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New Insight into the Performance and Self-Defensive Responses of the Algal–Bacterial Granular Sludge Process under Cr(VI)-Induced Stress

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Abstract: Algal–bacterial granular sludge, a new biological technology, has been widely recognized due to its highly effective pollutant treatment and energy efficiency. This study investigated the effects of environmental concentrations of Cr(VI) (0.5–2.5 mg/L) on the performance of algal–bacterial granular sludge and self-defensive responses after 90 days of cultivation. The results showed that Cr(VI) affected chemical oxygen demand (COD) decrease, ammonia-N and phosphate removal, with different trends being apparent. A linear decline in COD decrease was observed, whereas an initial decrease and then increase in ammonia-N and phosphate removal took place. Algal–bacterial granular sludge effectively removed Cr(VI) from wastewater through biological adsorption and reduction, showing the potential to treat Cr(VI)-contaminated wastewater. Cr(VI) affected the community abundance of the algal–bacterial granular sludge, in which *Chlorophyceae* and cyanobacteria were vulnerable under Cr(VI)-induced stress. To reduce the toxicity of Cr(VI), over-produced EPS-PN and antioxidant enzymes (MDA, SOD and CAT) acted as self-defensive responses to resist oxidative damage. This study aimed to conduct a comprehensive environmental sustainability assessment of the algal–bacterial granular sludge process in treating municipal wastewater containing Cr(VI). It is hoped that this study can provide useful information for improved engineering feasibility of algal–bacterial granular sludge.

Keywords: algal–bacterial granular sludge; hexavalent chromium; extracellular polymeric substances; antioxidant enzymes; self-defensive responses

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1. Introduction

As a new type of biological wastewater treatment approach, algal–bacterial granular sludge technology has attracted extensive attention [1]. It has been reported that the algal–bacterial granular sludge process can effectively remove nutrients and pollutants in wastewater under simulated light and dark cycling. In contrast to traditional wastewater treatment technologies, algal–bacterial granular sludge relies on the symbiotic relationship between algae and bacteria, i.e., aerobic bacteria use the oxygen released by algal photosynthesis to convert organic carbon into carbon dioxide, while algae use inorganic nitrogen and phosphate for photosynthesis to synthesize intracellular components for their own growth and reproduction. In this process, released oxygen is used as an electronic receptor for bacteria to degrade organic matter and phosphorus [2]. The synergistic cycle between algae and bacteria can effectively improve the removal efficiency of nutrients and reduce energy consumption and greenhouse gas (GHG) emissions [3,4].

At present, in the research focused on the application of algal–bacterial granular sludge for wastewater treatment, most of the influent water used in laboratory-scale experiments is simulated wastewater with simple and controlled compositions, i.e., the carbon source is provided by sodium acetate, glucose or sucrose, the nitrogen source is provided by ammonium chloride, and the phosphorus source is provided by phosphate. Under these conditions, the algal–bacterial granular sludge can operate stably for a long time and meet the effluent quality requirements [5]. In reality, the composition of actual wastewater is complex, and the understanding of the impact of some toxins in wastewater on the engineering feasibility of algal–bacterial granular sludge implementation is limited. As reported in our previous studies, the abundance of algae in algal–bacterial granular sludge was reduced, and the symbiotic relationship between algae and bacteria was destroyed under the stress of 1–10 mg/L of Cd(II) [6]. To cope with this, increased polysaccharide (PS) from 37 ± 4 mg/g VSS to 110 ± 5 mg/g VSS was secreted [6]. Research into algal–bacterial granular sludge effectiveness in the presence of other toxic heavy metals has also been conducted (Cr(VI) and Pb (II)) [7–11], but these studies mainly focused on the adsorption and bioremediation of heavy metals.

Increased industrial activities have resulted in increased discharge of heavy metal-containing wastewater. As one of the most widely used metals in the industry, chromium, mainly as Cr(VI), deserves attention. Cr(VI) can cause various acute and chronic diseases and affects the metabolism and normal function of living cells [12]. Due to the adverse effects of Cr(VI) on microorganisms, Cr(VI) may cause irreversible damage to wastewater biological treatment systems and can adversely influence effluent quality. The World Health Organization (WHO) and the United States Environmental Protection Agency (USEPA) have recommended limits of 0.05 mg/L and 0.10 mg/L for Cr(VI) concentrations in drinking and surface waters [13]. However, the concentration of Cr(VI) in wastewater is generally significantly higher than the specified threshold.

To enhance the feasibility of engineering implementation of algal–bacterial granular sludge, some questions need to be answered: (i) What is the performance of algal–bacterial granular sludge in treating wastewater containing Cr(VI)? (ii) Will the effluent water meet the standard of <0.05 mg Cr(VI)/L? (iii) How stable is the algal–bacterial granular sludge under prolonged operation time? Compared to the conventional bacterial aerobic granular sludge, algal–bacterial granular sludge has been used as an adsorbent for Cr(VI) from wastewater, showing advantages in both biosorption capacity and granular stability [10]. However, there is little information concerning the performance of algal–bacterial granular sludge under Cr(VI) stress and the resulting adaptive responses. Consequently, the main purpose of this study was to explore the impact of Cr(VI) at environmentally relevant concentrations on nutrient (carbon, nitrogen and phosphorus) removal and adaptive responses, including microbial community and physiological changes of the algal–bacterial granular sludge. It is expected that this study can provide useful information for advanced research and practical application of algal–bacterial granular sludge technology.

