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Supplementation of strigolactone analog for enhancing the removal of nutrient and heavy metal by microalgae-fungi symbionts

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ABSTRACT

The purification effects of *Chlorella vulgaris* and *Chlorella vulgaris-Clonostachys* symbionts on nutrients and heavy metals in wastewater were investigated to determine the optimal symbiotic treatment technology and the suitable concentration of GR24. The results showed that exogenous GR24 not only promoted the formation of uniform green spheres in microalgae-fungi symbionts, but also increased its photosynthetic performance and growth. At 10^{-7} M GR24 addition concentration, the specific growth rate of microalgae-fungi symbionts was 0.367 ± 0.04 d^{-1}, the maximum removal efficiency of chemical oxygen demand (COD), total nitrogen (TN) and total phosphorus (TP) were 81.96 ± 7.93 %, 80.26 ± 7.91 % and 82.96 ± 8.05 %, respectively. When the concentration of exogenous GR24 was 10^{-5} M, the removal efficiency of heavy metals was the best at both low and high pollution levels. It was worth noting that the removal efficiency of Zn at low concentration pollution levels (1 mg L^{-1}) was 99.32 ± 0.43 %, nearly 100 %. This study promoted the development of microalgae-fungal symbionts in the field of wastewater treatment.

1. Introduction

Heavy metal pollution is a global threat to water environment pollution due to its toxicity, non-degradability, and high bio-accumulation potential in aquatic organisms. The use of heavy metals in industry (electroplating, mining, printing, metal processing, etc.), agriculture (the use of pesticides and fertilizers) [1], and technological applications (the unreasonable treatment of solid waste) [2] have significantly increased the exposure of water bodies to heavy metals. The heavy metal pollution of aquatic environments has posed a threat to agricultural production and human health, and has become a highly concerned environmental issue in China [3]. It has been listed as the optimal control pollutant for water environment management.

The treatment methods of heavy metal pollution in water mainly include chemical method, physical method, and biological treatment [4]. Traditional methods (including adsorption, membrane separation and chemical precipitation method) have been proven to be very effective in removing heavy metals from wastewater, but the treatment costs are extremely high, which seriously limits its wide use [5]. Currently, Microalgae-based biological removal of heavy metals has attracted wide attention due to its advantages of economy and efficiency [6]. First of all, microalgae could accumulate and adsorb heavy metals on the surface of algal cells in a short time, which reduced the processing time [7]. Secondly, Microalgae had high sensitivity to heavy metals, and different microalgae had different adsorption capacity and selectivity to different heavy metals. It can treat wastewater containing a variety of heavy metals. Thirdly, microalgae treatment technology does not require the use of other expensive reagents, reducing the burden of subsequent treatment [8]. Therefore, using microalgae to treat heavy metal polluted water bodies can recover and reuse heavy metals, improving economic benefits. Research has shown that most algae species have high adsorption capacity for Pb [9]. Brown algae could adsorb more metals (Zn, Cu, Cr, Ni, etc.) due to the high content of alginate. Generally speaking, algae have the weakest ability to adsorb

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metal nickel, except for *Microcystis aeruginosa* [10].

However, the individual size and cell density of microalgae are relatively small, and traditional methods for microalgae separation and harvesting not only require high energy or consume a lot of time, but also have poor reliability in separation and yield [6]. Fungal assisted microbial flocculation technology could effectively solve the problem of microalgae separation and recovery in wastewater treatment [11]. At the same time, research has found that the functional groups on the surface of fungi have strong adsorption capacity for heavy metals in water [12]. Therefore, microalgae-fungi symbionts have great potential for application in the removal of heavy metals in wastewater [6]. *Clonostachys* was an important fungi that can effectively reduce the occurrence and harm of soil borne diseases, promote plant growth, and increase yield [13]. The construction of *Chlorella-Clonostachys* symbiotic system was beneficial for its stability and growth. Therefore, this study constructed a *Chlorella-Clonostachys* symbiotic system as a heavy metal adsorption material, which can not only provide more biomaterials for the biological treatment of heavy metal wastewater, but also fill the gap in the application of microalgae symbiotic system in heavy metal wastewater.

As a new type of plant hormone, strigolactone (SL) is extracted from plant root exudates and can be used to regulate plant physiological metabolic processes and promote plant growth [14]. The induction of SL can also stimulate fungal mitochondrial metabolism and promote the branching and reproduction of arbuscular mycorrhizal fungi [15]. GR24 as a synthetic SL is considered to be involved in the biochemical reaction of algal cells. Research has found that when an appropriate amount of GR24 was added to the system, the relevant growth and photosynthetic performance parameters of *Chlorella vulgaris* were enhanced, thereby improving the removal performance of nutrients from wastewater in the co-culture system [16].

The microalgae-fungi symbionts technology has ecological and economic benefits in treating wastewater, but improving the removal efficiency of pollutants in wastewater is the key to its application in aquaculture wastewater purification. At present, the algae and fungi symbiotic technology of mainly focuses on the selection of algae species in improving wastewater treatment [17], optimization of light cultivation conditions [18], and optimization of microalgae fungal symbiotic system [19]. At present, the study of exogenous addition of GR24 enhancement of microalgae fungal symbiotic system treatment of heavy metals in wastewater is rarely reported.

In this study, wastewater was treated using two types of algal and fungal systems, including *Chlorella vulgaris* (marked as Treatment 1) and *Chlorella vulgaris-Clonostachys* symbionts (marked as Treatment 2). By changing the amount of exogenous GR24 added, we investigated the effects of GR24 concentration on the growth, photosynthetic performance, and removal efficiency performance of nutrients and heavy metals (Co, Cr, Cu, Mn, Ni, and Zn) from wastewater in two treatment systems. Based on the purification effect of wastewater, the best treatment process and the optimal GR24 dosage were selected. It can provide a basis for the application of microalgae-fungi symbionts technology in the field of aquaculture wastewater purification.

