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Research on the Tolerance and Degradation of o-Cresol by Microalgae

Guangshuo Han [1,2,†], Longtan Ma [3,*,†], Caisong Zhang [1,2], Baihui Wang [1,2], Xianghao Sheng [1,2], Zhongpeng Wang [1,2], Xiuju Wang [1,2,*] and Liguo Wang [1,2,*]

[1] School of Water Conservancy and Environment, University of Jinan, Jinan 250022, China; hangs98@163.com (G.H.); s18561800216@163.com (C.Z.); w18766561979@163.com (B.W.); xh20212021@163.com (X.S.); chm_wangzp@ujn.edu.cn (Z.W.)

[2] Key Laboratory of Water Resources and Environmental Engineering in Universities of Shandong, University of Jinan, Jinan 250022, China

[3] Shandong Hancheng Construction Engineering Co., Ltd., Jinan 250000, China

* Correspondence: luckyly519@163.com (L.M.); chm_wangxj@ujn.edu.cn (X.W.); lgwang666@163.com (L.W.); Tel.: +86-18-605-311-686 (L.M.); +86-19-953-123-057 (X.W.); +86-13-698-613-168 (L.W.)

† These authors contributed equally to this work.

Abstract: o-Cresol shows high toxicity and stability. To explore a better degradation method of o-cresol, the degradation of o-cresol by three kinds of microalgae (*Selenastrum capricornutum*, *Scenedesmus obliquus*, and *Microcystis aeruginosa*) was researched in this paper. The growth status and degradation rate were used to reflect the tolerance and degradation effect of microalgae. The effects of the medium's initial pH, microalgal density, and different exogenous pollutants on the degradation of o-cresol by *Selenastrum capricornutum* were investigated. The results showed that *Selenastrum capricornutum* had the best degradation effect on o-cresol. microalgal density increased after adaptation to different concentrations of o-cresol for some time. At pH 7.0 as the initial condition, the microalgal exhibited the best results of degradation. When the microalgal density OD_{680} was 0.20, o-cresol was the first to be completely degraded within 5 days. At higher initial concentrations of o-cresol, the microalgae preferentially degraded glucose to promote the growth of the microalgae under mixotrophic cultivation. *Selenastrum capricornutum* could degrade phenol and o-cresol at the same time, and the degradation was completed within 8 days when the initial concentration of o-cresol and phenol were 100 and 120 mg/L. It was proven that the degradation of o-cresol by *Selenastrum capricornutum* is feasible under suitable conditions.

Keywords: phenolic; microalgae; growth behavior; degradation rate; co-degradation

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1. Introduction

Phenolic compounds are aromatic compounds including phenol and its derivatives. They are important raw materials for chemical production and have been widely used in metallurgy, machinery manufacturing, the petrochemical industry, medicine, and pesticides [1]. However, these compounds often occur as hazardous pollutants in aquatic ecosystems as their high toxicity to plants, animals, and aquatic life [2]. As early as 1977, the US Environmental Protection Agency (EPA) listed 11 phenolic compounds as priority pollutants [3]. Cresol comes from a wide range of sources, it can be released from landfills, hazardous waste landfills, and even livestock manure. [4]. o-Cresol shows high toxicity and stability [5]. Among the three isomers of cresol, the toxicity of o-cresol is higher than the other two cresols [6]. The harmfulness of o-cresol to the human body mainly depends on the concentration of o-cresol and the exposure time [7]. Studies have shown that o-cresol can cause several respiratory diseases, neurological diseases, and other serious harm to various organs of the human body [8]. Moreover, o-cresol has strong carcinogenic, teratogenic, and mutagenic effects on animals [9]. It is very important to find an effective method to

degrade o-cresol. To protect the environment, they are treated before being discharged into the environment. Compared with traditional physical or chemical treatment, biological treatment is characterized by being a simple process, low cost, and no harmful by-products. Biological treatment is gradually being widely used [10].

Microalgae is the primary producer of the ecosystem, the distribution is broad, the variety is high, the production time is short, and the adaptability is strong [11]. Research has shown that the potential of microalgae to degrade organic pollutants in wastewater and seawater can not be ignored. [12,13]. It has been experimentally proven that cyanobacteria and microalgae can make full use of renewable energy (solar energy) and are highly adaptable to autotrophic, heterotrophic, or mixotrophic culture conditions [5,14]. Since the first use of microalgae in the biodegradation process by Oswald and Gotaas, the role of microalgae in biodegradation has been gradually emphasized. The polycyclic aromatic hydrocarbons (PAHs) removal efficiency increased with the initial cell density, and 96% of phenanthrene (PHE), 100% of fluoranthene (FLA), and 100% of pyrene (PYR) in the medium were removed by live *Selenastrum capricornutum* [15]. A large number of studies have shown that *Chlorella* has an effective degradation effect on organic pollutants [16–21]. In addition, the pollution removal abilities of spirulina [22], diatoms [23], and *Chlamydomonas reinhardtii* [24] have also been studied. Most of the results have shown that the tolerance limits of microalgae to pollutants vary with different microbial species [25]. Studies have shown that 2-hydroxyhexaonic acid, 2-hydroxyglutaric acid, and 2-hydroxyadipic acid after ring-opening were detected in an o-cresol degradation experiment by *Pseudomonas stutzeri* N2 in China. Therefore, ring-opening was first generated from methyl carboxylation on the benzene ring and then decarboxylated to phenol,. In this way, ring-opening was conducted by way of ortho-cleavage.