2. Materials and Methods

2.1. Wastewater and Algal–Bacterial Granular Sludge

The simulated wastewater was composed of NaAc (446 mg/L), NH_4Cl (115 mg/L), KH_2PO_4 (22 mg/L), $FeCl_3$ (10 mg/L), $CaCl_2$ 10 (mg/L), $MgSO_4{\cdot}7H_2O$ (10 mg/L) and trace elements (1 mg/L). The stock solution of trace elements had the following components: 10 g/L of EDTA, 100 mg/L of $MnSO_4{\cdot}H_2O$, 30 mg/L of $CuSO_4{\cdot}5H_2O$, 120 mg/L of $ZnSO_4{\cdot}7H_2O$, 150 mg/L of H_3BO_3, 60 mg/L of $Na_2MnO_4{\cdot}2H_2O$, 180 mg/L of KI, and 150 mg/L of $CoCl_2{\cdot}6H_2O$. The algal–bacterial granular sludge used in the present study was cultivated using the methods described in our previous publications [14].

2.2. Batch Experiments

In the batch experiments conducted, in a series of microreactors with diameter of 47 mm and height of 60 mm, 5 mL of fresh algal–bacterial granular sludge was added into

50 mL of simulated wastewater, corresponding to a volatile suspended solids (VSS) concentration of 23.9 ± 0.2 g/L. The reactors were exposed to full-wavelength LED illumination of 10,000 lux. The LED light was 10 cm above the reactors. The initial Cr(VI) concentrations were 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L. Cr(VI)-free culture was set as the control group. The six series of batch experiments were done in triplicate and conducted under alternate light and dark cycles of 8 h/16 h at room temperature. The hydraulic retention time (HRT) was 24 h. The initial pH was 7.0 ± 0.2. Samples were taken after 8 h of light and were filtered through a microporous membrane (0.45 μm) for further analysis. No external aeration, stirring or shaking was provided.

2.3. Analysis Methods

Chemical oxygen demand (COD), ammonia-N and phosphate-P were determined according to standard methods [15]. A PHS-3E pH meter (INESA Scientific Instrument Co., Shanghai, China) was used to measure solution pH. The concentrations of Cr(VI) and total Cr were quantified using the diphenylcarbazide spectrophotometric method with a UV-Visible spectrophotometer (TU-1810, Puxi General. Instrument Co., Beijing, China) at 540 nm and a PinAAcle900F atomic absorption spectrophotometer (PerkinElmer, Waltham, MA, USA), respectively. Chlorophyll a and b were extracted using a mixture of acetone and ethanol (7:2) to quantify their contents in the algal–bacterial granular sludge [16].

Extracellular polymeric substances (EPS) were extracted by a modified heat extraction method, which was described in our previous study [17]. EPS-polysaccharide (EPS-PS) content was measured using the phenol-sulfuric acid method [18]. EPS-protein (EPS-PN) content was measured using C504031 Lowry Protein Assay Kits (Sangon Biotech, Shanghai, China) according to the instructions. EPS compositions were further analyzed using a DM4000B Fluorescence Spectrophotometer (Leica, Wetzlar, Germany) with 250–400 nm excitation wavelength and 280–550 nm emission wavelength to obtain three-dimensional excitation–emission (3D-EEM) spectrograms.

The algal–bacterial granular sludge samples were collected after 90 days of culture for microbial community analysis. DNA in samples was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The quality of total DNA was checked by 0.8% agarose gel electrophoresis and further quantified. The 16S rRNA and 18S rRNA genes were amplified using 515F/907R prokaryotic primers targeting the V4-V5 region of the 16S rRNA gene and 528F/706R eukaryotic primers targeting the V4 region of the 18S rRNA gene, respectively [6]. The purified amplicons were collected in an equimolar manner on the Illumina MiSeq platform and sequenced by Meiji Biopharmaceutical Technology Co., Ltd. (Shanghai, China).

Fresh algal–bacterial granular sludge was pretreated according to the method described in a previous study [6] to obtain dry powders. Morphology and surface compositions were analyzed using a JSM 7200F scanning electron microscope (SEM) (JEOL, Tokyo, Japan) equipped with energy-dispersive spectroscopy (EDS). The surface element compositions were analyzed using X-ray photoelectron spectroscopy (XPS. Axis-Ultra DLD) (Kratos, Manchester, UK) employing a monochromatic Kα X-ray source.

Algal–bacterial granular sludge was frozen in liquid nitrogen and ground to paste, then mixed with phosphate buffer and centrifuged for 10 min to obtain the supernatant as a crude enzyme solution. The contents of malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) were determined using A003-1, A001-1 and A007-1 kits (NanJing Jiancheng Bioengineering Institute, Nanjing, China), respectively, according to the instructions.

