water

MDPI

Article

Over-Produced Extracellular Polymeric Substances and Activated Antioxidant Enzymes Attribute to Resistance of Pb(II) for Algal–Bacterial Granular Sludge in Municipal Wastewater Treatment

Juanru Yang [1], Yu Zhang [1] and Shulian Wang [2,3,4,*]

[1] School of Civil Engineering, Architecture and Environment, Hubei University of Technology, Wuhan 430068, China
[2] Key Laboratory of Health Intelligent Perception and Ecological Restoration of River and Lake, Ministry of Education, Hubei University of Technology, Wuhan 430068, China
[3] Innovation Demonstration Base of Ecological Environment Geotechnical and Ecological Restoration of Rivers and Lakes, School of Civil and Environmental Engineering, Hubei University of Technology, Wuhan 430068, China
[4] Hubei Cooperative Innovation Center for Industrial Fermentation, Hubei University of Technology, Wuhan 430068, China
* Correspondence: wangshulian@hbut.edu.cn

Abstract: Algal–bacterial granular sludge technology is a new type of wastewater treatment and resource regeneration process, which has received widespread attention due to its excellent nitrogen and phosphorus removal performance, and energy-saving and emission reduction effects. Although algal–bacterial granular sludge technology has achieved an ideal nutrient removal ability, some pollutants in wastewater might affect the symbiotic relationship between algae and bacteria. This study investigated the impact of coexisting Pb(II) on the symbiosis of algal–bacterial granular sludge. It was found that 2.5–10.0 mg/L of Pb(II) exposure increased the relative abundance of Pro-teobacteria. In addition, more protein in extracellular polymeric substances (EPS-PN) was secreted at 2.5 mg/L of Pb(II) exposure while EPS-PN content reduced at a rate of 5.0–10.0 mg/L of Pb(II). Under different concentrations of Pb(II), the damage degree of algal–bacterial granular sludge was exacerbated, evidenced by increased malondialdehyde (MDA) content. To cope with these adverse circumstances, the antioxidant enzyme activity of both super-oxide dismutase (SOD) and peroxidase dismutase (CAT) was boosted. With the help of these adaptive strategies, the symbiosis of algal–bacterial granular sludge was stable. Moreover, the performance of algal–bacterial granular sludge in treating COD, ammonia-N and phosphate-P was kept at above 95%. This study approved that a Pb(II) concentration less than 10.0 mg/L had little effect on the performance of algal–bacterial granular sludge in wastewater treatment. It is hoped that this study can provide useful information for an improved engineering feasibility of algal–bacterial granular sludge process.

Keywords: algal–bacterial granular sludge; divalent lead ion; extracellular polymeric substance; antioxidant enzyme; resistance

check for updates

Citation: Yang, J.; Zhang, Y.; Wang, S. Over-Produced Extracellular Polymeric Substances and Activated Antioxidant Enzymes Attribute to Resistance of Pb(II) for Algal–Bacterial Granular Sludge in Municipal Wastewater Treatment. *Water* **2023**, *15*, 3833. https://doi.org/10.3390/w15213833

Academic Editor: Alejandro Gonzalez-Martinez

Received: 16 October 2023
Revised: 31 October 2023
Accepted: 1 November 2023
Published: 2 November 2023

1. Introduction

With the rapid development of industries such as electroplating, mining, fertilizer, tanning and papermaking, as well as the extensive use of products like batteries and pesticides, the direct and indirect discharge of heavy metal-containing wastewater into the environment has been increasing [1]. Heavy metals have high toxicity, are resistant to microbial degradation and can easily accumulate in organisms. They not only disrupt the ecological environment of water bodies but also pose risks of toxicity and carcinogenicity to organisms through the food chain [2,3]. Divalent lead (Pb(II)), in particular, has received

much attention due to its wide application in industries, and its inherent toxicity [4–6]. As one of the nonessential metal elements with biomagnification ability, excess intake of Pb(II) in the human body damages the central nervous system and causes organ failures [7]. The concentrations of total lead in polluted water are reported to be in the range of 3–35 mg/L [8], which is significantly higher than the specified threshold of 0.1 mg/L for municipal wastewater discharge [9] and 0.01 mg/L for drinking water quality [10] in China.

The increased amount of Pb(II) discharged into the water environment has become a significant threat to the ecological environment. By causing deficiencies or altering the distribution in biological cells, Pb(II) can disrupt membrane permeability and mineral nutrient balance, affect the catalytic activity of many enzymes and thereby interfere with biological growth and development [11,12]. Research has shown that Pb(II) has affected the morphology, physiological characteristics and biochemical functions of plants, and inhibited root growth and synthesis of photosynthetic pigments [13]. In addition, the toxic effects of Pb(II) on plants weakened their antioxidant stress capacity, leading to lipid peroxidation of chloroplast membranes and even cell damage. It was found that Pb(II) inactivated enzymes involved in photosynthesis, inhibited chlorophyll synthesis and even disrupted chloroplast integrity [14]. Moreover, the chlorophyll in plants and algae was easily degraded by Pb(II) exposure in the growth environment [15].

As a new type of biotechnology for wastewater treatment, algal–bacterial granular sludge achieves the purpose of wastewater purification or remediation by combining the powerful nutrient uptake function of non-cellular structures (e.g., prokaryotic cyanobacteria, eukaryotic algae, diatoms, etc.) and the efficient degradation ability of bacteria (e.g., nitrifying bacteria, denitrifying bacteria, polyphosphate bacteria, etc.) towards pollutants [16–19]. Compared to traditional wastewater treatment technologies (e.g., activated sludge process), algal–bacterial granular sludge relies on the symbiotic relationship between algae and bacteria, where aerobic bacteria use the oxygen released by algae 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 [20].

In theory, the synergistic cycle between algae and bacteria in algal–bacterial granular sludge can result in effectively improved removal efficiency of nutrients and reduced energy consumption and greenhouse gas emissions for actual wastewater treatment. However, up to now, the application of algal–bacterial granular sludge is still in its initial stages since some components (e.g., heavy metals, persistent organic pollutants and microplastics) in wastewater affect the symbiosis of microalgae and bacteria. Previous research has found that the coexistence of Cd(II) exerts toxic effects on microorganisms in algal–bacterial granular sludge, leading to changes in community structure and poor wastewater treatment efficiency [21]. Further studies have approved that algal–bacterial granular sludge exhibits a good adsorption capacity and can effectively treat Cr(VI)-containing wastewater through mechanisms of bio-reduction and bio-adsorption with a total removal efficiency of 89.1% [22,23]. In the symbiosis of microalgae and bacteria, algae can adsorb heavy metals, and bacteria are able to biotransform them to low toxicity forms [24]. The algal–bacterial granular sludge process has exhibited effective remediation efficiency for heavy metals and is hopeful to be widely used to treat heavy metal-containing wastewater. Currently, the heavy metal removal technology used for wastewater treatment has not yet focused on the algal–bacterial granular sludge process. Purebred algae are suitable for complex wastewater environments with cell breakage, and low concentrations of heavy metals cannot be highly selected. In addition, the actual application of the algal–bacterial granular sludge process is not widespread or accepted. Therefore, it is of vital importance to study the heavy metal-loaded algal–bacterial granular sludge process.