2. Methods and materials

2.1. Microalgae and fungi

Chlorella vulgaris (FACHB-8) was purchased from Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). In order to obtain a large number of *Chlorella vulgaris* cells in logarithmic growth phase, Blue-green 11 (BG11) liquid medium was inoculated in a light incubator (25 ± 2 °C), and cultured for 7 days under a cold white LED light source with a photosynthetic light quantum flux density of 6000 Lux. The L/D cycle was 12 h:12 h. The triangular flasks were shaken manually three times a day at 8 a.m., 3 p.m. and 10 p.m. in order to bring the cultured microalgae in full contact with the medium for faster and balanced growth.

Clonostachys was isolated from fresh soil collected from the Yushu region (96.6° e, 33.2° n) in Qinghai. In the ultra-clean bench, the soil was mixed with sterile water prepared in advance, and a small amount of supernatant diluent was inoculated into Potato Dextrose Broth (PDB) medium and cultured at 28 ± 1 °C for 48 h under sterile and lightless conditions. The main components of the PDB medium were 300 g L^{-1} of potato leaching powder, 20 g L^{-1} of glucose, and a final pH of 5.6 ± 0.2. Then, the fungi were purified to a single colony by a streaking method. After obtaining a single fungus, it was added to the PDB medium and cultured in a constant temperature shaker at 28 ± 1 °C and no light. The dominant strains were identified by Ezup column fungal genomic DNA extraction kit (No: SK8259, Sangon Bioengineering Shanghai, LTD.). The sequencing result was blasted by using NCBI BLAST software. Part of the mycelia of the identified pure strains were placed in PDB medium and cultured in a constant temperature shaker (28 ± 1 °C) with a speed of 160 rpm/min to obtain sufficient mycelia for subsequent experiments.

2.2. Preparation of microalgae-fungi symbionts

Chlorella vulgaris with a dry weight (DW) of 0.0227 ± 0.0002 g was centrifuged and added to BG11 medium. The microalgal DW was determined according to the following procedures: (1) 15 mL of the culture was filtered through a glass microfiber filter (GF/C, Whatman, USA); (2) the filter with attached microalgal cells was dried at 120 °C for 24 h and then cooled to room temperature in a desiccator; and (3) DW was determined by obtaining the differences between the filter weights before and after filtration. Then 75 strains of *Clonostachys* (dry weight ratio of 1:30) were added to the group medium. After the medium was adsorbed in the dark for 12 h,

Later, inoculum was cultivated in an illuminated incubator (28 ± 1 °C) for 7 days under LED light with a photosynthetic photon flux density of 6000 Lux, L/D cycle of 12 h:12 h.

2.3. Experiment of microalgal-fungal symbionts treating wastewater

2.3.1. Removal of conventional pollutants

Batch processing experiments were conducted in a 250 mL conical glass bottle. Swine wastewater was collected from Jiaxing, and the concentrations of total nitrogen (TN), total phosphorus (TP) and chemical oxygen demand (COD) were 99.66 ± 2.26 mg L^{-1}, 20.73 ± 0.66 mg L^{-1} and 1261.89 ± 88.26 mg L^{-1}, respectively. Firstly, 150 mL of swine wastewater was added into a conical flask. Subsequently, *Chlorella vulgaris* (marked as Treatment 1) and *Chlorella vulgaris-Clonostachys* symbionts (marked as Treatment 2) were added, with an initial concentration of 89.26 ± 3.17 mg L^{-1}. Then add different concentrations of GR24 (0 M (Control), 10^{-9} M, 10^{-7} M, and 10^{-5} M). The reactor was placed in a constant temperature shaker at 25 °C with the speed of 160 rpm, the illumination condition was 6000 Lux, and the illumination period was 12 h light and 12 h dark for 7 days. Three replicates were set for each group.

2.3.2. Removal of heavy metal

The removal of heavy metals was carried out in a 250 mL sterile conical flask. According to the tolerance of microalgae-fungi symbionts to heavy metal wastewater, two different heavy metal concentrations were set, and heavy metal wastewater was obtained by artificial preparation. Firstly, BG11 was prepared with ultrapure water and sterilized at 121 °C for 15 min. After cooling, the mother liquor of heavy metals was diluted to obtain two gradients of heavy metal mixed solution. In addition, sterile BG11 was added with ultrapure water to 200 mL as the experimental control group. Subsequently, *Chlorella vulgaris* and *Chlorella vulgaris-Clonostachys* symbionts were added, with an initial concentration of 89.26 ± 3.17 mg L^{-1}. Then, Culture systems exposed to concentrations of GR24 (0, 10^{-9}, 10^{-7}, and 10^{-5} M), and the experiment was performed under constant temperature (25 °C) with the incubated

at 160 rpm and 6000 Lux light (12 h/12 h light/dark cycle) conditions for 7 days to determine the removal of heavy metals. Three replicates were set for each group.

2.4. Analytical methods and definitions

2.4.1. Analysis of chlorophyll a (CHL-a)

The content of chlorophyll *a* (*CHL-a*) was assessed by Zhang et al.'s method [20]. First, 4 mL of microalgal-fungal mixture was centrifuged at 1000 ×*g* for 10 min. Then, the supernatant was discarded and the precipitate was washed with a vortex oscillator in 4 mL acetone (90 %). The precipitate was completely dissolved in acetone and placed in a dark environment at 4 °C for 24 h. After centrifugation (10 min, 10,000 ×*g*), the supernatant was collected and the absorbance was measured at 630, 645, 663 and 750 nm using a spectrophotometer (UV-1800PC, Aoyi Instrument Shanghai Co., Ltd), with acetone (90 %) used as a blank control. The content of *CHL-a* was obtained by Eq. (1):

$$Chl-a = [11.64 \times (OD_{663} - OD_{750}) - 2.16 \times (OD_{645} - OD_{750}) + 0.10 \times (OD_{630} - OD_{750})]V \quad (1)$$

where, OD_x represents the absorbance values of the test sample at different wavelength values X, and *V* represents the volume of the test sample.