3. Results and Discussion

3.1. Performance of the Algal–Bacterial Granular Sludge Process on Nutrient Removal under Cr(VI) Stress

The performance of the algal–bacterial granular sludge process in terms of COD decrease, ammonia-N and phosphate-P removal is shown in Figure 1. After ninety days

of operation, COD decrease was 75%, 36%, 15%, 11%, 6% and 4% ($p < 0.05$) at Cr(VI) concentrations of 0–2.5 mg/L, indicating that COD decrease was inhibited by Cr(VI). Compared to Cd(II), the tolerance threshold level of algal–bacterial granular sludge to Cr(VI) was less, evidenced by the result that 1 mg/L Cd(II) had an insignificant effect on COD decrease [6], while 0.5–1.0 mg/L Cr(VI) greatly inhibited COD decrease. Ammonia-N removal showed different trends in the control as compared to Cr(VI)-exposed experiments. In the first fifteen days in the control, ammonia-N removal increased from 62% to 97%. This may be due to self-adaptation to experimental conditions. Thereafter, ammonia-N removal was stable at about 95%. For the experimental groups, ammonia-N removal decreased in the first twenty-five days and then increased. It should be noted that ammonia-N removal decreased with increasing Cr(VI) concentrations (i.e., 0.5–2.5 mg/L) ($p < 0.05$), indicating a slightly inhibitory effect of Cr(VI) on ammonia-N removal. As for phosphate-P removal, a similar trend was observed in the control; that is, in the first ten days, phosphate-P removal increased from 35% to 74% and thereafter was stable at about 80%. In the Cr(VI)-added experiments, phosphate-P removal tended to linearly increase with cultivation time, with an insignificant difference between different Cr(VI) concentrations.

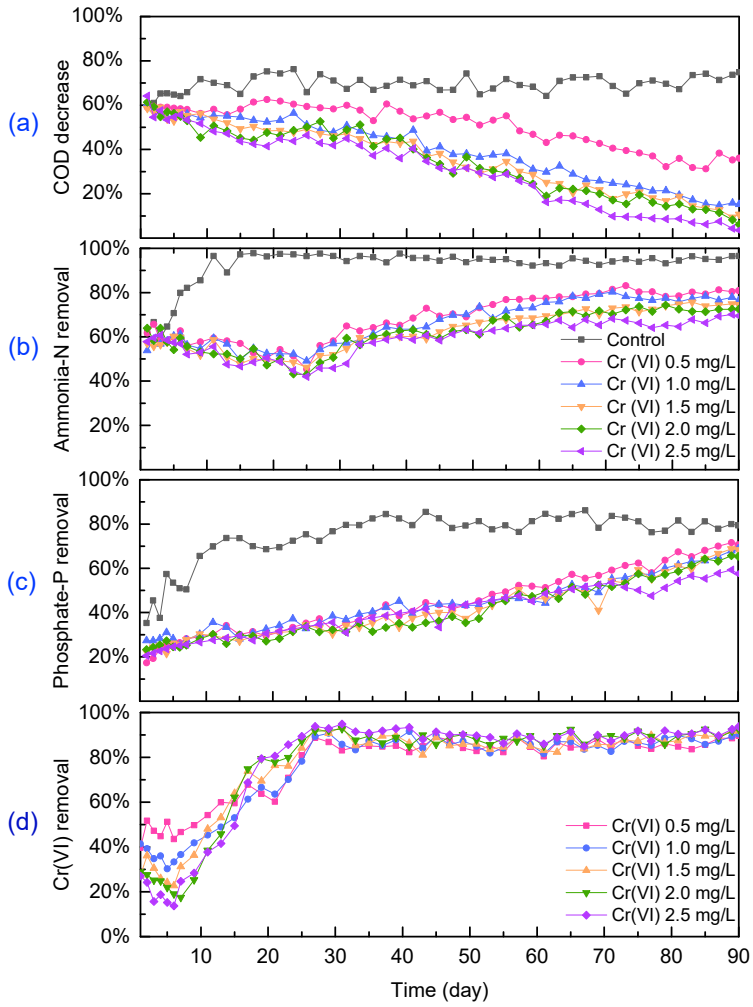

Figure 1. Decrease and removal profiles of COD (**a**), ammonia-N (**b**), phosphate-P (**c**) and Cr(VI) (**d**) across 90 days of operation.

Figure 1d shows the variations of Cr(VI) removal by algal–bacterial granular sludge across ninety days of operation. In the first seven days, Cr(VI) removal had fluctuations but with a general downward trend. Cr(VI) removal at 2.5–0.5 mg/L at the end of seven days was 25–47%, indicating that higher concentrations of Cr(VI) inhibited, relatively, Cr(VI) removal. Subsequently, Cr(VI) removal tended to increase linearly and became stable

at 80–90%. The quality of effluent water after ninety days of cultivation is shown in Table 1. As seen, COD, ammonia-N and phosphate-P in the control were 68, 1.04 and 0.98 mg/L, respectively. Ammonia-N and phosphate-P met water quality standards for China and Europe, while COD only met water quality standards for Europe. Cr(VI) at the set concentrations of 0.5–2.5 mg/L greatly affected the performance of the algal–bacterial granular sludge. At a Cr(VI) concentration of 0.5 mg/L, COD, ammonia-N and phosphate-P concentrations in the effluent water were 222, 5.74 and 1.47 mg/L, respectively, which were above the maximum permitted concentrations. Cr(VI) and total Cr concentrations in all groups were higher than or equal to the specified threshold.

Table 1. Quality of effluent water after 90 days of cultivation and typical discharge standards of WWTP effluent.