Therefore, it is possible to degrade o-cresol with microalgae. However, few investigations have focused on the microalgal removal of o-cresol from wastewater. In this study, three species of microalgae were selected to screen for pollution-resistant strains among different microalgae. Three microalgae were selected for o-cresol degradation, and the best strain was determined by measuring the biomass of the strain and the residual concentration of o-cresol in wastewater. By screening out the microalgae with the best degradation effect and exploring the influence of different pH values and different microalgal densities on the degradation effect. Different concentrations of nutrient carbon sources and pollution carbon sources were added under the optimal degradation conditions to explore the impact on the degradation effect. This information will be valuable for the ecological risk and removal of o-cresol in wastewater.

2. Materials and Methods

2.1. Microalgae Strain and Culture Medium

Selenastrum capricornutum (FACHB-271), *Microcystis aeruginosa* (FACHB-905) and *Scenedesmus obliquus* (FACHB-12) from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (Wuhan, China) were selected. Blue-Green Medium (BG11 medium) [26] was used for microalgae cultivation, consisting of 1.5 g/L sodium nitrate, 0.04 g/L dipotassium hydrogen phosphate, 0.075 g/L magnesium sulfate heptahydrate, 0.036 g/L calcium chloride dihydrate, 0.001 g/L ethylenediaminetetraacetic acid disodium salt, 0.02 g/L sodium carbonate, 0.006 g/L ammonium ferric citrate, and 0.006 g/L citric acid. The medium also included a 1mL per liter of trace elements solution consisting of 2.86 g/L boric acid, 1.86 g/L manganese tetrachloride tetrahydrate, 0.22 g/L zinc sulfate heptahydrate, 0.39 g/L sodium molybdate dihydrate, 0.08 g/L copper sulfate pentahydrate, and 0.05 g/L cobaltous nitrate hexahydrate. The pH of the medium was adjusted to 7.1 using hydrochloric acid (HCl) or sodium hydroxide (NaOH) and then autoclaved at 121 °C for 20 min. Phenol, o-cresol with a certified purity >99%, and other reagents were all of analytical grade and obtained from Shanghai Sinopharm Group Corporation. Methanol was of HPLC grade from Agilent Technologies Inc (Agilent, Santa Clara, CA, USA).

2.2. Experimental Set-Up

2.2.1. Biodegradation of Single Pollutant

The degradation of o-cresol at different concentrations by three microalgae was investigated. Different concentrations of o-cresol were added to the BG11 medium to achieve concentrations of 50, 100, 200, 300, and 400 mg/L of o-cresol. Additionally BG11 medium without o-cresol was the control group. Microalgae with similar algal densities were inoculated into 200 mL of BG11 medium containing different concentrations of o-cresol. All microalgae were cultured in a light incubator with the following culture conditions: light intensity of 3000 lx, temperature of 25 ± 0.5 °C, and a light-dark cycle of 12 h: 12 h (culture conditions were the same for all experiments below). The initial pH of the solution, which was alkaline, was not changed at the beginning of the experiment. The samples were shaken every six hours. Algal density and o-cresol content in each medium were measured every other day. Screening experiments were carried out on the biodegradation of o-cresol by different algae to compare the general performance of different microalgae and screen out the dominant algal species that degrade o-cresol.

2.2.2. Biodegradation Experiments

In this section, the effects of other factors on the degradation of o-cresol by dominant algal species were investigated. The concentration of o-cresol in the medium was fixed at the optimal concentration measured in Section 2.2.1, and the dominant algal species was inoculated into the medium with different pH values (pH 5.0–9.0). After studying the experimental results under different conditions and considering the growth of the microalgae and degradation of o-cresol, the optimal initial pH value was selected. Next, different initial microalgal densities OD_{680} (OD_{680} of 0.10, 0.20, 0.45, 0.65, 0.85, and 1.05) were set under the optimal initial pH and at 100 mg/L of o-cresol. The optimal initial pH value and initial microalgae density OD_{680} were selected as the basic conditions for future experiments.

2.2.3. Co-Degradation of o-Cresol and Glucose/Phenol

To study the co-degradation of the dominant algal species, the medium was modified by adding phenol and glucose to the medium containing o-cresol. The dominant algal species was inoculated into the modified medium according to the optimal culture conditions selected in Section 2.2.2. The algal density, glucose, phenol, and o-cresol contents were determined. In the co-degradation study of o-cresol and glucose, glucose was added to 200 mL of BG11 medium as an organic carbon source for the mixed culture (with final concentrations of 2, 5, and 8 g/L), and the concentration of o-cresol at this time was 300 mg/L or 400 mg/L. In the co-degradation study of o-cresol and phenol, the concentration of o-cresol was fixed at 100 mg/L, while the concentration of phenol was 120, 220, or 320 mg/L.