So far, most studies have focused on Pb(II) removal techniques [8]. Many researches have reported that filtration, precipitation, adsorption, ion exchange and electrodialysis could be applied as effective methods to remove Pb(II) in wastewater [8]. The detailed removal mechanisms have been thoroughly studied. On the other hand, the algal–bacterial

granular sludge process has been expected to be a new type of biotechnology in treating municipal wastewater [18]. However, limited information regarding the effect of Pb(II) exposure on the removal efficiency of nutrients (e.g., COD, ammonia nitrogen and phosphates) and the corresponding metabolic responses is available. Further research is necessary to address this knowledge gap. The main objective of this experimental study is to investigate the influence of Pb(II) on the treatment efficiency of algal–bacterial granular wastewater, and analyze its impact mechanism regarding metabolic reactions and microbial community structure. It is hoped that this study can provide more information for future research on the engineering feasibility of algal–bacterial granular technology.

2. Materials and Methods

2.1. Synthetic Wastewater and Algal–Bacterial Granular Sludge

The synthesized wastewater used in this study was simulated domestic sewage, which was composed of 527 mg/L of NaAc ($CH_3COONa \cdot 3H_2O$), 114.6 mg/L of NH_4Cl, 10 mg/L of KH_2PO_4, 10 mg/L of $CaCl_2$, 10 mg/L of $MgSO_4 \cdot 7H_2O$, 10 mg/L of $FeSO_4 \cdot 7H_2O$ and 1 mL/L of trace elements. The trace solution contained 10 g/L of EDTA, 150 mg/L of H_3BO_3, 100 mg/L of $MnSO_4 \cdot H_2O$, 30 mg/L of $CuSO_4 \cdot 5H_2O$, 120 mg/L of $ZnSO_4 \cdot 7H_2O$, 60 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 180 mg/L of KI and 150 mg/L of $CoCl_2 \cdot 6H_2O$.

In this study, algal–bacterial granular sludge was prepared from the sludge of a wastewater treatment plant in Wuhan, China, using aerobic granular sludge cultivated using the calcium salt method as raw materials [25]. Briefly, aerobic granules fed with the synthetic wastewater described above were exposed to a light-emitting diode (LED) light at a photosynthetic photon flux density (PPFD) of 10,000 lux under aerobic conditions. After one month of cultivation, algal–bacterial granular sludge was gradually matured with a typical green color, and an average size of 2 mm. Mature algal–bacterial granular sludge with a 5 min sludge volume index (SVI_5) of 77.25 mL/g was obtained [20,21,26]. The harvested mature algal–bacterial granular sludge was used in subsequent experiments.

2.2. Experimental Design

In this study, in a 60 mL glass serum bottle with a height/diameter of 2, 5 g of fresh algal–bacterial granular sludge was added to 30 mL of synthetic wastewater. The volatile suspended solids (VSS) concentration was 17.6 $\pm$ 0.2 g/L. A series of certain volumes of 500 mg/L of Pb(II) nitrate stock solution were added to obtain 2.5, 5.0, 7.5 and 10.0 mg/L of Pb(II) in stimulated wastewater. No additional Pb(II) was set as the control. The pH of the stimulated wastewater was kept at 6.4 $\pm$ 0.2. The experimental light intensity provided by an LED was controlled at 10,000 lux; the light cycle was 8 h of light and 16 h of dark to simulate natural lighting. Since nutrient removal efficiencies peaked at the end of the daytime, sampling was carried out at 8 h. The influent and effluent water samples were filtered through a 0.45 μm filter membrane for further analysis.

2.3. Analysis Methods

In this study, COD, ammonia-N, phosphate-P and VSS were determined according to the standard methods [27]. The pH of the solution was measured using a PHS-3E (INESA Scientific Instrument Co., Shanghai, China) pH meter. The concentration of total lead in the water samples was determined by an AAnalyst 800 atomic absorption spectrometry (Perkin Elmer, Waltham, MA, USA). Chlorophyll in algal–bacterial granular sludge was extracted using 95% ethanol according to the following procedures. Firstly, 0.2 g of fresh algal–bacterial granular sludge was placed in a 10 mL centrifuge tube. Then, 5 mL of 95% ethanol was added and the centrifuge tube was urgently wrapped in tin foil to prevent light transmission. The tube was then placed in a dark refrigerator at 4 °C for 24 h, followed with centrifugation at 4000 rpm/min for 10 min. The total chlorophyll (total Chl) content, including chlorophyll a (Chl a) and chlorophyll b (Chl b), was extracted using an acetone extraction method [28].

Extracellular polymeric substances (EPS) were extracted by a modified heat extraction method [29]. Briefly, 0.5 g of algal–bacterial granular sludge was placed into a 50 mL centrifuge tube, followed with washing with deionized water three times. Sodium chloride solution (0.05%, 10 mL) was added to suspended algal–bacterial granular sludge in the solution. The centrifuge tube containing the above solution was placed in an ultrasonic cleaning machine and sonicated at 20 KHz for 3 min and then heated at 60 °C in a water bath for 30 min. Finally, the centrifuge tube was frozen and centrifuged at 12,000× *g* for 30 min. The supernatant was collected and filtrated with a 0.45 μM membrane for the following determination. The extracted EPS was measured using a DM4000B LED fluorescence microscope (Leica, Heidelberg, Germany), with an emission wavelength of 250–600 nm and an excitation wavelength of 250–550 nm. The polysaccharide concentration was measured by the phenol–sulfuric acid method with glucose as the standard [30] and the Lowry–Folin method was used for protein determination [31].

After being frozen in liquid nitrogen, algal–bacterial granular sludge was ground to a paste using a high-speed grinder and mixed with 0.2 mol/L of phosphate buffer (pH 7.8) (1:9), followed with centrifugation (4 °C, 4000 r/min) for 10 min to obtain a crude enzyme solution from the supernatant for enzyme activity measurement. The activities of superoxide dismutase (SOD), peroxidase dismutase (CAT) and malondialdehyde (MDA) were tested by using kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

The algal–bacterial granular sludge samples were collected after 90-day 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 [21]. The purified amplicons were collected in an equimolar manner on the Illumina MiSeq platform and sequenced by Meiji Biopharmaceutical Technology Co., Ltd. (Shanghai, China).

2.4. Statistical Analyses

In this study, all experiments were performed in three replicates. All measurements were repeated three times, and the data were represented as mean ± standard deviation (SD). The SPSS V19.0 (IBM, New York, NY, USA) software was used to conduct one-way analysis variance, and $p < 0.05$ stands for statistical significance.