Parameters (mg/L)	Cr(VI) Concentrations in this Study (mg/L)						China Class I-A [19] [a]	Europe [20]
	0	0.5	1.0	1.5	2.0	2.5		
COD	68	222	292	308	324	333	50	125
Ammonia-N	1.04	5.74	7.07	7.63	8.25	9.16	5 (8) [b]	NA [c]
Phosphate-P/Total phosphate [d]	0.98	1.47	1.56	1.58	1.73	2.12	1	1 (2) [e]
Cr(VI)	/	0.05	0.10	0.12	0.15	0.16	0.05	NA
Total Cr	/	0.26	0.37	0.63	0.81	0.85	0.1	NA

[a] Refer to the amendment sheet of Discharge Standard of Pollutants for Municipal Wastewater Treatment Plants (GB 18918-2002 [19]). [b] Ammonia-N is 10 mg/L if the temperature is above 12 °C; ammonia-N is 15 mg/L if the temperature is less than or equal to 12 °C. [c] NA: not available. [d] Phosphate-P concentration from this study and total phosphate concentration from the typical discharge standards. [e] TP is 2 mg/L if the population is between 10,000 and 100,000; TP is 1 mg/L if the population is more than 100,000.

3.2. Fate of Cr(VI) in Algal–Bacterial Granular Sludge

The algal–bacterial granular sludge cultured for 90 days was examined using EDS to explore the distribution of Cr (Figure S1). It was found that the accumulated Cr content increased from 0.35% to 2.98% for the Cr(VI) concentrations of 0.5–2.5 mg/L. SEM images and elemental mapping further revealed the distribution of Cr(VI) in algal–bacterial granular sludge (Figure S2). An XPS survey scan of the algal–bacterial granular sludge cultivated for 90 days in the presence of 2.5 mg/L of Cr(VI) showed the algal–bacterial granular sludge to be composed of C, N, O and Cr (Figure S3a). In the high-resolution XPS spectrum of Cr 2*p* (Figure S3b), two strong peaks centered at 586.9 eV and 576.6 eV can be attributed to the binding energy of Cr 2*p*1/2 and Cr 2*p*3/2, respectively, suggesting that the aqueous Cr(VI) had been converted into Cr(III). As reported previously, algal–bacterial granular sludge could be used as a new type of biosorption material to remove Cr(VI) through biosorption, biochemical reduction and intracellular accumulation [7,9–11].

Algal–bacterial granular sludge has significant potential in the treatment of Cr-containing wastewater due to its abundant surface adsorption sites and high tolerance to toxic substances [21]. Yang et al [10] reported that the maximum biosorption capacity could reach as much as 51.0 mg Cr(VI)/g MLSS at pH 2. Secreted extracellular polymeric substances (EPS), polysaccharides, proteins and lipids in algal cell walls, and peptidoglycan in bacterial cell walls can all provide many functional groups contributing to the biosorption of Cr(VI) [22]. Algal biomass can supply different functional groups (e.g., carbonyl, carboxyl and hydroxyls) capable of binding Cr(VI) [23]. Cr(VI) adsorption on the surface of algal–bacterial granular sludge occurs through different paths, e.g., covalent bond formation between functional groups of EPS and cell walls and ionic exchange of Cr(VI) with light metal ions (e.g., Ca (II) and Mg (II)) [10].

Rapid biosorption, considered to be the first stage for Cr(VI) removal, occurs extracellularly and is a passive process. The second stage is bioaccumulation, which is a positive and intracellular process [24]. Considering trace concentrations of free Cr(VI) detected during 30–90 days of cultivation in this study (Figure 1d), it can be assumed that Cr(VI) adsorbed on the surface of algal–bacterial granular sludge is transformed to Cr(III). The

addition of reducible carbon compounds (e.g., acetate) in the simulated wastewater was responsible for this transformation. Cr(VI) is adsorbed on the surface of algal–bacterial granular sludge, can be actively transported across the cell membranes, and can be reduced by reductase to form an organic Cr(III) complex [9]. Compared to Cr(VI), Cr(III) is less soluble and hardly permeates cell membranes [25]. Therefore, Cr(III) aggregates on the outside of the cell membranes and changes the cell surface morphology [25].

3.3. Changes in Microbial Communities under Cr(VI) Stress

Figure 2 shows the relative abundance of eukaryotic diversity at the species level and prokaryotic diversity at the phylum level in algal–bacterial granular sludge after 90 days of cultivation. *Trebouxiophyceae* and *Chlorophyceae* were the main eukaryotic algae. In the control, their relative abundances were 78.7% and 21.2%, respectively. With increased Cr(VI) from 0.5 mg/L to 2.5 mg/L, the relative abundance of *Trebouxiophyceae* increased from 85.2% to 99.6%, while *Chlorophyceae* decreased from 21.2% to 0.2%, indicating that Cr(VI) affected the composition of eukaryotic algae. *As Trebouxiophyceae* is a Cr-tolerant strain [26], *Chlorophyceae* accounted for a small proportion (0.2%), while *Trebouxiophyceae* was the predominant eukaryotic microalgae at a Cr(VI) concentration of 2.5 mg/L.