2.2.4. Measurement Methods

The density of algal cells and o-cresol concentration were measured according to the above experimental requirements. First, the number of algal cells at each stage was accurately counted by a hemocytometer under a microscope (JS-750T, LIOO, Beijing, China). The corresponding absorbance was then detected by a UV-Vis spectrophotometer (752N, Shimadzu, Japan) at a wavelength of 680 nm [27]. Finally, the linear relationship between algal density and OD_{680} was obtained. For the measurement of o-cresol concentration, the algae liquid was centrifuged in a high-speed centrifuge at 8000 rpm for 10 min and then the supernatant was collected. The supernatant was then filtered through a 0.45 μm membrane and detected by high performance liquid chromatography (HPLC) (1260 II, Agilent, Santa Clara, CA, USA). The detection conditions were as follows: the chromatographic column used was a C18 column (4.6 mm × 250 mm, 5 μm); the mobile phase was methanol and water, at a ratio of 4:6; the flow rate was 1 mL/min; the injection volume was 20 μL; the

wavelength was 270 nm; and the running time was about 30 min. Standard curves were established using standard o-cresol samples at known concentrations [4].

2.2.5. Date Analysis

Each treatment included three independent bottles with two samplings carried out for each individual bottle. Standard deviations of the average values are presented om the figures.

3. Results and Discussion

3.1. Bioremoval of o-Cresol by Microalgae

The work began with screening tests comparing the effectiveness of three different microalgae in degrading o-cresol. The growth of three different microalgae and the degradation of o-cresol over 7 days, with o-cresol as the only carbon source, is shown in Figure 1. After many previous experiments, the residual concentration of pollutants in the control bottle, without microalgae inoculation did not significantly change, indicating that the natural loss of o-cresol could be ignored.

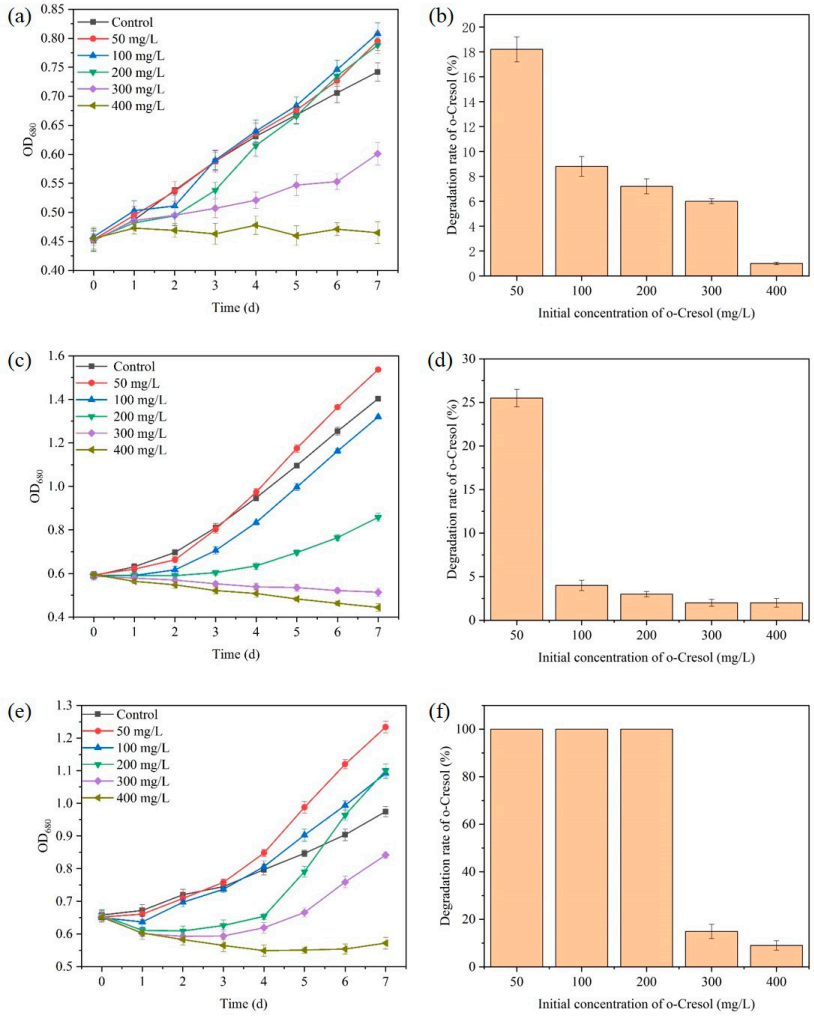

Figure 1. Growth of three microalgae strains of *Scenedesmus obliquus*, *Microcystis aeruginosa*, and *Selenastrum capricornutum* and changes in the concentration of o-cresol. (**a**) Growth curve of *Scenedesmus obliquus*; (**c**) growth curve of *Microcystis aeruginosa*; (**e**) growth curve of *Selenastrum capricornutum*; (**b**) o-cresol degradation rate by *Scenedesmus obliquus*; (**d**) o-Cresol degradation rate by *Microcystis aeruginosa*; (**f**) degradation curve of o-cresol by *Selenastrum capricornutum*.