2.4.2. Growth of two treatments

The 10 mL treatment solution was collected and filtered by PES membrane. The PES filter membrane and the blank filter membrane were dried at 105 °C for 6 h to constant weight. The dry weight of the dried cells was the weight of the filter membrane when the sample was stored minus the weight of the blank filter membrane. The specific growth rate (u, d^{-1}) and average daily productivity (P, g L^{-1} d^{-1}) of biomass were calculated according to Eqs. (2) and (3), respectively.

$$u = \frac{lnC_t - lnC_0}{t} \rightarrow lnC_t = ut + lnC_0 \quad (2)$$

$$P = \frac{DW_k - DW_0}{t_k - t_0} \quad (3)$$

where, C_t was biomass content (g L^{-1}) at the time point t (d), C_0 was the initial biomass content (g L^{-1}). DW_k was the biomass content at the time point of t_k (g L^{-1}) and DW_0 was biomass content at t_0 (g L^{-1}).

2.4.3. Photosynthetic performance test

Photosynthetic performance parameters, including F_V/F_M, PI_{ABS}, Ψ_O, Φ_{Eo}, Φ_{Do}, was measured by Photon Systems Instrument Aquapen (AP-C 100, Brno, Czech Republic). The 4 mL sample was placed in a cuvette equipped with the equipment for dark adaptation for 5 min, and then placed in a chlorophyll fluorescence tester to measure the rapid chlorophyll fluorescence kinetics (OJIP) parameters.

2.4.4. Calculation of nutrient and heavy metal removal efficiency

The treatment effects of wastewater were determined using samples on the 3rd, 7th, and 10th days of the experiment. Nutrients (COD, TN and TP) in wastewater were determined according to the standard analysis method [21].

The concentrations of heavy metals (Co, Cr, Cu, Mn, Ni, and Zn) were determined by ICP-MS. The details were as follows: the sewage cultured for 7 days was centrifuged at 10,000 ×*g* for 10 min, and the supernatant was filtered with a 0.45 μm filter membrane. The high concentration group was diluted 100 times with ultrapure water, and the low concentration group was diluted 10 times with ultrapure water (the detection limit of ICP-MS was 500 ppb). Concentrated nitric acid was added to make the test solution contain 5 % nitric acid to stabilize the heavy metal content of the sample, and then the sample was sequentially determined by ICP-MS.

Removal efficiency (RE) of nutrient and heavy metal were measured using Eq. (4):

$$RE = \left(1 - \frac{C_t}{C_0}\right) \times 100 \quad (4)$$

In Eq. (4), C_t and C_0 were the final concentration and initial concentration of COD, TN, TP and different heavy metals.

2.5. Statistical analyses

Each treatment group was measured three times in parallel. SPSS 19.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Duncan's multiple comparison method was used to determine the significant difference between treatments ($P < 0.05$).

3. Results and discussion

3.1. Effect of various GR24 treatments on pelletization

The effect of various GR24 treatments on pelletization of algal-fungal symbionts was showed in Table 1. Which showed that the addition of GR24 could effectively promote the formation of microalgae-fungi symbionts. When GR24 was not added, the symbiotic balls were white and green in color. After adding GR24, microalgae-fungi symbionts form uniform green balls, and the addition of GR24 could promote the reduction of the diameter of the balls. When the concentration of GR24 was 10^{-7} M, the diameter of the microalgae fungal symbionts ball formed is the smallest (3–4 mm). Therefore, GR24 with a concentration of 10^{-7} M was the optimal addition concentration for ball forming. A previous study showed that SLs as a class of potent phytohormones, can effectively promote fungal growth. As a more active SLs (GR24), its promotion effect on the growth of fungal branch number was obvious. The exogenous GR24 could effectively shorten the pelleting time of *Chlorella vulgaris* and fungi and showed stable pelleting effect [22].

3.2. CHL-a for the two strains under GR24 treatments

CHL-a is not only an important substance for photosynthesis of *Chlorella vulgaris*, but also one of the most commonly used indicators for judging microalgae biomass [23]. The change of *CHL-a* content under two algae-bacteria techniques is shown in Fig. 1. The results showed that the content of *CHL-a* in different treatments with GR24 was higher than that without GR24 on the 3rd, 7th and 10th day, indicating that GR24 promoted the synthesis of *CHL-a* in microalgae, which was inconsistent with previous studies [24]. With the increase of GR24 content (0, 10^{-9}, 10^{-7} and 10^{-5} M), the *CHL-a* content of the two treatments increased first and then decreased, reaching the maximum at GR24 concentration of 10^{-7} M. Under different concentrations of GR24, the *CHL-a* content of each experimental group reached the peak on the 7th day. With a GR24 concentration of 10^{-7} M, the *CHL-a* content of treatment 1 and treatment 2 reached the maximum values of 195.43 ± 17.42 μg L^{-1} and 241.17 ± 22.06 μg L^{-1}, respectively. Compared with the control group, it increased by 50.76 % and 50.88 % respectively. Therefore, the optimal concentration of GR24 to obtain the highest *CHL-a* content was 10^{-9} to

Table 1
Effect of various GR24 treatments on pelletization of algal-fungal symbionts.