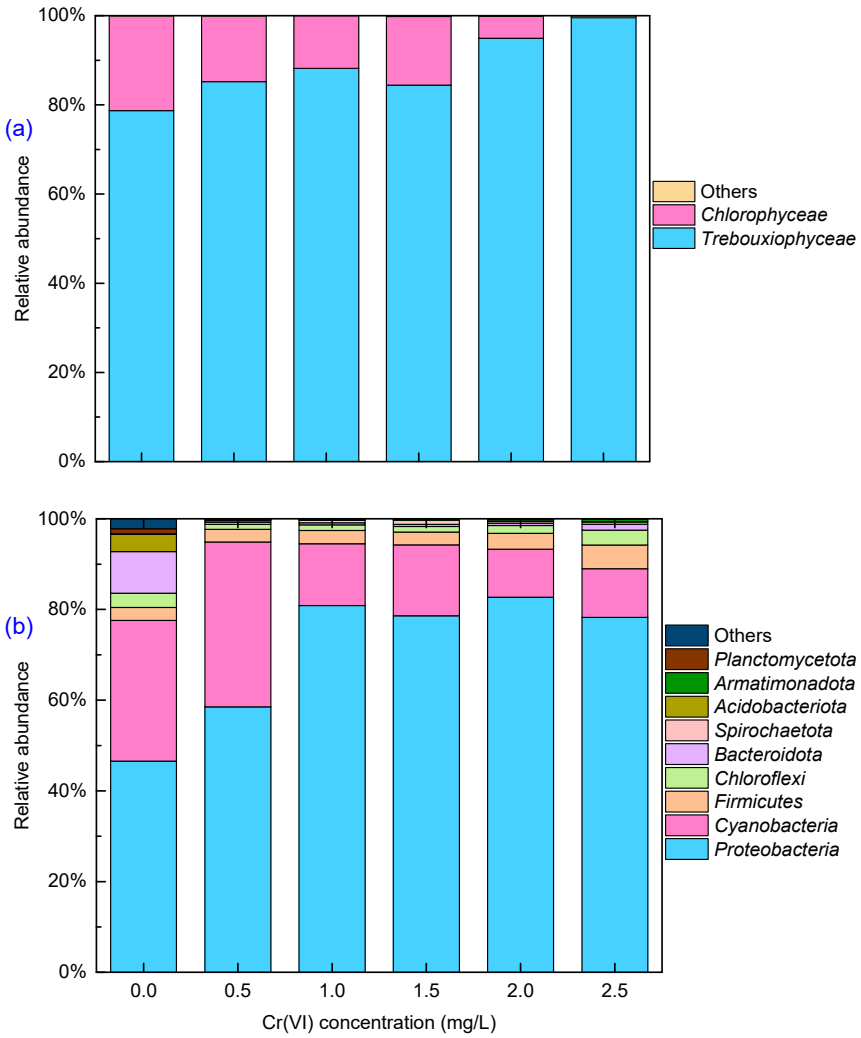

Figure 2. Relative abundance of eukaryotic diversity at species level (**a**) and prokaryotic diversity at phylum level (**b**) in algal–bacterial granular sludge after 90 days with various concentrations of Cr(VI).

The dominant bacteria in the prokaryotic microbial community were *Proteobacteria*, cyanobacteria and *Bacteroidota*, accounting for 46.5%, 31.0% and 9.2%, respectively, in

the control. The relative abundance of *Proteobacteria* increased with Cr(VI) concentration from 0.5 mg/L to 2.5 mg/L. Several bacteria have been identified as being able to reduce very toxic Cr(VI) to less toxic Cr(III) under aerobic and anaerobic conditions, which is considered an alternative approach to removal of Cr(VI). *Proteobacteria* can be isolated from activated sludge containing Cr(VI) and are regarded as a strain with both Cr(VI) resistance and reducing ability [27]. Therefore, the presence of Cr(VI) does not have an adverse effect on the viability of *Proteobacteria*, which are known to be electroactive bacteria involved in extracellular electron transfer [28]. Despite toxic stress due to Cr(VI), *Proteobacteria* that tolerate toxic stress conditions can rapidly decompose nutrients and pollutants and tenaciously survive. In addition, *Firmicutes*, which play an important role in converting refractory substrates into simple organic compounds [28], increased at a Cr(VI) concentration of 2.5 mg/L. Conversely, the relative abundance of *Bacteroidota* decreased from 9.2% in the control to 1.4% at a Cr(VI) concentration of 2.5 mg/L after 90 days of cultivation.

Photosynthetic pigments are usually used to quantify microalgae species. Generally, chlorophyll a exists in all phototrophic microorganisms, including green algae, diatoms and cyanobacteria, and chlorophyll b only exists in green algae [29]. As shown in Figure 3a, the total chlorophyll content decreased from 28.9 mg/g VSS in the control to 21.3 mg/g VSS at 2.5 mg/L of Cr(VI), indicating reduced algal biomass due to Cr(VI) interference with electron transport in respiration and photosynthesis. As for cyanobacteria, they decreased under Cr(VI) stress, evidenced by reduced relative abundance (31.0% to 10.8%) with increased Cr(VI) concentration (Figure 2b). Chlorophyll a decreased, while the ratio of chlorophyll a to chlorophyll b showed an increasing trend with Cr(VI) concentration (Figure S4b), indicating that the reduction of green algae was less than that of cyanobacteria.