The microalgal growth and o-cresol degradation by *Scenedesmus obliquus* is presented in Figure 1a,b. The initial concentrations of o-cresol were 50, 100, and 200 mg/L, which had a small promotional effect on the growth of microalgae (Figure 1a). When the initial concentration of o-cresol was 400 mg/L, the growth ofmicroalgae was completely inhibited. The degradation effect of the microalgae on o-cresol was sub-optimal (Figure 1b). When the initial concentration of o-cresol was 50 mg/L, the degradation rate of o-cresol within seven days was only 18.2%. The experimental results showed that o-cresol inhibited the growth of *Microcystis aeruginosa* more obviously than *Scenedesmus obliquus* (Figure 1c). Compared with the control group, the initial concentration of o-cresol was 50 mg/L, which promoted the growth of the microalgae. Nevertheless, when the concentration of o-cresol was more than 300 mg/L, the biomass of the microalgae decreased gradually. The degradation effect of *Microcystis aeruginosa* on o-cresol was also very poor. When the initial concentration of o-cresol was 50 mg/L, the degradation rate of o-cresol after seven days was 25.5%. When the initial concentration of o-cresol was in the range of 200 to 400 mg/L, the degradation of o-cresol by *Microcystis aeruginosa* was almost negligible.

On the seventh day, we observed that the absorbance of *Selenastrum capricornutum* was significantly higher than that of the control when the initial concentration of o-cresol was 50, 100, or 200 mg/L. However, when the initial concentration of o-cresol was 200 mg/L the absorbance of *Selenastrum capricornutum* showed a trend of decreasing and then increasing (Figure 1e). According to Figure 1e it is clear that atlow concentrations o-cresol was almost completely degraded, while at high concentrations o-cresol was less degraded. Combining Figure 1e,f we can conclude that o-cresol act as a carbon source for the growth of *Selenastrum capricornutum*, so low concentrations of o-cresol will promote the growth of *Selenastrum capricornutum*, while at high concentrations *Selenastrum capricornutum* needs some time to adapt to the abundance of o-cresol. Once adapted to the high concentration, it can continue to be used as a carbon source for its growth and development. Previous studies have shown that microalgae have a good degradation effect on benzo(a)pyrene (BaP), where adsorption was an important phenomenon for *Selenastrum capricornutum* to remove BaP, and biodegradation was the principal means of removing BaP from living cells [28].

3.2. Degradable o-Cresol Concentration by Selenastrum Capricornutum

Firstly, to investigate the effect of pH on the degradation efficiency of o-cresol, the biodegradation of o-cresol at an initial concentration of 100 mg/L under the same conditions was experimentally studied, with initial pH values ranging from 5.0 to 9.0. Many studies have shown that higher or lower pH values significantly affect the efficiency of microbial degradation of organic pollutants [29].

The absorbance of the microalgae increased under different pH values (Figure 2a). It was seen that, compared with other pH values, the growth speed of the microalgae had the highest inhibition rate at a pH of 5.0. At a pH of 9.0, its final biomass was lower than at pH 6.0–8.0. When the pH was 7.0 and 8.0, the growth rate and biomass of the microalgae were close to each other within six days. It was most obvious that the absorbance of the microalgae decreased during the day after the addition of o-cresol at a pH of 6, but after a day for adaptation, the growth rate increased and reached its maximum on days 3 to 4. In experiments with the microalgae removal of carbon dioxide from flue gases, Olaizola [30] showed that the maximum photochemical efficiency occurs at dissolved CO_2 concentrations above two orders of magnitude (0.7–70 mg/L) and at pH values of 6.5–8.5. Controlling pH in a microalgae system is essential for photochemical processes and microalgal growth [31].

The degradation effect of o-cresol by *Selenastrum capricornutum* at different pH values when o-cresol was used as the sole carbon source, is shown in Figure 2b. The results showed that the growth of the microalgae was directly related to the degradation of o-cresol. The degradation rate of o-cresol was inhibited under both low and high pH conditions, most obvious at a pH of 5.0. At pH 5.0, the degradation rate of o-cresol by the microalgae after six days was only 4%, deemed almost negligible. The degradation rate of o-cresol at a pH of 9.0 was 76.5%. Studies have shown that when the pH increased to 6.0, the H^+

concentration is more appropriate, and phenol is more easily accessible to the biosorption sites. Whereas, when the pH reaches8.8, bionanoparticles are negatively charged, and phenol is more likely to dissociate [32]. In the experiment, the microalgae had the best degradation effect under neutral conditions. At pH 6.0 and 7.0, o-cresol was completely degraded on the fifth day; however, the degradation effect of o-cresol was better at pH 6.0 (90.7%) on day 4 than at pH 7.0 (52.8%). At pH 8.0, o-cresol was completely degraded on the sixth day of the experiment.