GR24 treatments	0 M	10^{-5} M	10^{-7} M	10^{-9} M
Pelletization	Yes	Yes	Yes	Yes
Diameter (mm)	5–7	4–5	3–4	5–6
Color	White and green	Green	Green	Green
Pellets				

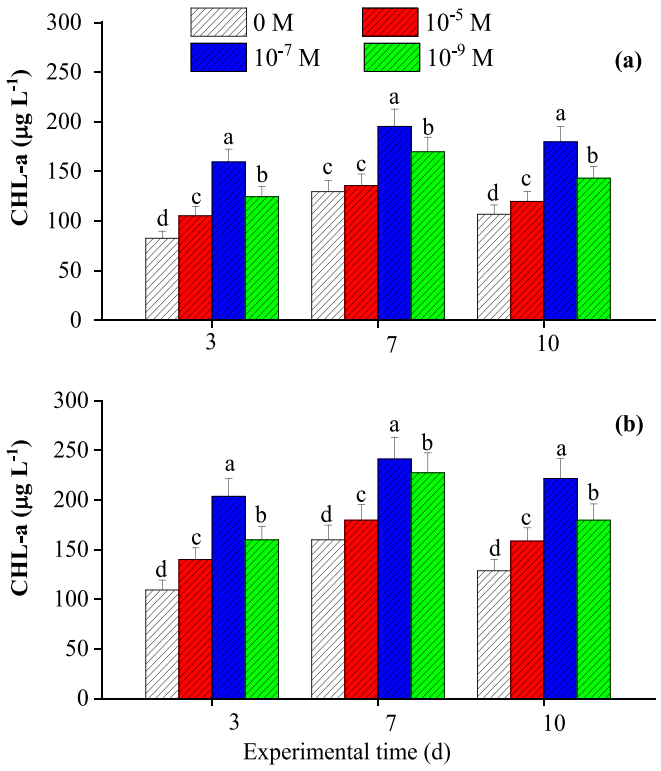

Fig. 1. The chlorophyll *a* content for the two strain under different GR24 concentrations at the 3rd, 7th and 10th days. Treatments 1, (b) Treatments 2.

10^{-7} M, which was consistent with a previous study [24]. This result may be due to the fact that exogenous GR24 could promote fungal mycelium branching and microalgae nutrient uptake, and was more conducive to the growth of symbiotic system.

3.3. Growth performances of the two strains under GR24 treatments

Growth rate and average daily productivity can reflect the growth of algae-bacteria system [25]. The growth rate and average daily productivity of the two treatments at different GR24 concentrations for 7 days are shown in Table 2. Compared with the control, the addition of GR24 significantly increased the average daily yield and growth rate of the two treatments ($P < 0.05$). Under different GR24 exogenous addition concentrations, the daily yield and growth rate of treatment 2 were higher than that of treatment 1. At the optimal GR24 level (10^{-7} M), treatment 1 and treatment 2 reached the maximum average daily yield and growth rate. Experiments showed that GR24 can promote the growth of microalgae. This result was very consistent with the change of *CHL-a* content (Fig. 1). It may be because GR24 could improve the electron transfer efficiency and increase the electron transfer yield by promoting the absorption of light by microalgae [26]. It could also promote the elongation of crown and root and the growth of fungal mycelium by promoting the action of auxin and hormone, and further increased the chance of contact between algae and fungi [27], which was conducive to the rapid formation of algal fungal symbionts.

3.4. Photosynthetic performances of the two strains under GR24 treatments

OJIP test is usually used to study the photosynthesis and stress of plants [28]. The photosynthetic performance of the two treatment systems was determined by OJIP method. Table 2 showed that in the two treatment systems, the F_V/F_M and PI_{ABS} values increased first and then decreased with the increase of GR24 concentration, and exceeded that of

Table 2
The Growth rates, average daily and photosynthetic parameters of the two treatments during the operational periods.

Treatments	Growth rate (d^{-1})	Average daily output (g L^{-1} d^{-1})	F_V/F_M	PI_{ABS}	Ψ_O	Φ_{Eo}
Treatment 1						
0 M	0.158^{d} ± 0.02	0.094^{c} ± 0.009	0.63^{b} ± 0.06	5.79^{b} ± 0.56	0.65^{b} ± 0.07	0.37^{b} ± 0.04
10^{-5} M	0.205^{c} ± 0.02	0.108^{c} ± 0.010	0.65^{b} ± 0.06	5.91^{b} ± 0.60	0.68^{b} ± 0.07	0.41^{b} ± 0.04
10^{-7} M	0.296^{a} ± 0.03	0.149^{a} ± 0.012	0.74^{a} ± 0.07	6.35^{a} ± 0.61	0.89^{a} ± 0.09	0.59^{a} ± 0.06
10^{-9} M	0.271^{b} ± 0.03	0.132^{b} ± 0.011	0.71^{a} ± 0.07	6.19^{ab} ± 0.62	0.85^{a} ± 0.09	0.54^{a} ± 0.05
Treatment 2						
0 M	0.214^{c} ± 0.02	0.113^{d} ± 0.010	0.75^{b} ± 0.08	6.09^{b} ± 0.58	0.77^{b} ± 0.08	0.56^{b} ± 0.06
10^{-5} M	0.246^{c} ± 0.02	0.134^{c} ± 0.012	0.79^{b} ± 0.08	6.15^{b} ± 0.62	0.84^{b} ± 0.08	0.61^{b} ± 0.06
10^{-7} M	0.367^{a} ± 0.04	0.177^{a} ± 0.016	0.92^{a} ± 0.09	7.28^{a} ± 0.71	0.98^{a} ± 0.09	0.71^{a} ± 0.07
10^{-9} M	0.313^{b} ± 0.03	0.154^{b} ± 0.014	0.89^{a} ± 0.09	6.94^{a} ± 0.68	0.95^{a} ± 0.09	0.69^{a} ± 0.07

Note: The data in the table were represented mean ± standard deviation ($n = 3$). Data with different superscript letters showed obvious difference ($P < 0.05$).

blank control. Ψ_O and Φ_{Eo} showed similar trends to F_V/F_M and PI_{ABS}, indicating that GR24 not only promoted the absorption of light by microalgae, but also improved the electron transfer efficiency and maximum electron transfer yield. The photosynthetic performance parameters of the two treatments reached the maximum when the GR24 concentration was 10^{-7} M. The photosynthetic performance parameters showed that treatment 2 > treatment 1, with significant difference ($P < 0.05$). This may be because GR24 could promote the absorption of light by microalgae, improve electron transfer efficiency and maximum electron transfer yield [29].