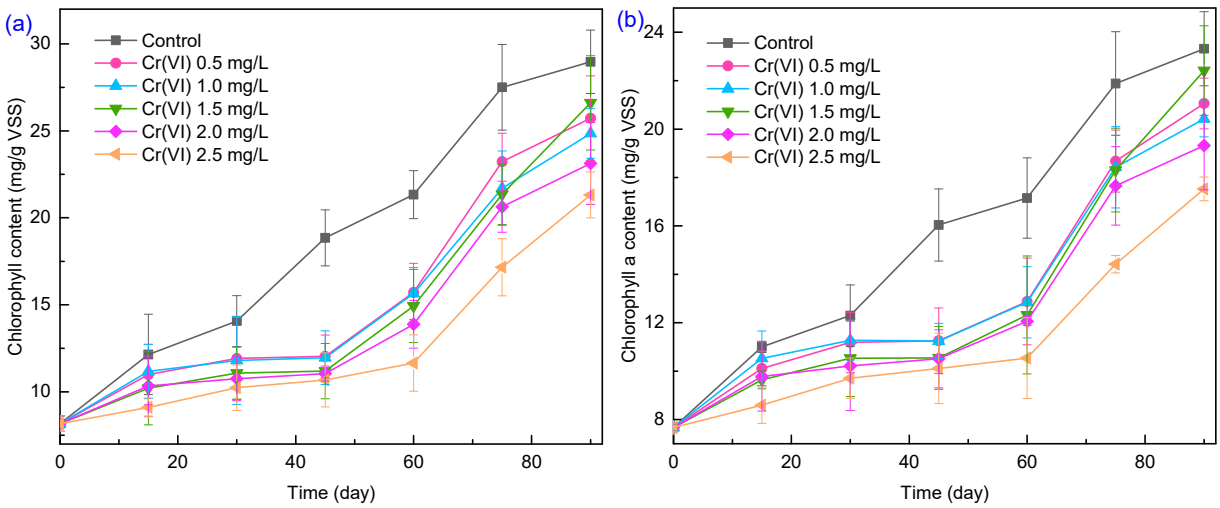

Figure 3. Total chlorophyll (**a**) and chlorophyll a (**b**) content of the algal–bacterial granular sludge.

Photosynthetic pigment is one of the physiological indicators of microalgae under stress and can directly reflect the degree of damage [30]. Therefore, under Cr(VI) stress, green algae are more robust than cyanobacteria. Cyanobacteria are commonly used to adsorb and reduce Cr(VI) to Cr(III) and have been considered as an alternative bioremediation treatment due to their environmental friendliness and cost efficiency [31]. However, it should be noted that it is a suicidal approach to treat Cr(VI), i.e., the relative abundance of cyanobacteria decreased with increasing Cr(VI) concentration (Figure 3b). A similar phenomenon has been observed in the presence of cadmium [6], indicating cyanobacteria are more susceptible to heavy metal stress than eukaryotes.

3.4. Defensive Responses of Algal–Bacterial Granular Sludge under Cr(VI) Stress

3.4.1. EPS Variations

EPS are complex polymers existing in pure bacteria, activated sludge, granular sludge and microalgae and are mainly composed of protein (EPS-PN) and polysaccharide (EPS-PS). Biosorption of Cr(VI) onto EPS has been reported as the major mechanism contributing to Cr bioremediation [32]. As shown in Figure 4, EPS-PN content in the experimental control did not change significantly across 90 days of cultivation. The observed variations in the EPS-PN content at Cr(VI) concentrations of 0.5–2.5 mg/L were significant. EPS-PN content increased from 120.4 to 189.8 mg/g VSS with increasing Cr(VI) concentration from 0.5–2.5 mg/L after 60 days of cultivation, indicating that the overproduction of EPS-PN was a protective mechanism to eliminate or reduce adverse effects. In contrast, EPS-PN then decreased from 79.1 to 67.8 mg/g VSS after 90 days of cultivation. In contrast, EPS-PS content decreased with increasing Cr(VI) concentration during the whole cultivation duration. This might be due to EPS-PS being an energy source that was largely consumed for the overproduction of EPS-PN due to Cr(VI) stress [6,33].

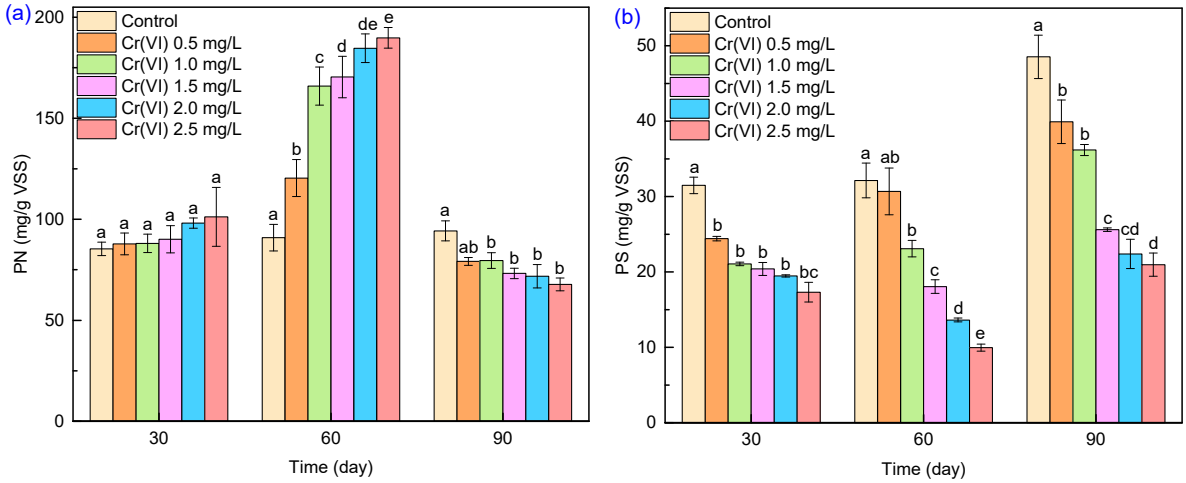

Figure 4. The content of EPS-PN (**a**) and EPS-PS (**b**) in algal–bacterial granular sludge at Cr(VI) concentrations of 0–2.5 mg/L. Different letters mean significant difference between treatments ($p < 0.05$).