3. Results and Discussion

3.1. Changes in Microbial Communities

The microbial community of eukaryote at species level in algal–bacterial granular sludge after 90-day culture is shown in Figure 1a. Eukaryote were mainly composed of *Fungi_unclassified*, *Ascomycota* and *Leucosporidiella*, accounting for 98.6%, 0.5% and 0.5% in the control. In the presence of Pb(II) from 2.5 to 10.0 mg/L, the relative abundance of *Fungi_unclassified* decreased from 97.8% to 61.4%, while the relative abundance of *Ascomycota* increased from 0.5% to 36.4%. In addition, the relative abundance of *Leucosporidiella* was kept at 0.9–1.7%. As reported, *Ascomycota* were highly tolerant to heavy metals [32,33]. The increased relative abundance of *Ascomycota* might be a way for microorganisms in algal–bacterial granular sludge to adapt to Pb(II). As seen in Figure 1b, prokaryotic diversity at the phylum level in microbial communities of algal–bacterial granular sludge was mainly composed of *Proteobacteria*, *Chloroflexi*, *Bacteroidota*, *Cyanobacteria* and *Firmicutes*, accounting for 51.8%, 31.1%, 2.8%, 7.8% and 1.3%, respectively. After a 90-day culture with 2.5–10.0 mg/L of Pb(II), the relative abundance of *Proteobacteria* increased from 62.0% to 77.8%. As a Pb-resistant strain, *Proteobacteria* was tolerant to Pb(II). Therefore, when algal–bacterial granular sludge was exposed to Pb(II) for a long time, the relative abundance of *Proteobacteria* increased [34].

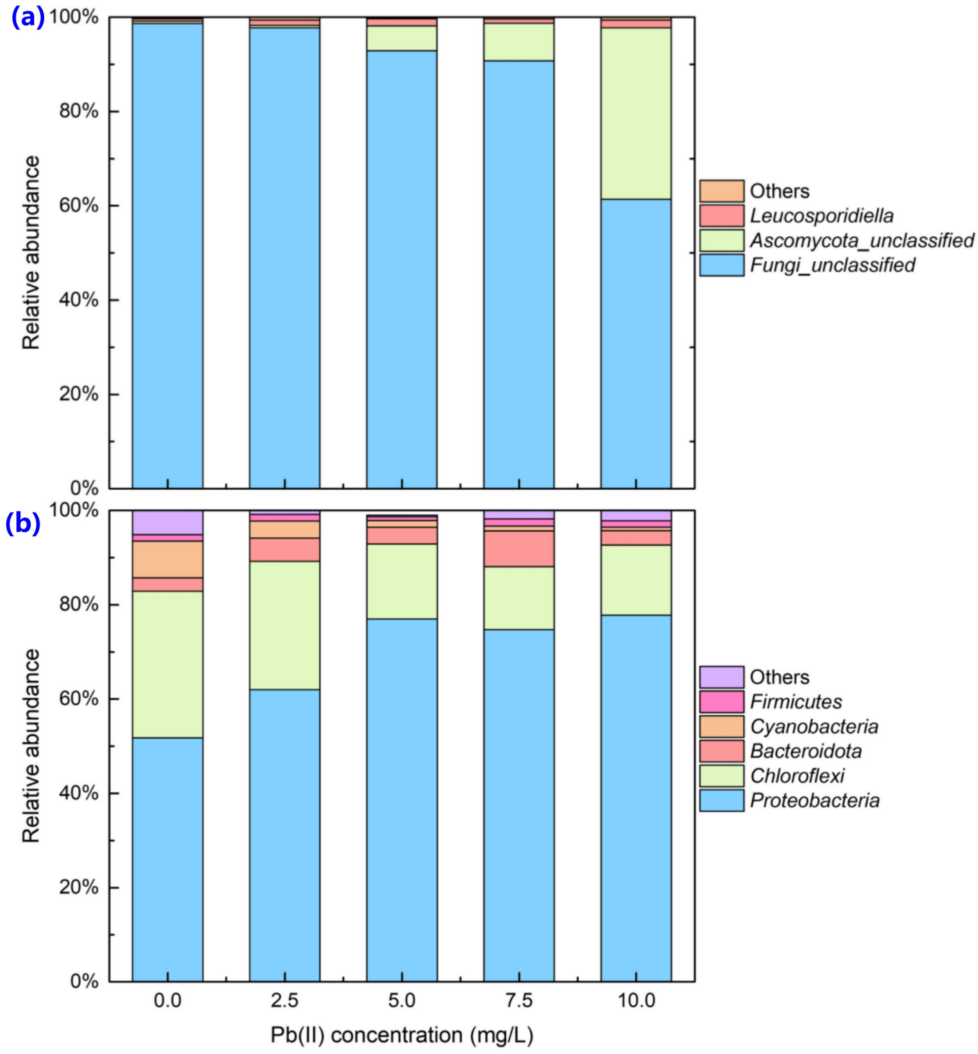


Figure 1. Distributions of eukaryotic diversity at species level (**a**) and prokaryotic diversity at phylum level (**b**) in microbial communities of algal–bacterial granular sludge after 90-day culture. The others named in the figures indicated the minority fractions that the sum of different eukaryotic species and prokaryotic phylum accounted for less than 1% of its total sequences in each sample.

The algae in the algal–bacterial granular sludge used in this study were cyanobacteria. It was found that the relative abundance of cyanobacteria in prokaryotic diversity was 7.8% in the control, which reduced to 0.7% with an increased Pb(II) concentration of 10.0 mg/L. In fact, photosynthetic pigments are one of the most commonly used indicators to determine the algae. Heavy metals can impair the biosynthesis of photosynthetic pigments in algae with varying degrees. As seen in Figure 2, insignificant chlorophyll a content was shown with a Pb(II) concentration of 0–7.5 mg/L during 60-day culture ($p > 0.05$). However, chlorophyll a content was reduced from 60–to 90 days. At 90 days, chlorophyll a content was 21.39 ± 0.48, 17.86 ± 0.22, 16.25 ± 0.14 and 13.58 ± 0.49 mg/g VSS, respectively. As for the 10.0 mg/L of Pb(II) concentration, chlorophyll a content decreased from 21.39 ± 0.48 mg/g VSS to 11.32 ± 0.41 mg/g VSS at 90 days. As the only chlorophyll in cyanobacteria, chlorophyll a was an index of cyanobacteria biomass [35]. However, when the concentration of heavy metals in the environment where cyanobacteria live was high, the growth of cyanobacteria was affected and the biomass was reduced [36]. It was reported that heavy metals may affect the electron transfer reaction of the photosystem through irreversible combination with intracellular components, which led to the obstruction of the photoreaction stage and seriously impacted photosynthesis [37].

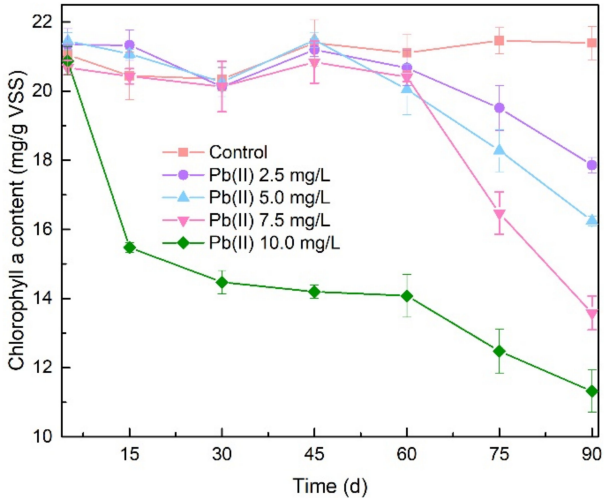


Figure 2. Chlorophyll a content in algal–bacterial granular sludge.