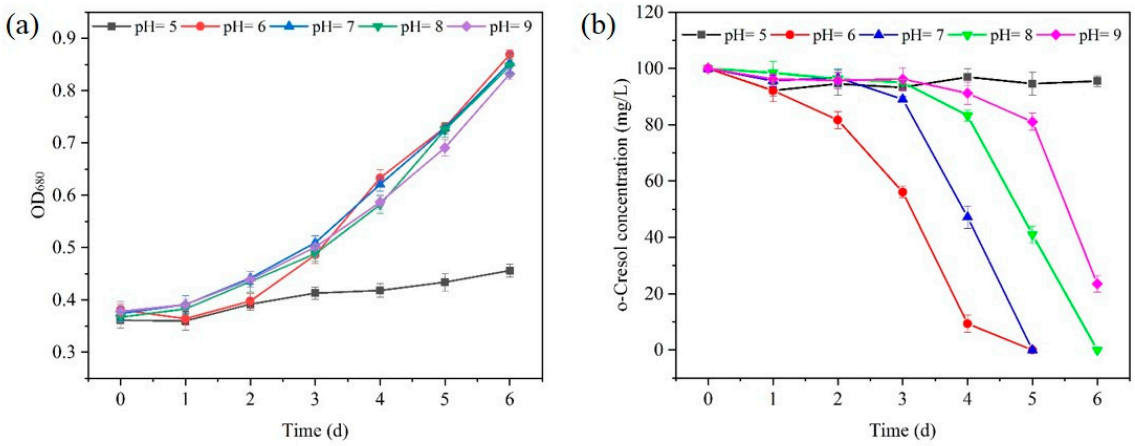

Figure 2. Growth of *Selenastrum capricornutum* and the degradation of o-cresol at different pH values. (**a**) Growth curve of *Selenastrum capricornutum*; (**b**) degradation curve of o-cresol.

To better study the effect of different microalgal densities on the degradation effect of o-cresol, further experiments were carried out at pH 6.0 and 7.0 (Figure 3). It was evident that under acidic conditions, microalgae with a pH of 6.0 had an adaptation period (Figure 3a,c). No matter the microalgae density, the microalgae density would decrease within one day of the experiment. The higher the microalgae density was, the more obvious the effect was (Figure 3a), while under a neutral pH, the microalgae density continued to increase (Figure 3c). The growth of the microalgae with various initial microalgae densities at pH 7.0 was better than that at pH 6.0. The results showed that the maximum growth rate was observed at days 6 under both pH conditions with an OD_{680} of 0.20.

Different microalgae densities showed a good degradation effect on o-cresol within six days of the experimental study (Figure 3b,d). In terms of the growth status of the microalgae, the degradation effect was the best when the initial microalgae density OD_{680} was 0.20, regardless of whether the pH was 7.0 or 6.0. Different microalgae densities had different degradation effects under different pH values. Generally speaking, the microalgae had a better degradation effect on o-cresol when the pH was 7.0 (Figure 3d). At pH 6.0, when the microalgae density OD_{680} values were 0.10, 0.20, and 0.65, o-cresol was completely degraded within five days, and when the OD_{680} values were 0.45 and 0.85, o-cresol was completely degraded within six days, When the OD_{680} was 1.05, the degradation rate of o-cresol on the 6th day was 76.9%. Comparatively speaking, at pH 7.0, when the microalgae density OD_{680} was 0.10, 0.20, 0.45, and 0.65, o-cresol was completely degraded within five days, and when the OD_{680} values were 0.85 and 1.05, o-cresol was completely degraded within six days.

The overall data of this experiment included the growth of *Selenastrum capricornutum* and the degradation of o-cresol under different conditions. An OD_{680} of 0.20 and a pH of 7.0 were selected as the conditions for the following experiments.

