivation of microalgae. Furthermore, phytohormone supplementation with IAA and GA3 increased the biomass of *O. borgei* and nutrient removal from MW in a cost-effective manner.

4. Conclusions

The present study demonstrated that the supplementation of phytohormones enhanced the photosynthetic activity (photosynthetic pigments, Fv/Fm and φPSII), facilitated nitrogen metabolism (NR and GS), mitigated oxidative stress damage (SOD, CAT, and carotenoid contents) in *O. borgei*, and augmented *O. borgei* biomass production, thereby improving the N and P assimilation efficiency of *O. borgei*. Specifically, the supplementation with 10^{-6} M of IAA, GA3, and ZT significantly enhanced the removal of N and P from SMW under sequential batch operation ($p < 0.01$), although there was no difference between the three. The average removal rates of NH_4^+-N and PO_4^{3-}-P were 0.73 mg$\cdot L^{-1} \cdot d^{-1}$ and 1.28 mg$\cdot L^{-1} \cdot d^{-1}$, respectively, which were 78% and 198% higher than those of the control group. Furthermore, considering its cost-effectiveness relative to GA3, ZT, and the control treatment, this study recommends IAA as a promising option for practical application in mariculture wastewater treatment. In summary, this study proposes an environmentally friendly and cost-effective approach for the treatment of high-salinity mariculture wastewater, which can also be used as a bioprocess to increase microalgae biomass.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w16040552/s1, Table S1: Chemical composition and concentration of Zhanshui 107-13 medium and artificial seawater; Table S2: Chemical composition and concentration of artificial aquaculture wastewater; Table S3: Effect of phytohormones on the Nutrient Removal Rate of *O. borgei*; Table S4: Effect of phytohormones on photosynthetic pigments and chlorophyll fluorescence parameters of *O. borgei*; Table S5: Effect of phytohormones on activities of NR, GS, SOD and CAT in *O. borgei* after 4-day stimulation.

Author Contributions: Y.L.: Writing—Original Draft Preparation, Methodology, Data Curation, Visualization. C.D.: Methodology, Writing—Original Draft Preparation, Investigation, Project Administration. X.S.: Data Curation, Investigation, Writing—Original Draft Preparation. Z.H.: Investigation, Formal Analysis, Writing—Original Draft Preparation. F.L.: Project Administration, Resources, Writing—Original Draft Preparation. Y.Z.: Data Curation, Validation, Writing—Original Draft Preparation. C.L.: Resources, Supervision, Writing—Original Draft Preparation. X.H.: Resources, Writing—Original Draft Preparation, Validation. N.Z.: Funding Acquisition, Writing—Review and Editing, Conceptualization, Methodology, Writing—Original Draft Preparation. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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