3.2. Extracellular Polymeric Substance Content

Extracellular polymeric substances (EPS) are macromolecular polymers secreted by microorganisms, mainly consisting of proteins, polysaccharides and a small amount of nucleic acid [38]. EPS can form a protective barrier for cells to resist the adverse effects of the external environment. The proteins in EPS play an important role in the bioremediation of heavy metals [39,40]. The changes in the content of EPS-PN and EPS-PS in algal–bacterial granular sludge is shown in Figure 3. It was found that more EPS-PN was secreted at 2.5 mg/L of Pb(II) exposure while EPS-PN content reduced at 5.0–10.0 mg/L of Pb(II). Take the 2.5 mg/L Pb(II)-exposure concentration as an example, EPS-PN content increased from 63.11 ± 2.37 mg/g VSS in the control to 68.93 ± 1.13 mg/g VSS after 30 days. At a Pb(II)-exposure concentration of 5.0–10.0 mg/L, EPS-PN content decreased from 53.69 ± 3.26 mg/g VSS to 20.01 ± 3.13 mg/g VSS after 30 days. Similar trends for EPS-PS change were observed. At a 2.5 mg/L of Pb(II)-exposure concentration after 90 days, EPS-PS content increased from 74.92 ± 4.93 mg/g VSS in the control to 94.27 ± 1.93 mg/g VSS while EPS-PN content reduced from 69.37 ± 3.86 mg/g VSS to 18.71 ± 5.79 mg/g VSS at 5.0–10.0 mg/L of Pb(II).

According to the changes in EPS-PN and EPS-PS content, low concentrations of Pb(II) (0–2.5 mg/L) promoted algal–bacterial granular sludge to secrete more EPS, while high concentrations of Pb(II) (5.0–10.0 mg/L) inhibited EPS secretion, presenting a phenomenon of low concentration promotion–high concentration inhibition. It was concluded that algal–bacterial granular sludge synthesized more EPS to cope with this adverse environment under low concentrations of Pb(II), while cell activity was inhibited to some extent under high concentrations of Pb(II).

To further verify the changes in the types and concentrations of organic substances in EPS, EPS samples from the experimental and control groups were measured using a three-dimensional fluorescence spectrometer. Figure 4 shows the fluorescence spectra of EPS from samples exposed under different Pb(II) concentrations. All the spectra showed two obvious characteristic peaks, namely peak A (Ex/Em = 290/335–338) and peak B (Ex/Em = 360/420). Peak A belonged to tryptophan protein substances and peak B was attributed to humic substances [41]. An insignificant shift in the positions of the two characteristic peaks of EPS indicated no change in the organic components in EPS of algal–bacterial granular sludge exposed to 2.5–10.0 mg/L of Pb(II). It should be noted that the fluorescence intensity of peak A increased from 19,549 in the control to 27,897, and then decreased to 10,570. The intensity of peak B decreased from 20,268 to 745 at 0–10.0 mg/L of Pb(II). Studies have shown that decreased fluorescence intensity can be attributed to fluorescence quenching [42,43]. The experimental results indicated that humic substances formed complexes with Pb(II), leading to a decrease in fluorescence intensity of peak B.

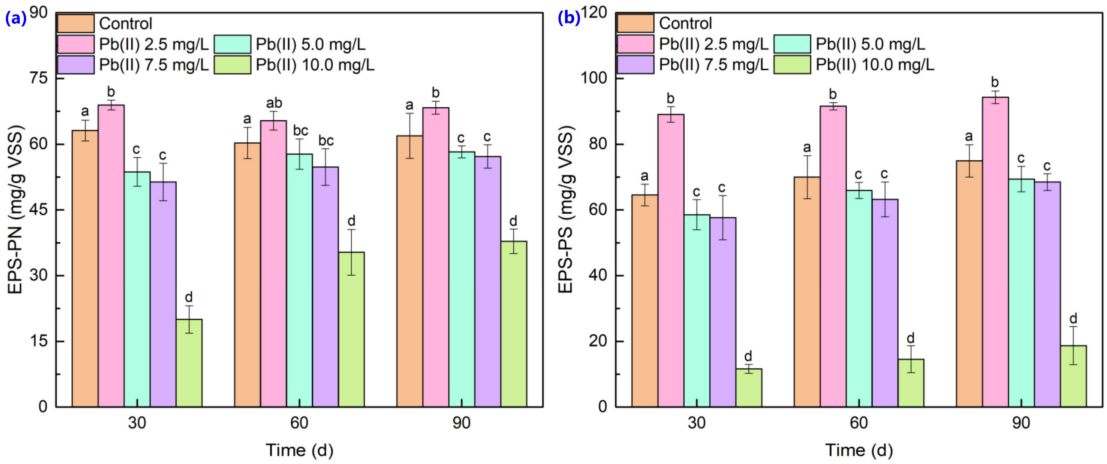
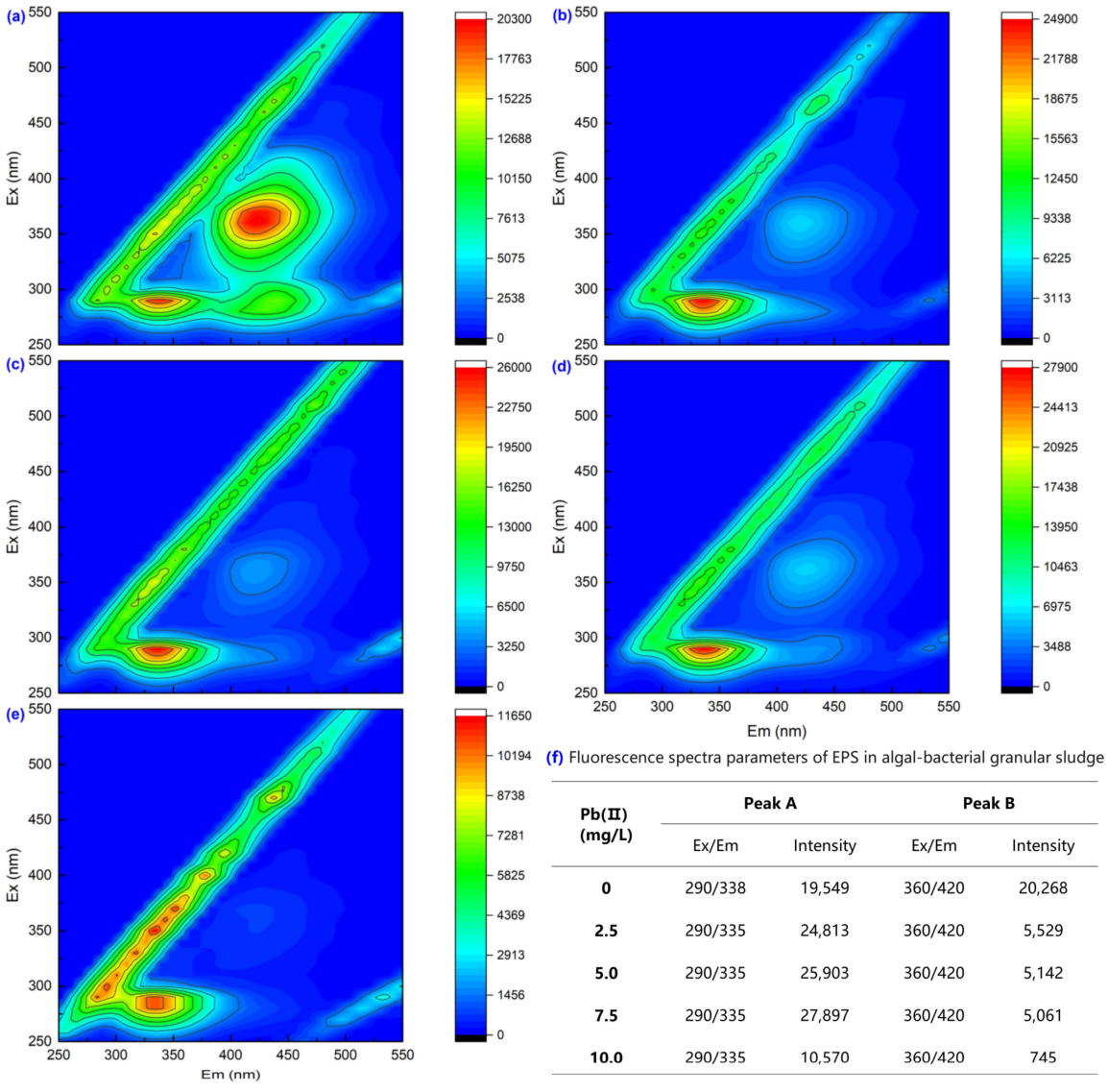