3.5. Removal of nutrient under different GR24 concentrations

Under different GR24 addition concentrations, the RE-COD, RE-TN and RE-TP by treatment 2 on the 3rd, 7th and 10th days were shown in Fig. 2. As shown in Fig. 2(a), the addition of GR24 significantly increased the RE-COD of treatment 2. With the increase of GR24 content (0, 10^{-9}, 10^{-7}, and 10^{-5} M), the RE-COD of treatment 2 increased first and then decreased, and reached the maximum at a GR24 concentration of 10^{-7} M. Under different concentrations of GR24 exogenous addition, the RE-COD of each experimental group reached the peak on the 7th day, and the RE-COD reached the maximum (78.25 %) at a GR24 concentration of 10^{-7} M, which was 22.22 % higher than the control. Table 3 showed that the average ER-COD in treatment 2 was better than that in treatment 1. Therefore, the RE-COD based on the microalgae-fungi symbionts was significantly better than that of single microalgae. The results of RE-COD were similar to that observed by Zhang et al. [16]. The main mechanism for removing COD was carbon assimilation, *Chlorella vulgaris* produces O_2 through photosynthesis under light conditions, which was used by fungi [30]. The mutual synergy between the two accelerates the removal of COD in the system; At the same time, *Chlorella vulgaris* can use heterotrophic mode to remove some organic carbon under dark conditions [31], but the utilization rate of organic carbon source in a single *Chlorella vulgaris* system without fungi was lower than that of microalgae-fungi symbionts [32].

The TN and TP removal efficiency in treatment 2 are shown in Fig. 2b and c. RE-TN and RE-TN of treatment 2 showed the same trend as the change of COD. And RE-TN and RE-TN of treatment 2 first increased and then decreased, reaching its maximum (TN 82.24 %, TP 84.18 %) on the

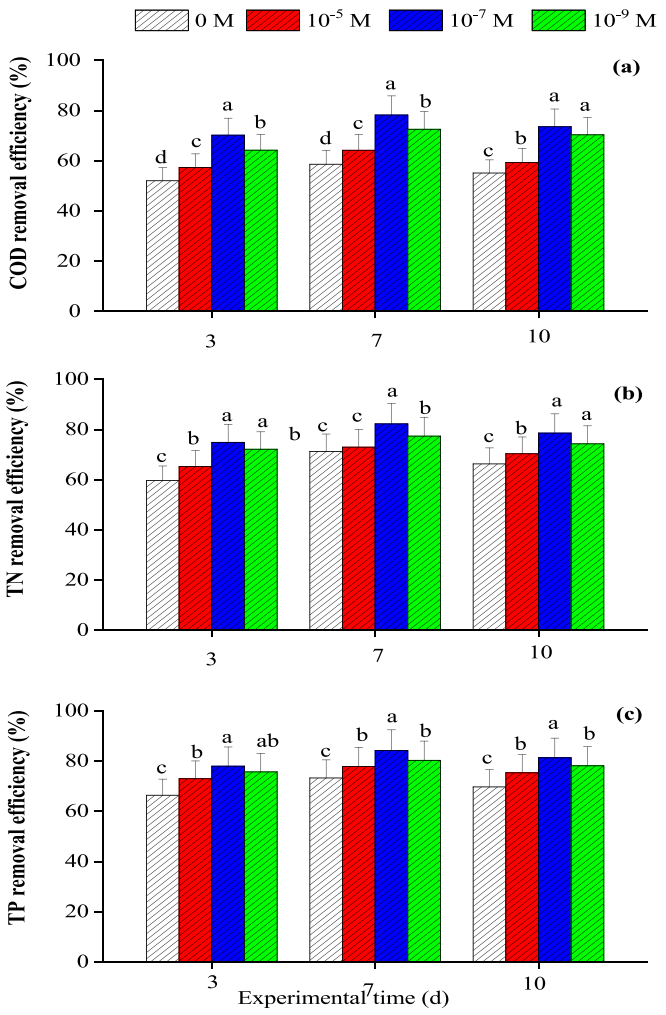

Fig. 2. Removal efficiencies of COD, TN and TP under different GR24 concentrations for Treatment 2 on the 3rd, 7th and 10th days. (a) COD removal efficiency, (b) TN removal efficiency, and (c) TP removal efficiency.

Table 3
Average values ± standard deviation of the RE-COD, TN and TP of the two treatments with different contents of GR24.

Treatments	Removal efficiency (%)		
	COD	TN	TP
Treatment 1			
0 M	63.19^{c} ± 5.98	64.05^{b} ± 6.13	65.26^{b} ± 6.37
10^{-5} M	68.56^{b} ± 6.34	66.18^{b} ± 7.16	68.35^{b} ± 6.58
10^{-7} M	75.25^{a} ± 6.97	76.14^{a} ± 7.32	76.19^{a} ± 7.32
10^{-9} M	70.51^{b} ± 6.82	73.26^{a} ± 7.17	73.08^{a} ± 7.11
Treatment 2			
0 M	69.74^{b} ± 6.68	71.59^{b} ± 6.75	72.63^{b} ± 7.09
10^{-5} M	73.57^{b} ± 6.89	74.34^{b} ± 7.28	75.09^{b} ± 7.34
10^{-7} M	81.96^{a} ± 7.93	80.26^{a} ± 7.91	82.96^{a} ± 8.05
10^{-9} M	79.48^{a} ± 7.72	78.53^{a} ± 7.65	79.28^{a} ± 7.83

Note: Data with different superscript letters showed obvious difference ($P < 0.05$) on the basis of the Duncan's multiple range tests to the same selected strains under different treatments.