3D-EEM was used to further analyze the EPS produced, with the results shown in Figure S4 and Table S1. Two distinct peaks, A and B, at the Ex/Em of 290 nm/352 nm and Ex/Em 360 nm/444 nm, were observed. Peak A corresponds to the tryptophan protein-like substances, and peak B is identified as being due to humic acid-like substances [34]. Compared to the control, no obvious wavelength shift was observed in these two peaks, indicating the chemical similarity of EPS-PN produced at different Cr(VI) concentrations. However, the intensities of both peaks A and B increased with Cr(VI) concentrations of 0–2.5 mg/L, implying more adsorption sites in the presence of higher Cr(VI). It should be noted that the fluorescence intensity of peak B was reduced at 2.5 mg/L of Cr(VI)-exposed concentration. Cyanobacteria have been reported to release humic acid-like substances to defend against environmental stress [35], which further proves the decreased abundance of cyanobacteria (Figure 2b).

3.4.2. Antioxidant Enzyme Activity

Generally, algal cells produce a large amount of reactive oxygen species (ROS) under Cr(VI) stress, which affects the content of photosynthetic pigments in algal cells and damages them [36]. In a study by Chen, et al [37], a large amount of ROS was found to cause and aggravate the oxidative decomposition of membrane lipids and proteins. During this process, a small molecular organic compound, MDA, which is generally used to evaluate oxidative stress, was produced [37]. The MDA content in the control changed insignificantly during the 90 days of cultivation (Figure 5a) but did increase with Cr(VI)

concentrations across 0.5–2.5 mg/L. When the Cr(VI) concentration was 2.5 mg/L, the MDA content was 102.9 nmol/mg protein after 90 days of cultivation, suggesting serious oxidative stress and damaged cell membranes caused by Cr(VI).

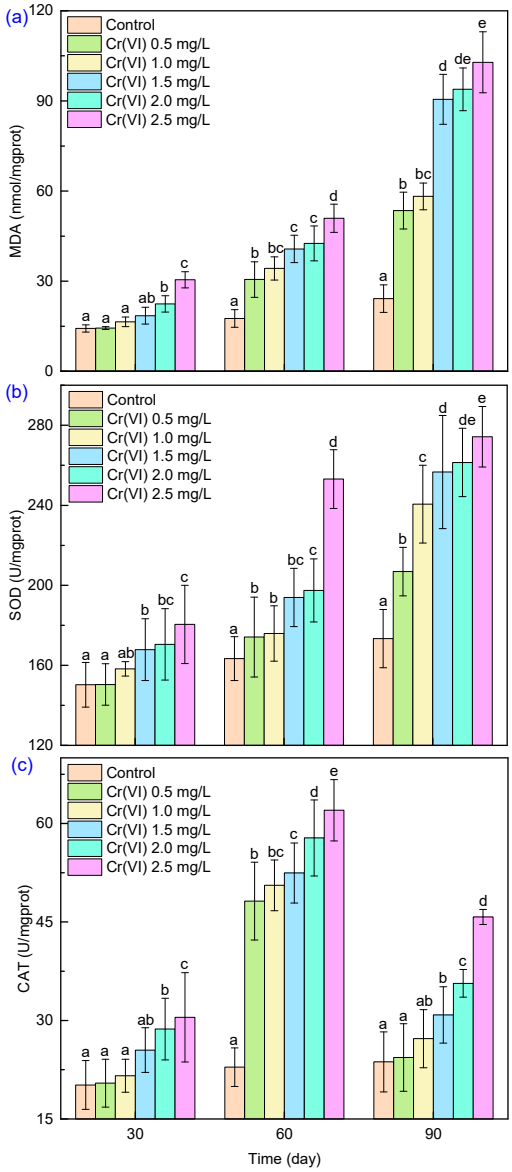

Figure 5. The content of MDA (**a**), SOD (**b**) and CAT (**c**) in algal–bacterial granular sludge at Cr(VI) concentrations of 0–2.5 mg/L. Different letters mean significant difference between treatments ($p < 0.05$).

It should be noted that ammonia-N and phosphate-P removal recovered with time, as shown in Figure 1, indicating effective adaptive strategies. Microalgae synthesize antioxidant enzymes (e.g., SOD and CAT) and nonenzymatic antioxidants to counteract ROS released by heavy metals during adsorption [38]. SOD acts as the first line of defense against the superoxide anion by breaking it down into oxygen molecules and hydrogen peroxide [39]. The hydrogen peroxide is further degraded by CAT into water and oxygen molecules [40,41]. In this study, SOD content increased with Cr(VI) concentration in contrast to the control experiment (Figure 5b). At a Cr(VI) concentration of 2.5 mg/L, SOD content reached 274.3 U/mg protein after 90 days of cultivation, demonstrating significant defensive responses to Cr(VI) oxidative stress. A similar trend is apparent for CAT content with Cr(VI) concentration for up to 60 days (Figure 5c). However, CAT content was decreased at 90 days of cultivation, showing a unique self-protection strategy against over-produced ROS. This observation was inconsistent with the literature [41].