Figure 3. Content of EPS-PN (**a**) and EPS-PS (**b**) in algal–bacterial granular sludge. Different letters mean significant difference between treatments ($p < 0.05$).



(f) Fluorescence spectra parameters of EPS in algal-bacterial granular sludge

Pb(II) (mg/L)	Peak A		Peak B	
	Ex/Em	Intensity	Ex/Em	Intensity
0	290/338	19,549	360/420	20,268
2.5	290/335	24,813	360/420	5,529
5.0	290/335	25,903	360/420	5,142
7.5	290/335	27,897	360/420	5,061
10.0	290/335	10,570	360/420	745

Figure 4. 3D-EEM spectra of EPS from algal–bacterial granular sludge after 90 day-culture in the presence of Pb(II) (**a**)—0; (**b**)—2.5 mg/L; (**c**)—5.0 mg/L; (**d**)—7.5 mg/L; (**e**)—10.0 mg/L) and the fluorescence spectra parameters (**f**).

3.3. Antioxidant Enzyme Activities and MDA Content Analysis

It was reported that cells produced active free radicals under adverse environment, including superoxide free radicals, carboxyl free radicals and hydrogen peroxide. The existence of these free radicals could disrupt the balance of intracellular oxidation, leading to permanent dysfunction and cell death [44]. In addition, the massive production of these reactive oxygen species could trigger or intensify the membrane lipid peroxidation, causing serious damage to the membrane system. The product of the membrane lipid peroxidation, MDA, could be used as an indicator to reflect the oxidative pressure faced by algae cells [45]. As seen in Figure 5, MDA content was insignificantly different at 1–7.5 mg/L of Pb(II) exposure concentration ($p > 0.05$) while increased at 10.0 mg/L of Pb(II)-exposed concentration ($p < 0.05$) at 30 days. A similar trend was obtained at 60 and 90 days. It was found that MDA content was increased from 167.48 ± 11.27 nmol/mgprot in the control to 325.47 ± 16.59, 355.68 ± 10.79, 418.23 ± 16.71 and 453.79 ± 18.53 nmol/mgprot at 2.5, 5.0, 7.5 and 10.0 mg/L of Pb(II) exposure concentration, respectively. These results indicated that the damage degree caused by Pb(II) to algal–bacterial granular sludge was exacerbated with the cultivation time and exposure concentration.

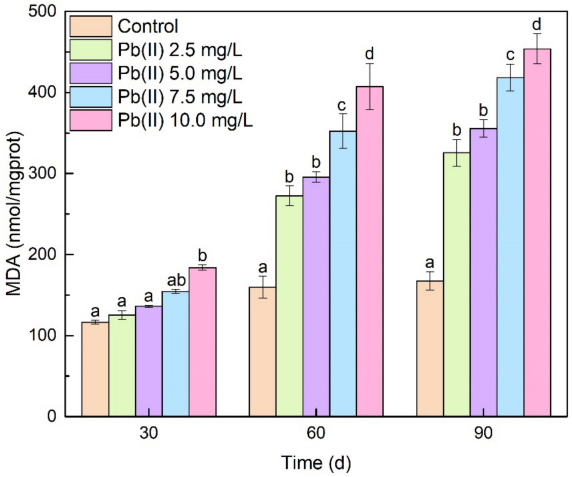


Figure 5. Antioxidant enzyme activity of MDA. Different letters mean significant difference between treatments ($p < 0.05$).

To cope with the oxidative damage caused by Pb(II) and ensure the normal metabolism of cells, several enzyme activities (e.g., SOD and CAT) could form an antioxidant enzyme system. The presence of antioxidant enzymes can effectively remove the accumulated active free radicals in organisms, ensuring the normal life activities of cells. SOD is the primary antioxidant enzyme that plays a role in scavenging free radicals in cells. Its role is to catalyze the disproportionation of free radicals and convert them into hydrogen peroxide (H_2O_2) and superoxide radicals. The accumulation of H_2O_2 further promotes the synthesis of CAT, which catalyzes H_2O_2 to decompose into H_2O and O_2 [46–48]. Figure 6 shows the antioxidant enzyme activity of SOD and CAT. At 30 days, insignificant differences in SOD activity at 0–10.0 mg/L of Pb(II) exposure concentration ($p > 0.05$) can be seen. SOD activity increased from 217.75 ± 14.57 U/mgprot in the control to 380.78 ± 17.11 U/mgprot at 10.0 mg/L of Pb(II) exposure concentration at 60 days. A similar trend for SOD activity was observed at 90 days. On the other hand, CAT activity increased with Pb(II) exposure concentration. The maximum CAT activity (i.e., 49.37 ± 2.63 U/mgprot) was obtained at 60 days with 10.0 mg/L of Pb(II). However, CAT activity decreased at 90 days. This phenomenon was similar to previous research. Algal–bacterial granular sludge effectively eliminates the free radicals produced by oxidative stress through antioxidant enzymes in the cells to maintain the metabolic balance of reactive oxygen species.