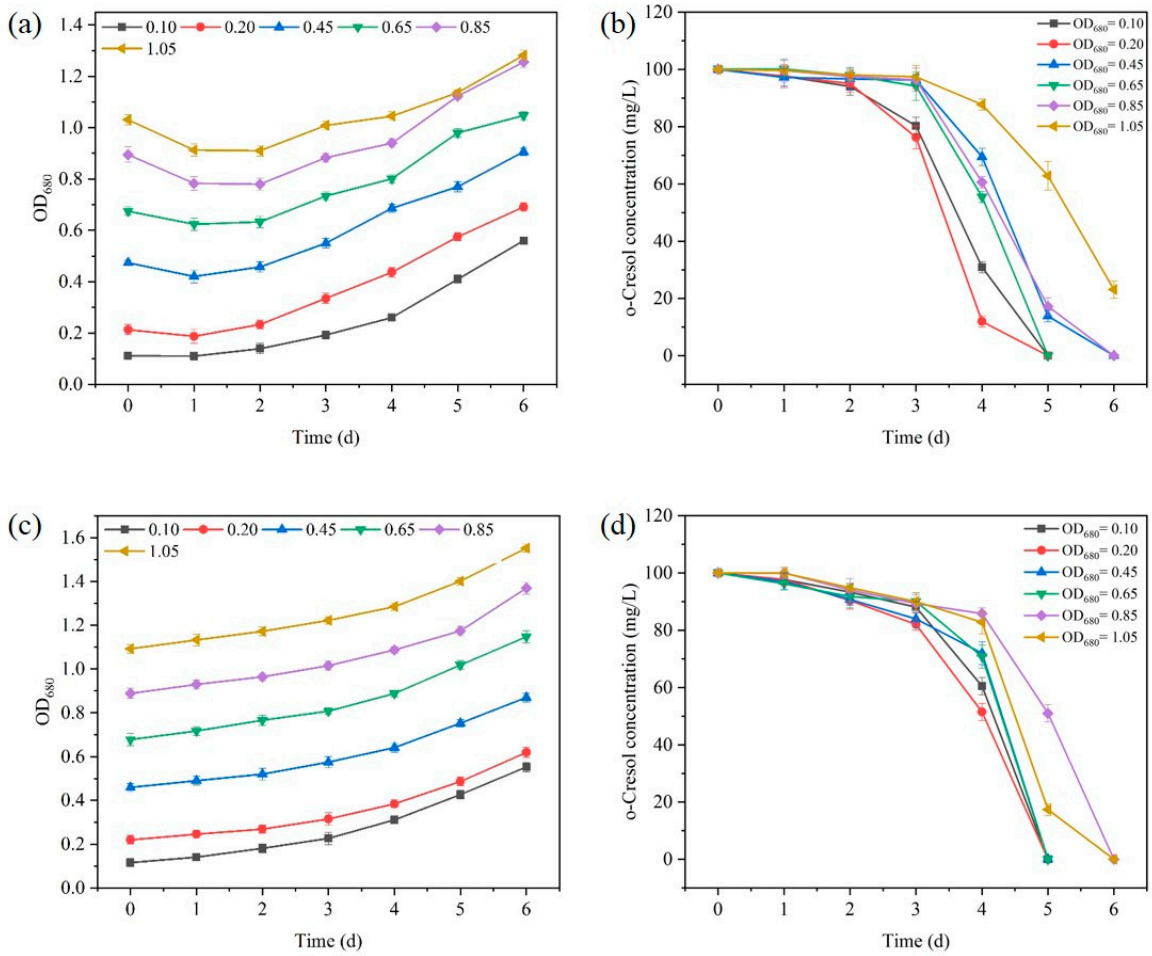

Figure 3. Growth of *Selenastrum capricornutum* and the degradation of o-cesol under different microalgae densities. (**a**) Growth curve of *Selenastrum capricornutum* at pH of 6.0; (**b**) degradation curve of o-cresol at pH 6.0; (**c**) growth curve of *Selenastrum capricornutum* at pH 7.0; (**d**) degradation curve of o-cresol at pH 7.0.

3.3. Co-Degradation Experiment

3.3.1. Co-Degradation of o-Cresol and Glucose

The previous results showed that under the mixed nutrient conditions of adding phenol and glucose at the same time, the growth of *Chlorella* was inhibited, but adding an appropriate amount of glucose can enhance the tolerance of microalgae to phenol and affect the removal rate of phenol [16]. *Selenastrum capricornutum* can completely degrade low concentrations of o-cresol. Therefore, a higher initial concentration of o-cresol was selected to explore the effect of the addition of glucose on the degradation of o-cresol by microalgae. Experiments were carried out at a a microalgae density OD_{680} of 0.20 and a pH of 7.0. Loh et al. [33] reported that when the concentration of glucose was higher than 1.0 g/L, the removal rate of phenol decreased with increasing glucose concentration, to a rate even lower than that of the control without glucose. In the presence of phenol and glucose, microbial utilization of these two organics was competitive. From the data obtained, in the two higher o-cresol concentration experimental groups, the biomass of the microalgae increased significantly after glucose was added, but the degradation rate did not change much. The results showed that the co-existence of glucose and o-cresol would lead to the preferential degradation of glucose.

Under the same pollutant concentration and different glucose concentrations, the growth of the microalgae was significantly different (Figure 4a,c). The results showed that the biomass of the microalgae increased significantly after the addition of glucose, however,

it was not the case that when more glucose was added, the better the growth of microalgae. During the eight-day experimental study, the final biomass of the microalgae was highest at either 300 or 400 mg/L of o-cresol after the addition of 5 g/L of glucose. The final biomass contents in the mixotrophic groups with the addition of 300 mg/L of o-cresol and 2, 5, and 8 g/L of glucose were 2.6, 4.3, and 4.1 times higher than in the autotrophy groups without o-cresol, respectively. The growth of the microalgae was significantly different on the fourth day of the experiment. Thecorresponding final biomass contents in the 400 mg/L group with the addition of 2, 5, and 8 g/L of glucose were 2.5, 3.1, and 2.3 times higher than those without glucose, respectively. Previous research results have shown that compared with autotrophs, microalgae cultured under mixed culture conditions could significantly increase biomass production [34].