3.5. Engineering Implications and Perspectives

Biological treatment using the activated sludge method as its core strategy is generally adopted for wastewater treatment in China. The conventional activated sludge (CAS) process is often accompanied by a large amount of GHG emissions, including carbon dioxide, generated by the oxidation of organic substances in the wastewater, nitrous oxide produced as the intermediate product of biological denitrification, and dissolved methane generated during anaerobic digestion. Additionally, the CAS process requires high energy input to achieve the removal of organic and nutrient substances from wastewater [42]. GHG emissions generated from wastewater treatment have a negative impact on the global climate. Faced with these challenges and driven by widespread concern for public health and ecological sustainability, wastewater discharge standards have been continuously improved. To date, evidence suggests that effluent water from the proposed algal–bacterial granular sludge process used in bench-scale experiments was able to meet the stringent discharge standards in many countries [43]. However, the removal rate of pollutants and nutrients may be slowed under nonideal conditions. For example, ammonia nitrogen removal was reduced by nearly 15% by the algal–bacterial granular sludge process after 30 days of cultivation in the presence of 1 mg/L of Cd(II) [6]. A similar inhibitory effect was observed in the presence of sulfamethoxazole [44]. In this study, 0.5–2.5 mg/L of Cr(VI) resulted in higher COD to differing extents, such that effluent discharge standards were not met. Cr(VI), a Class I pollutant, was in the effluent and met the discharge standards, with Cr(VI) being reduced to relatively innocuous Cr(III). Therefore, when algal–bacterial granular sludge is used to treat Cr(VI)-containing wastewater, further processes need to be superimposed to solve the problem of effluent COD not meeting the discharge standards.

A variety of defense mechanisms, including reduced intracellular glycogen content, promoted chlorophyll production and increased EPS, were found under simulated conditions [6,44]. However, little attention has been paid to the effects of long-term operation. The microbiome of algal–bacterial granular sludge is dynamic and determined by wastewater characteristics and operating conditions. Due to the uneven microbial growth cycle, the role of algae and bacteria in their defense mechanisms against stress is unclear. Although the proposed algal–bacterial granular sludge process is resistant to Cr(VI) in this study, further work is required to explore the specific mechanisms. In addition, Cr(VI) tolerance tests could be helpful for understanding engineering feasibility.

As a single-cell prokaryote, cyanobacteria have obvious competitive advantages in adapting to environmental factors (e.g., light, temperature, and nutrient intake). Therefore, in some situations, cyanobacteria may eventually evolve into the dominant photosynthetic species in algal–bacterial granular sludge [45]. Since cyanobacteria are mainly distributed in the outer layer of algal–bacterial granular sludge, they are more vulnerable to the impact of the external environment. Algal growth and metabolite production are affected by light, temperature, carbon dioxide concentration, and components in wastewater. So far, the succession of cyanobacteria in algal–bacterial granular sludge is confusing. When the cultivation medium is rich in nutrients and harmful substances, cyanobacteria tend to overpropagate and may release cyanotoxins, adversely affecting water quality. Among the cyanotoxins, microcystins (MCs) are the prevalent class and have a number of variants. Microcystin-LR (MC-LR) is resistant to extreme conditions. In Chinese hygienic standards for drinking water (GB 5749-2022 [46]), the upper limit of 1.0 μg L for MC-LR is strictly specified. Therefore, for the safe, practical application of the algal–bacterial granular sludge process, serious concern about cyanotoxins should be raised.

4. Conclusions

This study aimed to conduct a comprehensive environmental sustainability assessment of the algal–bacterial granular sludge process. The results have shown the ability of algal–bacterial granular sludge to remove nutrients and pollutants from wastewater. It was found that the symbiotic relationship between microalgae and bacteria was destroyed in the presence of Cr(VI), evidenced by decreased COD and a changed microbial

population. Although the ammonia-N and phosphate removal rates were affected at the initial cultivation stage, they did recover gradually. To cope with the stress of Cr(VI), algal–bacterial granular sludge secreted more EPS-PN to provide more adsorption sites for the biological adsorption of Cr(VI) and transfer of Cr(VI) to the low-toxicity form of Cr(III). The activities of SOD and CAT enzymes increased to maintain the stability of microalgal cells and negated the oxidative damage caused by Cr(VI) stress. New insight into the performance and self-defensive responses of algal–bacterial granular sludge under Cr(VI) stress for a long-term operation was shown for the first time. Further research on how to maintain performance is necessary for the sustainable operation and engineering feasibility of algal–bacterial granular sludge in wastewater processing. It is hoped that this study can provide a useful reference.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su152416754/s1, Figure S1: Energy-dispersive X-ray spectra (EDS) of algal–bacterial granular sludge; Figure S2: SEM images of algal–bacterial granular sludge at Cr(VI) concentrations of 0 (a), 0.5 (b), 1.0 (c), 1.5 (d), 2.0(e) and 2.5 mg/L (f); Figure S3: Full spectrometric surveying (a) and Cr 2*p* XPS spectra of algal–bacterial granular sludge cultivated with 2.5 mg/L of Cr(VI) for 90 days (b); Figure S4: Chlorophyll b content (a) and the ratio of chlorophyll a and chlorophyll b (b) of the algal–bacterial granular sludge; Figure S5: 3D-EEM fluorescence spectra of EPS after 90 days of operation at Cr(VI) concentrations of 0 (a), 0.5 (b), 1.0 (c), 1.5 (d), 2.0 (e) and 2.5 mg/L (f); Table S1: Fluorescence spectra parameters of EPS in algal–bacterial granular sludge.

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