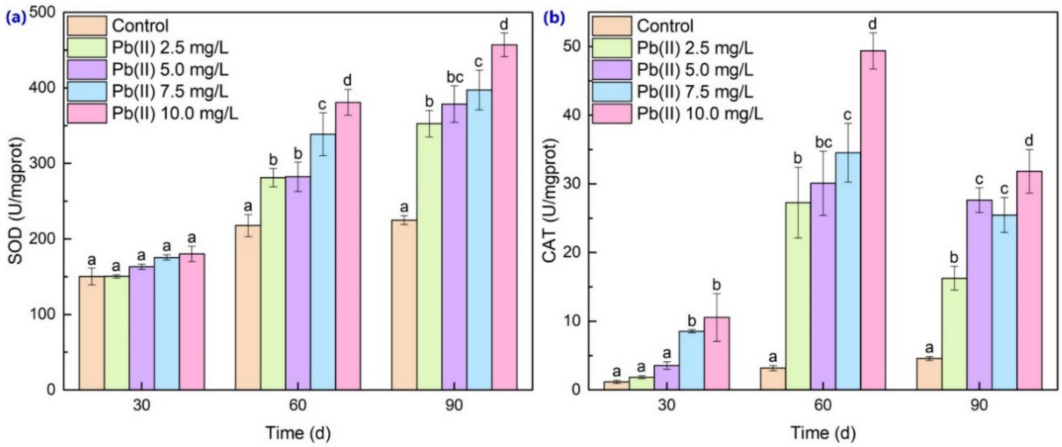


Figure 6. Antioxidant enzyme activity of SOD (**a**) and CAT (**b**). Different letters mean significant difference between treatments ($p < 0.05$).

3.4. Performance in Treating Municipal Wastewater

With different concentrations of Pb(II) exposure, insignificant differences in the removal rate of COD between the experimental groups and the control group were observed ($p > 0.05$), and both had stable removal rates of about 95%. After 90-day culture, the average COD removal rates were 95.83%, 95.51%, 95.41%, 95.09% and 95.08%, respectively, at a Pb(II) exposure concentration of 0–10.0 mg/L (Figure 7a). Moreover, algal–bacterial granular sludge maintained a high removal rate of ammonia-N, which was stable at around 97% with an insignificant difference among environmental groups ($p > 0.05$). After 90-day culture, average ammonia-N removal rates were 97.72%, 97.44%, 97.33%, 97.39% and 97.48%, respectively (Figure 7b). A similar trend was observed in the removal rate of phosphate-P, which remained around 94% ($p > 0.05$). After 90-day culture, the average phosphate-P removal rates were 94.79%, 94.06%, 94.04%, 93.55% and 93.68%, respectively (Figure 7c).

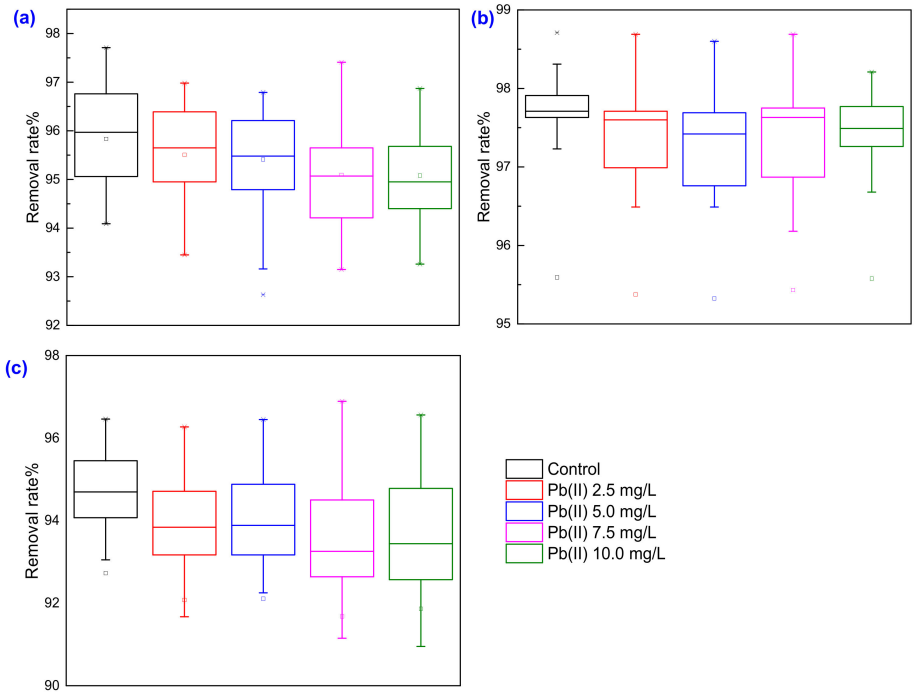


Figure 7. Removal profiles of COD (**a**), ammonia-N (**b**) and phosphate-P (**c**) across ninety days of operation. The symbols of '□' and '*' in the figures stand for average value and outside point, respectively.

It could be concluded that no inhibition of COD, ammonia-N and phosphate-P removal occurred with Pb(II) exposure less than 10.0 mg/L after 90-day culture. Unlike the presence of Cd(II) [21] and Cr(VI) [22], the synergistic effect of microalgae and bacteria could eliminate the toxic effects of Pb(II) on microorganisms and resist the adverse impact of Pb(II), evidenced by the almost unchanged nutrient removal abilities. COD, ammonia-N and phosphate-P from the control were 15, 0.80 and 0.26 mg/L, respectively, meeting the water quality standards for China, which were 75, 10 and 1 mg/L [9]. At a Pb(II) concentration of 10.0 mg/L, COD, ammonia-N and phosphate-P concentrations in the effluent water were 17, 0.88 and 0.32 mg/L, which were less than the specified threshold. Based on these results, 0–10.0 mg/L of Pb(II)-containing wastewater could be handled by the algal–bacterial granular sludge process and meet the discharge requirements. Therefore, the algal–bacterial granular sludge process could be considered as an alternative approach for treating low concentration of Pb(II)-containing wastewater.

The variations in Pb(II) removal by algal–bacterial granular sludge across 90 days of operation were obtained. It was found that 88.5%, 89.9%, 90.5% and 89.1% of Pb(II) (2.5–10.0 mg/L) was removed from the solution. In fact, the Pb(II) ions formed a stable compound, lead acetate, with sodium acetate in the feed. Lead acetate is easily soluble in water and generally generates lead hydroxide precipitates under alkaline conditions with a pH above 12. It was found that the pH of the solution varied in the range of 6.4 ± 0.2–8.5 ± 0.3, therefore Pb(II) was unlikely to be removed in the form of precipitation. The removal of Pb(II) may be attributed to electrostatic interaction with EPS, ion exchange with metal ions and bonding to functional groups [8]. Therefore, algal–bacterial granular sludge was able to effectively adsorb Pb(II) to meet the discharge requirements and could be considered as an alternative approach for treating Pb(II)-containing wastewater.

4. Conclusions

This study demonstrated the performance and the adaptive strategies of algal–bacterial granular sludge technology in wastewater treatment. Under Pb(II) stress for 90 days, algal–bacterial granular sludge secreted more EPS, which could adsorb Pb(II) through the rich negatively charged functional groups in EPS. In addition, the activities of SOD and CAT enzymes in microbial cells showed an upward trend to eliminate reactive oxygen species produced in cells. Both enzymes alleviated the oxidation burden by removing excess free radicals. In addition, the performance of algal–bacterial granular sludge maintained efficient COD, ammonia-N and phosphate-P removal capabilities. This article elaborates upon the response of algal–bacterial granular sludge to cope with Pb(II) exposure in the environment, and proposes a new and feasible approach for treating wastewater containing Pb(II).