7 day when the concentration of GR24 was 10^{-7} M, which were 12.11 %, and 14.22 % higher than those without the addition of GR24, respectively. Table 3 showed that the average RE-TN and RE-TP in treatment 2 was better than treatment 1. Compared with a single cultivation system for microalgae, RE-TN and RE-TP based on microalgae-fungi symbionts were very significant. After adding GR24, the metabolism and microalgae photosynthesis of the two treatments were enhanced, and the system grew rapidly, which further improved the purification performance of the symbionts [24]. This may be related to the diversity of fungal algae particles and the conditions induced by GR24. The fungal-microalgae combined system provides better wastewater purification effect than the single microalgae system, which may be due to the interaction between fungal hyphae and microalgae cells during the treatment process [16]. In addition, GR24 could promote the growth of microalgae and fungal filaments in microalgae fungal complexes, improving the purification effect of the complex on wastewater [33].

Overall, the removal of nutrients from wastewater can be attributed to the physiological synergies between algae and fungi in the symbiotic system [24]. Algae can absorb inorganic nutrients such as nitrogen and phosphorus to synthesize organic matter and release some inorganic matter to the surrounding; as an important decomposer. Fungi can decompose and utilize the organic substances secreted by algae as well as dead algal cells, and their decomposition products can be absorbed and utilized by algae. In addition, the oxygen released by algal photosynthesis provided a rich source of oxygen for the growth of the fungus, while the CO_2 released by the fungus metabolism became the main source of carbon for the algae, which in turn promoted the photosynthesis of the algae [27]. The synergistic effect between fungi and algae can be utilized to construct an algal purification system, which can realize efficient nutrient removal.

3.6. *Effects of the heavy metal removal under different GR24 concentrations*

3.6.1. *Co removal*

Fig. 3a shows the effect of exogenous GR24 with different concentrations on Co in the two treatments at different heavy metal concentration levels. Regardless of the low concentration level (1 mg L^{-1}) and high concentration level (10 mg L^{-1}), the RE-Co by treatment 2 was better than treatment 1. The GR24 concentration of 10^{-9} M propelled the RE-Co to 65.23 %. When the concentration of GR24 was 10^{-9} M and 10^{-5} M, the absorption of Co by algae-bacteria symbiosis reached the highest, reaching 25.520 % and 25.645 %, respectively (Fig. 3a). Table 4 further analyzed the average RE-Co in two treatments with different concentrations of GR24 added. Which showed that the RE-Co by treatment 2 was better than treatment 1, and the average value of RE-Co reached its maximum at both low and high concentration levels when the addition amount of GR24 was 10^{-9} M, with 68.36 % and 32.44 %, respectively. Study showed that the RE-Co by *Chlamydomonas reinhardtii* with cell wall and *Chlamydomonas reinhardtii* without cell wall reached 0.89 mg g^{-1} and 1.3 mg g^{-1} [34]. In this paper, the removal effect of *Chlorella vulgaris* on Co was better than that of *Chlamydomonas reinhardtii*. Due to the different types of microalgae used, *Chlorella* had stronger purification ability to Co than *Chlamydomonas reinhardtii*.

3.6.2. *Cr removal*

The RE-Cr in the two treatments is shown in Fig. 3b. Regardless of the low concentration level (1 mg L^{-1}) and high concentration level (10 mg L^{-1}), the RE-Cr by treatment 2 was better than that of treatment 1. After 7 days of measurement, with a GR24 concentration of 10^{-5} M, the highest RE-Cr were achieved in both the low concentration and high concentration groups, with 60.12 % and 78.38 %, respectively. With the increase of GR24 concentration (0, 10^{-9}, 10^{-7}, and 10^{-5} M) the average RE-Cr in treatment 1 increased gradually. Treatment 2 had no obvious regularity and reached the maximum when GR24 concentration was 10^{-5} M (Table 4). Therefore, with a GR24 concentration of 10^{-5} M, the treatment effect of microalgae and algae-bacteria symbionts on Cr could be greatly improved. Therefore, the removal effect of Cr was optimal at this concentration. It was speculated that metal ions could undergo biosorption and bioaccumulation with microalgae. Metal ions could undergo adsorption reactions with functional groups on the surface of

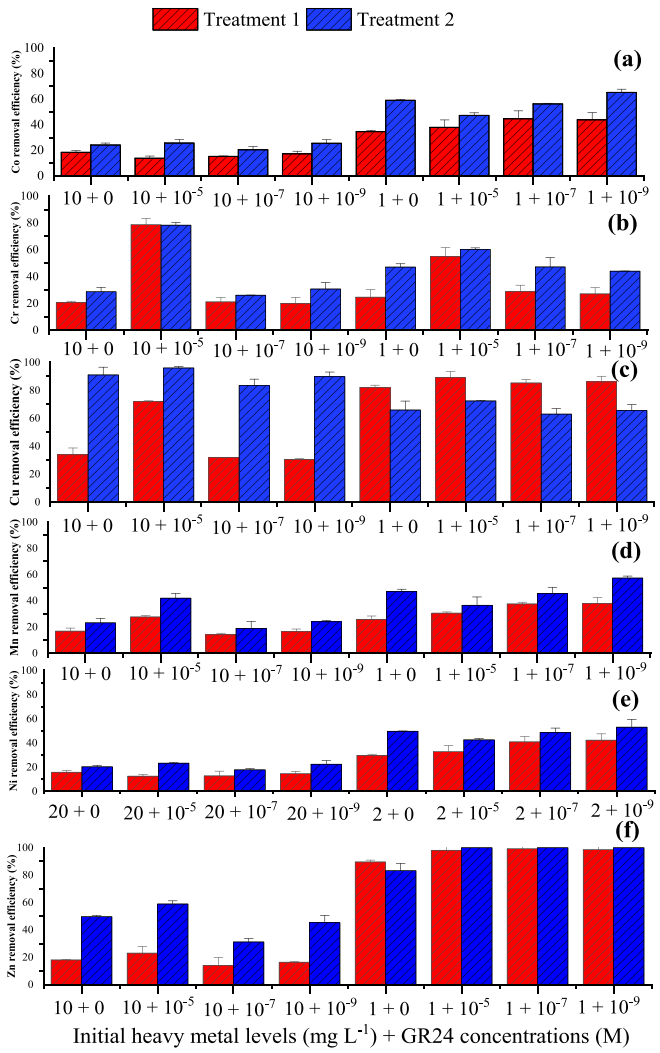

Fig. 3. The removal efficiency of Co, Cr, Cu, Mn, Ni and Zn under different GR24 concentrations for Treatment 2 on the 7th days. (a) Co removal efficiency, (b) Cr removal efficiency, (c) Cu removal efficiency, (d) Mn removal efficiency, (e) Ni removal efficiency, (f) Zn removal efficiency.

microalgae cells, and actively transfer to cells through cells, accumulating in organisms [35].