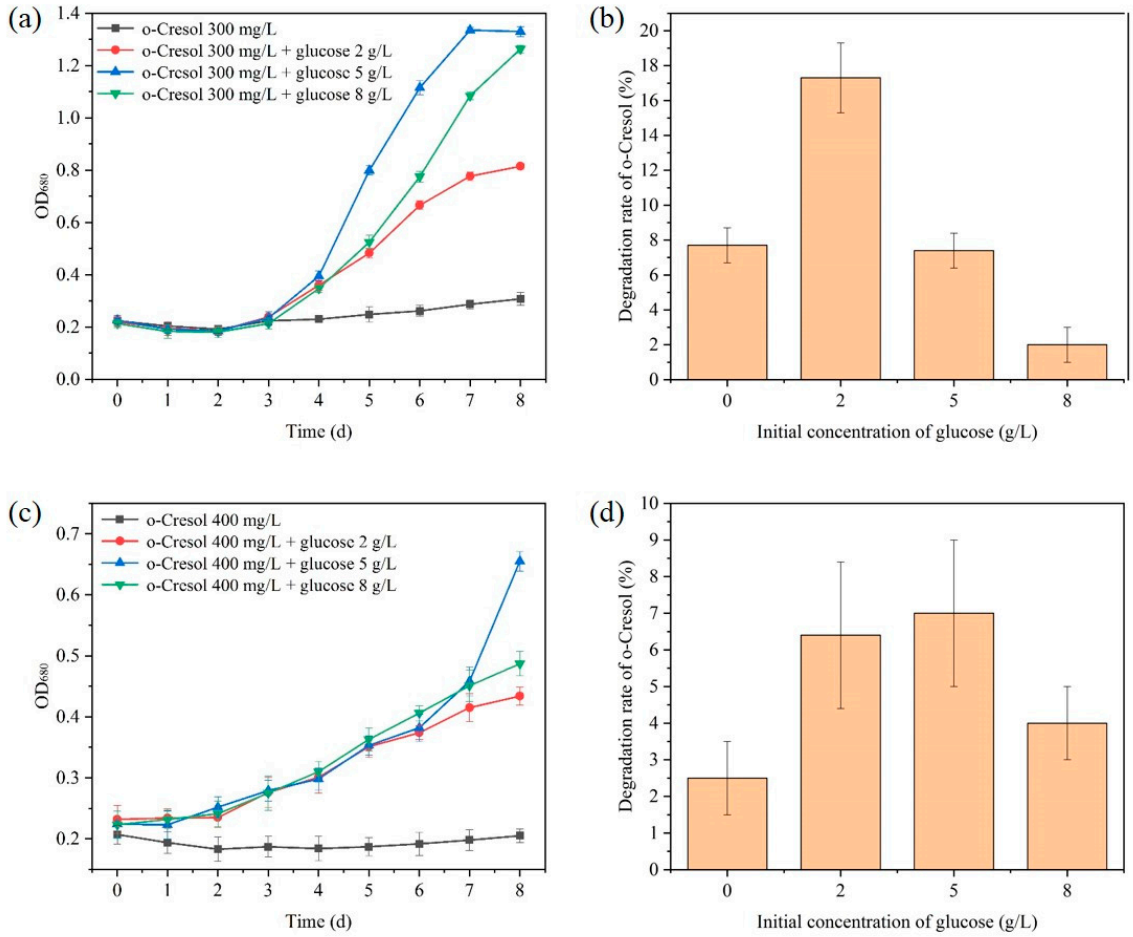

Figure 4. Co-degradation of glucose and o-cresol. (**a**,**c**) growth curve in the co-degrading system; (**b**,**d**) o-cresol degradation rate.

The results showed that 300 mg/L o-cresol was more easily degraded by the microalgae in the presence of low glucose and with the highest o-cresol removal rate at 17.3% (Figure 4b). Although the biomass of the microalgae was significantly increased after 5 g/L of glucose was added, the degradation rate of o-cresol was close to that of the control group without glucose; at 7.4% and 7.7%,respectively. However, the degradation rate of o-cresol was significantly inhibited after the addition of a high concentration of glucose, and the degradation rate of o-cresol was only 2% after the addition of 8 g/L of glucose, which was ignored. At high concentrations of pollutants, the degradation rate of o-cresol with different concentrations of glucose was higher than that of the control group without glucose (Figure 4d). The degradation rate of o-cresol was close to 6.4% and 7% after adding 2 and 5 g/L glucose, respectively, at 400 mg/L of o-cresol. The degradation rate of o-cresol

in the two experimental groups with 8 g/L and without glucose was lower: 4% and 2%, respectively. Rolle [35] et al. found that the highly active tricarboxylic acid cycle (TCA cycle) plays an important role in the biodegradation of phenol. This suggests that appropriate glucose addition in the medium may stimulate the TCA cycle in the algae, thereby promoting the degradation of o-cresol.

3.3.2. Co-Degradation of o-Cresol and Phenol

Considering that o-cresol and phenol are both highly toxic pollutants, we conducted a co-degradation experiment using low- concentrations of o-cresol and three different concentrations of phenol. The highest biomass was observed when the medium contained 120 mg/L of phenol and 100 mg/L of o-cresol (Figure 5a) with the highest degradation rate of both reaching 100% after eight days (Figure 5d). Microalgae had an obvious degradation effect on the two kinds of pollutants (Figure 5b,c). However, the toxicity to the microalgae increased with the increase in the phenol concentration, inhibiting the growth of the microalgae and led to a decrease in the o-cresol removal rate. When the initial phenol concentration was 220 and 320 mg/L, the degradation rate of o-cresol after eight days was 68.1 and 17%, while phenol was 88 and 19.3%, respectively. In addition, some studies on the co-degradation of phenol and cresol have shown that low concentrations of phenol improve the degradation effect of cresol, whereas high concentrations could inhibit the degradation effect of cresol [36]. Similarly, Xiao [27] et al. showed that phenol at low concentrations of phenol (100 mg/L) promote the removal of o-cresol by *Chlorella vulgaris*.