Author Contributions: Conceptualization, J.Y. and S.W.; methodology, J.Y. and Y.Z.; validation, J.Y.; investigation, Y.Z.; data curation, J.Y. and Y.Z.; writing—original draft preparation, J.Y.; writing—review and editing, S.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

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

References

1. Shrestha, R.; Ban, S.; Devkota, S.; Sharma, S.; Joshi, R.; Tiwari, A.P.; Kim, H.Y.; Joshi, M.K. Technological trends in heavy metals removal from industrial wastewater: A review. *J. Environ. Chem. Eng.* **2021**, *9*, 105688. [CrossRef]
2. Fu, Z.; Xi, S. The effects of heavy metals on human metabolism. *Toxicol. Mech. Methods* **2020**, *30*, 167–176. [CrossRef] [PubMed]
3. Jaishankar, M.; Tseten, T.; Anbalagan, N.; Mathew, B.B.; Beeregowda, K.N. Toxicity, mechanism and health effects of some heavy metals. *Interdiscip. Toxicol.* **2014**, *7*, 60–72. [CrossRef] [PubMed]
4. Kushwaha, A.; Hans, N.; Kumar, S.; Rani, R. A critical review on speciation, mobilization and toxicity of lead in soil-microbe-plant system and bioremediation strategies. *Ecotoxicol. Environ. Saf.* **2018**, *147*, 1035–1045. [CrossRef]

5. Kalita, D.; Joshi, S.R. Study on bioremediation of Lead by exopolysaccharide producing metallophilic bacterium isolated from extreme habitat. *Biotechnol. Rep.* **2017**, *16*, 48–57. [CrossRef]
6. Das, S.; Dash, H.R.; Chakraborty, J. Genetic basis and importance of metal resistant genes in bacteria for bioremediation of contaminated environments with toxic metal pollutants. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 2967–2984. [CrossRef]
7. Abbaszadeh, S.; Wan Alwi, S.R.; Webb, C.; Ghasemi, N.; Muhamad, I.I. Treatment of lead-contaminated water using activated carbon adsorbent from locally available papaya peel biowaste. *J. Clean. Prod.* **2016**, *118*, 210–222. [CrossRef]
8. Wang, Z.; Wang, H.; Nie, Q.; Ding, Y.; Lei, Z.; Zhang, Z.; Shimizu, K.; Yuan, T. Pb(II) bioremediation using fresh algal-bacterial aerobic granular sludge and its underlying mechanisms highlighting the role of extracellular polymeric substances. *J. Hazard. Mater.* **2023**, *444*, 130452. [CrossRef]
9. *GB 18918-2002*; Discharge Standard of Pollutants for Municipal Wastewater Treatment Plants. China Environment Press: Beijing, China, 2002.
10. *GB 5749-2022*; Standards for Drinking Water Quality. State Administration for Market Regulation: Beijing, China, 2022.
11. Leung, P.T.Y.; Yi, A.X.; Ip, J.C.H.; Mak, S.S.T.; Leung, K.M.Y. Photosynthetic and transcriptional responses of the marine diatom *Thalassiosira pseudonana* to the combined effect of temperature stress and copper exposure. *Mar. Pollut. Bull.* **2017**, *124*, 938–945. [CrossRef]
12. Lian, W.; Yang, L.; Joseph, S.; Shi, W.; Bian, R.; Zheng, J.; Li, L.; Shan, S.; Pan, G. Utilization of biochar produced from invasive plant species to efficiently adsorb Cd (II) and Pb (II). *Bioresour. Technol.* **2020**, *317*, 124011. [CrossRef]
13. Alkhatib, R.; Maruthavanan, J.; Ghoshroy, S.; Steiner, R.; Sterling, T.; Creamer, R. Physiological and ultrastructural effects of lead on tobacco. *Biol. Plant.* **2012**, *56*, 711–716. [CrossRef]
14. Dubey, S.; Chen, C.W.; Haldar, D.; Tambat, V.S.; Kumar, P.; Tiwari, A.; Singhania, R.R.; Dong, C.-D.; Patel, A.K. Advancement in algal bioremediation for organic, inorganic, and emerging pollutants. *Environ. Pollut.* **2023**, *317*, 120840. [CrossRef] [PubMed]
15. Wang, B.; Xiao, L.; Xu, A.; Mao, W.; Wu, Z.; Hicks, L.C.; Jiang, Y.; Xu, J. Silicon fertilization enhances the resistance of tobacco plants to combined Cd and Pb contamination: Physiological and microbial mechanisms. *Ecotoxicol. Environ. Saf.* **2023**, *255*, 114816. [CrossRef]
16. Zhang, B.; Li, W.; Guo, Y.; Zhang, Z.; Shi, W.; Cui, F.; Lens PN, L.; Tay, J.H. Microalgal-bacterial consortia: From interspecies interactions to biotechnological applications. *Renew. Sustain. Energy Rev.* **2020**, *118*, 109563. [CrossRef]
17. Ji, B.; Zhang, M.; Gu, J.; Ma, Y.; Liu, Y. A self-sustaining synergetic microalgal-bacterial granular sludge process towards energy-efficient and environmentally sustainable municipal wastewater treatment. *Water Res.* **2020**, *179*, 115884. [CrossRef] [PubMed]
18. Wang, S.; Zhu, L.; Ji, B.; Hou, H.; Ma, Y. Microalgal-bacterial granular sludge process in non-aerated municipal wastewater treatment under natural day-night conditions: Performance and microbial community. *Water* **2021**, *13*, 1479. [CrossRef]
19. Purba LD, A.; Zahra, S.A.; Yuzir, A.; Iwamoto, K.; Abdullah, N.; Shimizu, K.; Lei, Z.; Hermana, J. Algal-bacterial aerobic granular sludge for real municipal wastewater treatment: Performance, microbial community change and feasibility of lipid recovery. *J. Environ. Manag.* **2023**, *333*, 117374. [CrossRef]
20. Wang, S.; Ji, B.; Zhang, M.; Ma, Y.; Gu, J.; Liu, Y. Defensive responses of microalgal-bacterial granules to tetracycline in municipal wastewater treatment. *Bioresour. Technol.* **2020**, *312*, 123605. [CrossRef]
21. Wang, S.; Ji, B.; Cui, B.; Ma, Y.; Guo, D.; Liu, Y. Cadmium-effect on performance and symbiotic relationship of microalgal-bacterial granules. *J. Clean. Prod.* **2021**, *282*, 125383. [CrossRef]
22. Yang, X.; Zhao, Z.; Zhang, G.; Hirayama, S.; Nguyen, B.V.; Lei, Z.; Shimizu, K.; Zhang, Z. Insight into Cr(VI) biosorption onto algal-bacterial granular sludge: Cr(VI) bioreduction and its intracellular accumulation in addition to the effects of environmental factors. *J. Hazard. Mater.* **2021**, *414*, 125479. [CrossRef]
23. Yang, X.; Zhao, Z.; Yu, Y.; Shimizu, K.; Zhang, Z.; Lei, Z.; Lee, D.-J. Enhanced biosorption of Cr(VI) from synthetic wastewater using algal-bacterial aerobic granular sludge: Batch experiments, kinetics and mechanisms. *Sep. Purif. Technol.* **2020**, *251*, 117323. [CrossRef]
24. Zhao, D.; Cheah, W.Y.; Lai, S.H.; Ng, E.-P.; Khoo, K.S.; Show, P.L.; Ling, T.C. Symbiosis of microalgae and bacteria consortium for heavy metal remediation in wastewater. *J. Environ. Chem. Eng.* **2023**, *11*, 109943. [CrossRef]
25. Zhang, Y.; Dong, X.; Nuramkhaan, M.; Lei, Z.; Shimizu, K.; Zhang, Z.; Adachi, Y.; Lee, D.-J.; HwaTay, J. Rapid granulation of aerobic granular sludge: A mini review on operation strategies and comparative analysis. *Bioresour. Technol. Rep.* **2019**, *7*, 100206. [CrossRef]
26. Wang, S.; Ji, B.; Zhang, M.; Gu, J.; Ma, Y.; Liu, Y. Tetracycline-induced decoupling of symbiosis in microalgal-bacterial granular sludge. *Environ. Res.* **2021**, *197*, 111095. [CrossRef] [PubMed]
27. APHA. *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; American Public Health Association: Washington, DC, USA, 2005.
28. Zhou, X.; Zhang, Y.; An, X.; De Philippis, R.; Ma, X.; Ye, C.; Chen, L. Identification of aqueous extracts from Artemisia ordosica and their allelopathic effects on desert soil algae. *Chemoecology* **2019**, *29*, 61–71. [CrossRef]
29. Zhang, W.; Cao, B.; Wang, D.; Ma, T.; Xia, H.; Yu, D. Influence of wastewater sludge treatment using combined peroxyacetic acid oxidation and inorganic coagulants re-flocculation on characteristics of extracellular polymeric substances (EPS). *Water Res.* **2016**, *88*, 728–739. [CrossRef] [PubMed]
30. Herbert, D.P.; Phipps, P.J.; Strange, R.E. Chapter III Chemical Analysis of Microbial Cells. *Methods Microbiol.* **1971**, *5*, 209–344.