3.6.3. Cu removal

The results of Fig. 3c indicated that under low Cu concentration conditions, the RE-Cu by Treatment 1 was higher than that of Treatment 2. At a GR24 concentration of 10^{-5} M, the highest RE-Cu by single microalgae reached 89.37 %. In the high concentration heavy metal experimental group, treatment 2 had better RE-Cu than treatment 1 with a GR24 concentration of 10^{-5} M, the RE-Cu of microalgae-fungi symbionts was the highest, reaching 96.04 %. From Table 4, it can be seen that the average value of RE-Cu showed that treatment 2 was significantly better than treatment 1, and the maximum average value of RE-Cu reached 96.32 % with a GR24 of 10^{-5} M. it can be found that the GR24 concentration of 10^{-5} M can significantly improve the copper removal efficiency of single microalgae in high concentrations of heavy metals. There have been many studies on the use of microalgae for Cu purification in the past. One study used ordinary microalgae to remove Cu up to 76.71 mg g^{-1} within 1 h [36]. The construction of microalgae-fungi symbionts could further improve the RE-Cu in heavy metal wastewater under long-term high concentration conditions.

3.6.4. Mn removal

The RE-Mn by *Chlorella vulgaris* and *Chlorella vulgaris-Clonostachys symbionts* is shown in Fig. 3d. It was found that treatment 2 had better RE-Mn than treatment 1. When the concentration of exogenous GR24 was 10^{-9} M and 10^{-5} M, the highest RE-Mn reached 57.42 % and 42.03 %. It was worth noting that when the concentrations of GR24 were 10^{-7} M and 10^{-9} M, the effects on the removal efficiency of microalgae-fungal symbionts and single *Chlorella* were different. It can be seen from Table 4, when different concentrations of GR24 were added externally, the average value of RE-Mn in treatment 2 was significantly better than that in treatment 1 at both low and high concentration levels. Moreover, the average value of RE-Mn reached its maximum (58.74 %) at a low concentration (10^{-9} M) of GR24, and reached its maximum (46.27 %) at a high concentration (10^{-5} M) of GR24. It was speculated that the addition of plant hormones affects the physiological processes of *Chlorella* or algal fungal symbionts, thereby affecting their absorption of cobalt in the environment, which was very similar with the RE-Co.

3.6.5. Ni removal

The RE-Ni by Treatment 1 and Treatment 2 is shown in Fig. 3e. The research results showed that at low concentration levels, the RE-Ni by microalgae-fungi symbionts was better than that of single microalgae. When the concentration of GR24 was 10^{-9} M, RE-Ni reached the highest value of 53.13 %. At high concentrations (GR24 concentration of 10^{-5} M), the RE-Ni by the Treatment 2 reached the highest (23.42 %). As shown in Table 4, when different concentrations of GR24 were added externally, the average value of RE-Ni in treatment 2 was significantly better than that in treatment 1 at both low and high concentration levels. Moreover, the average value of RE-Ni reached its maximum (56.17 %) at a low concentration (10^{-9} M) of GR24, and reached its maximum (29.25 %) at a high concentration (10^{-5} M) of GR24. Previous studies had shown that ordinary *Chlorella* can achieve a removal effect of 15.4 mg g^{-1} of Ni [37]. The treatment results obtained from a single-microalgae in this experiment were slightly lower than this value, possibly due to the stress of other toxic metals on the microalgae. The construction of algal fungal symbionts and the addition of GR24 resulted in better treatment effects than reported in literature.

3.6.6. Zn removal

According to Fig. 3g, the RE-Zn by adding GR24 to the low concentration heavy metal experimental group was basically 100 % for *Chlorella vulgaris* and *Chlorella vulgaris-Clonostachys* symbionts. RE-Zn of microalgae-fungi symbionts was the best at the GR24 of 10^{-5} M, reaching the highest treatment rate for Zn. As shown in Table 4, when different concentrations of GR24 were added, the average value of RE-Cu showed that treatment 2 was significantly better than treatment 1 at both low and high concentration levels. Moreover, when the amount of GR24 added was 10^{-5} M, the average value of RE-Zn reached its maximum, reaching 62.35 % and 99.32 %, respectively. Compared to the previous use of ordinary *Chlorella* for Zn removal, the highest removal efficiency of 24.5 mg g^{-1} was achieved through different treatment conditions [38]. The treatment effect of a single microalgae on Zn in this experiment was also lower than the reported value in literature. Due to the stress of other heavy metals on *Chlorella vulgaris* in the experiment, the treatment effect of Zn after constructing a microalgae-fungi symbionts system and adding GR24 was once again demonstrated by previous reports, which demonstrated that exogenous GR24 and constructing an algal fungal symbiotic system can promote the removal of pollutants.

Compared with the previous study [1,39], the removal of heavy metals by microalgae-fungi symbiosis under GR24 addition was significantly enhanced. The possible reasons were: (1) a more stable symbiotic system was formed between microalgae and fungi, and the ability of co-metabolism and resistance to heavy metal stress was enhanced; (2) GR24 could promote the branching of fungal hyphae and increase the probability of mycelial encapsulation of algal cells, which played a key

Table 4
The removal efficiency of heavy metal for 7 days of the two treatments.