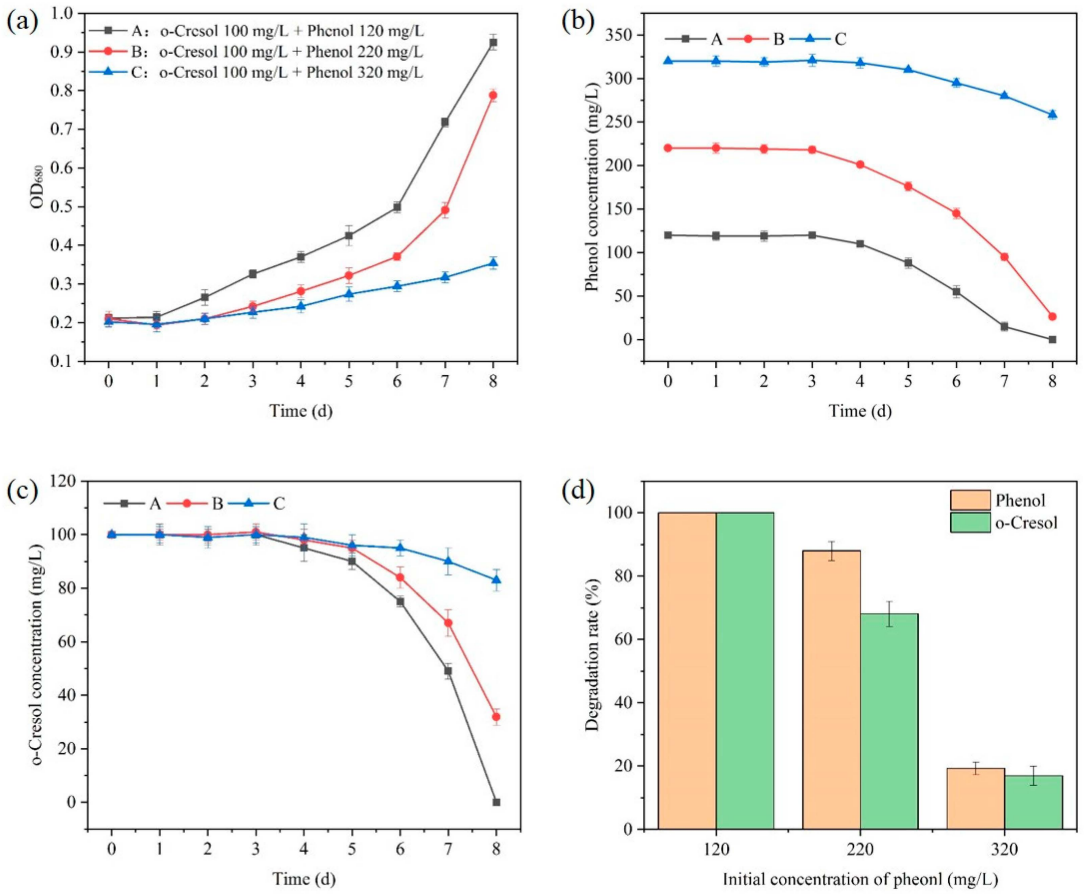

Figure 5. Co-degradation of phenol and o-cresol. (**a**) The growth curve in the co-degrading system; (**b**) degradation curve of phenol; (**c**) degradation curve of o-cresol; and (**d**) degradation rate of phenol and o-cresol.

The results of this experiment are similar to the results of Niesler et al. [37]. showing that the biodegradation of pentachlorophenol in the presence of glucose was less efficient than in the presence of phenol.

4. Conclusions

In this paper, the degradation of o-cresol by three types of microalgae (*Selenastrum capricornutum*, *Scenedesmus obliquus*, and *Microcystis aeruginosa*) was studied, and the growth status and degradation rate were used to reflect the tolerance and degradation effect of the microalgae. The ability of *Selenastrum capricornutum* to remove o-cresol from the environment was first reported in this paper. When the initial concentration of o-cresol was low, *Selenastrum capricornutum* grew well, and o-cresol was completely degraded in the experiment. The optimum degradation conditions of o-cresol were determined by studying the factors affecting the degradation of o-cresol. Under optimum degradation conditions, o-cresol was degraded entirely for the first time. Under high concentrations of o-cresol, adding glucose accelerated the growth of microalgae, while adding the appropriate amount of glucose improved the degradation rate of o-cresol. *Selenastrum capricornutum* can degrade phenol and o-cresol simultaneously. In this study, the possibility of o-cresol degradation by *Selenastrum capricornutum* was verified, demonstrating that *Selenastrum capricornutum* has great potential for the degradation of organic pollutants. The research results also provide a theoretical basis for the microalgae degradation of o-cresol in industrial applications.

Author Contributions: G.H.: Methodology, investigation, formal analysis, data curation, and Writing original draft. L.M.: investigation, software, validation, resources, and data curation. C.Z., B.W., and X.S.: data curation. Z.W.: visualization, supervision. X.W. and L.W.: conceptualization, visualization, supervision, writing, review and editing, project administration, and gfunding acquisition. All authors have read and agreed to the published version of the manuscript.

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