31. Raunkj, R.K.; Hvitved-Jacobsen, T.; Nielsen, P.H. Measurement of pools of protein, carbohydrate and lipid in domestic wastewater. *Water Res.* **1994**, *28*, 251–262. [CrossRef]
32. Liang, X.; Chen, L.; Liu, Z.; Jin, Y.; He, M.; Zhao, Z.; Liu, C.; Niyungeko, C.; Arai, Y. Composition of microbial community in pig manure biochar-amended soils and the linkage to the heavy metals accumulation in rice at harvest. *Land Degrad. Dev.* **2018**, *29*, 2189–2198. [CrossRef]
33. Lin, Y.; Ye, Y.; Hu, Y.; Shi, H. The variation in microbial community structure under different heavy metal contamination levels in paddy soils. *Ecotoxicol. Environ. Saf.* **2019**, *180*, 557–564. [CrossRef]
34. Wang, L.; Gong, L.; Gan, D.; Li, X.; Yao, J.; Wang, L.; Qu, J.; Cong, J.; Zhang, Y. Diversity, function and assembly of the Trifolium repens L. root-associated microbiome under lead stress. *J. Hazard. Mater.* **2022**, *438*, 129510. [CrossRef]
35. Abouhend, A.S.; Milferstedt, K.; Hamelin, J.; Ansari, A.A.; Butler, C.; Carbajal-González, B.I.; Park, C. Growth progression of oxygenic photogranules and its impact on bioactivity for aeration-free wastewater treatment. *Environ. Sci. Technol.* **2020**, *54*, 486–496. [CrossRef]
36. De Souza, P.O.; Sinhor, V.; Crizel, M.G.; Pires, N.; Sanches Filho, P.J.; Picoloto, R.S.; Duarte, F.A.; Pereira CM, P.; Mesko, M.F. Bioremediation of chromium and lead in wastewater from chemistry laboratories promotes by cyanobacteria. *Bioresour. Technol. Rep.* **2022**, *19*, 101161. [CrossRef]
37. Ventrella, A.; Catucci, L.; Piletska, E.; Piletsky, S.; Agostiano, A. Interactions between heavy metals and photosynthetic materials studied by optical techniques. *Bioelectrochemistry* **2009**, *77*, 19–25. [CrossRef]
38. Tsai, B.; Chang, C.; Lee, D. Fractionation of soluble microbial products (SMP) and soluble extracellular polymeric substances (EPS) from wastewater sludge. *Environ. Technol.* **2008**, *29*, 1127–1138. [CrossRef] [PubMed]
39. Yin, Y.; Hu, Y.; Xiong, F. Sorption of Cu(II) and Cd(II) by extracellular polymeric substances (EPS) from Aspergillus fumigatus. *Int. Biodeterior. Biodegrad.* **2011**, *65*, 1012–1018. [CrossRef]
40. Wang, J.; Li, Q.; Li, M.-M.; Chen, T.-H.; Zhou, Y.-F.; Yue, Z.-B. Competitive adsorption of heavy metal by extracellular polymeric substances (EPS) extracted from sulfate reducing bacteria. *Bioresour. Technol.* **2014**, *163*, 374–376. [CrossRef] [PubMed]
41. Wen, C.; Paul, W.; AL, J.; Karl, B. Fluorescence excitation−emission matrix regional integration to quantify spectra for dissolved organic matter. *Environ. Sci. Technol.* **2003**, *37*, 5701–5710. [CrossRef]
42. Wei, D.; Wang, B.; Ngo, H.H.; Guo, W.; Han, F.; Wang, X.; Du, B.; Wei, Q. Role of extracellular polymeric substances in biosorption of dye wastewater using aerobic granular sludge. *Bioresour. Technol.* **2015**, *185*, 14–20. [CrossRef]
43. Kumari, S.; Mangwani, N.; Das, S. Interaction of Pb(II) and biofilm associated extracellular polymeric substances of a marine bacterium *Pseudomonas pseudoalcaligenes* NP103. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2017**, *173*, 655–665. [CrossRef]
44. Gill, S.S.; Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* **2010**, *48*, 909–930. [CrossRef] [PubMed]
45. Mo, L.; Yang, Y.; Zhao, D.; Qin, L.; Yuan, B.; Liang, N. Time-dependent toxicity and health effects mechanism of cadmium to three green algae. *Int. J. Environ. Res. Public Health* **2022**, *19*, 10974. [CrossRef] [PubMed]
46. Verma, S.; Dubey, R.S. Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. *Plant Sci.* **2003**, *164*, 645–655. [CrossRef]
47. Li, H.; Yao, J.; Duran, R.; Liu, J.; Min, N.; Chen, Z.; Zhu, X.; Zhao, C.; Ma, B.; Pang, W.; et al. Toxic response of the freshwater green algae *Chlorella pyrenoidosa* to combined effect of flotation reagent butyl xanthate and nickel. *Environ. Pollut.* **2021**, *286*, 117285. [CrossRef]
48. Shao, W.; Li, M.; Teng, Z.; Qiu, B.; Huo, Y.; Zhang, K. Effects of Pb(II) and Cr(VI) stress on phosphate-solubilizing bacteria (*Bacillus* sp. Strain MRP-3): Oxidative stress and bioaccumulation potential. *Int. J. Environ. Res. Public Health* **2019**, *16*, 2172. [CrossRef]