	RE-Co (%)		RE-Cr (%)		RE-Cu (%)		RE- Mn (%)		RE-Ni (%)		RE-Zn (%)	
Contents	10 mg L^{-1}	1 mg L^{-1}	10 mg L^{-1}	1 mg L^{-1}	10 mg L^{-1}	1 mg L^{-1}	10 mg L^{-1}	1 mg L^{-1}	20 mg L^{-1}	2 mg L^{-1}	10 mg L^{-1}	1 mg L^{-1}
Treatment 1												
0 M	$22.15^{b} \pm 1.83$	$34.25^{c} \pm 2.77$	$22.19^{c} \pm 1.87$	$26.39^{c} \pm 2.42$	$38.29^{b} \pm 2.99$	$83.09^{c} \pm 7.96$	$18.46^{b} \pm 1.63$	$29.35^{b} \pm 2.64$	$17.32^{a} \pm 1.63$	$32.28^{b} \pm 3.01$	$22.57^{ab} \pm 2.06$	$88.06^{b} \pm 8.12$
10^{-5} M	$32.96^{a} \pm 2.18$	$37.56^{c} \pm 3.24$	$81.05^{a} \pm 7.26$	$58.65^{a} \pm 5.37$	$75.21^{a} \pm 7.25$	$91.48^{a} \pm 8.96$	$28.09^{a} \pm 2.51$	$31.39^{b} \pm 2.75$	$12.09^{bc} \pm 1.06$	$32.57^{b} \pm 3.14$	$23.95^{a} \pm 2.21$	$96.29^{a} \pm 2.91$
10^{-7} M	$16.03^{c} \pm 1.24$	$46.87^{a} \pm 4.32$	$26.78^{b} \pm 2.32$	$37.84^{b} \pm 3.53$	$33.28^{c} \pm 3.12$	$86.32^{bc} \pm 8.43$	$13.27^{c} \pm 1.25$	$36.92^{a} \pm 3.21$	$11.26^{c} \pm 0.87$	$41.27^{a} \pm 3.71$	$13.82^{c} \pm 1.26$	$99.02^{a} \pm 0.84$
10^{-9} M	$19.05^{bc} \pm 1.52$	$42.16^{b} \pm 3.95$	$23.54^{bc} \pm 2.21$	$28.44^{c} \pm 2.65$	$28.36^{d} \pm 2.64$	$88.19^{ab} \pm 8.62$	$16.45^{bc} \pm 1.42$	$38.24^{a} \pm 3.67$	$15.93^{ab} \pm 1.44$	$43.28^{a} \pm 4.19$	$18.67^{b} \pm 1.75$	$95.32^{a} \pm 4.19$
Treatment 2												
0 M	$29.63^{a} \pm 2.25$	$61.28^{b} \pm 5.73$	$33.25^{b} \pm 3.06$	$52.39.^{c} \pm 4.87$	$91.35^{b} \pm 8.77$	$69.57^{bc} \pm 6.71$	$25.04^{b} \pm 2.19$	$45.39^{b} \pm 4.26$	$22.73^{b} \pm 2.15$	$52.31^{b} \pm 4.98$	$44.39^{b} \pm 4.22$	$84.27^{b} \pm 8.15$
10^{-5} M	$24.06^{b} \pm 1.87$	$49.39^{d} \pm 4.45$	$78.29^{a} \pm 7.24$	$82.53^{a} \pm 7.62$	$96.32^{a} \pm 9.41$	$75.68^{a} \pm 7.45$	$46.27^{a} \pm 4.35$	$36.07^{c} \pm 3.28$	$29.25^{a} \pm 2.69$	$43.55^{c} \pm 3.87$	$62.35^{a} \pm 5.84$	$99.32^{a} \pm 0.43$
10^{-7} M	$22.76^{b} \pm 1.68$	$57.23^{c} \pm 5.21$	$27.25^{c} \pm 2.53$	$55.32^{c} \pm 5.21$	$82.73^{c} \pm 8.02$	$65.26^{c} \pm 6.24$	$20.34^{c} \pm 1.69$	$43.55^{b} \pm 4.06$	$17.38^{c} \pm 1.56$	$48.94^{bc} \pm 4.62$	$33.64^{c} \pm 3.02$	$99.19^{a} \pm 0.58$
10^{-9} M	$32.44^{a} \pm 2.41$	$68.36^{a} \pm 6.34$	$37.19^{b} \pm 5.98$	$47.59^{b} \pm 4.37$	$89.27^{b} \pm 8.56$	$70.56^{b} \pm 6.87$	$27.05^{b} \pm 2.58$	$58.74^{a} \pm 5.62$	$26.57^{a\ b} \pm 2.34$	$56.17^{a} \pm 5.36$	$42.26^{b} \pm 4.15$	$98.17^{a} \pm 1.14$

Note: The data in the table were represented mean ± standard deviation (n = 3). Data with different superscript letters showed obvious difference ($P < 0.05$).

role in the process of removing heavy metals by the algal-fungal symbiosis, and enhanced the removal efficiency of the heavy metals in the system.

4. Conclusions

In this study, the removal efficiency of nutrients and heavy metals in wastewater by two treatment systems was evaluated by exogenous GR24. The photosynthesis and biomass of the *Chlorella vulgaris-Clonostachys* symbionts were significantly improved. In addition, GR24 enhanced the removal effect of nutrients and heavy metals, especially when GR24 was supplemented at 10^{-5} M concentration. This study provided data support and theoretical basis for the construction of algal fungal symbionts and their application in wastewater treatment.

CRediT authorship contribution statement

Chunzhi Zhao: Writing – original draft, Writing – review & editing. **Zhengfang Wang:** Investigation, Writing – original draft. **Huayun Yang:** Methodology, Data curation, Formal analysis. **Bei Lu:** Methodology, Formal analysis. **Hui Zhang:** Conceptualization, Data curation, Formal analysis. **Yongjun Zhao:** Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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