

## Article

# Sustainable Treatment of Swine Wastewater: Optimizing the Culture Conditions of *Tetrademus cf. obliquus* to Improve Treatment Efficiency

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**Abstract:** To enhance the sustainability of microalgae-based swine wastewater treatment, this study aims to address the challenges of low efficiency in treating raw swine wastewater, collection difficulties, and high energy consumption treatment processes. The microalgae with strong environmental tolerance were first screened from swine wastewater, and its cultivation conditions were optimized to examine the effect of microalgae treatment on swine wastewater under optimal cultivation conditions. Additionally, the flocculation efficiency and mechanism of microalgae were analyzed. The results showed that *Tetrademus cf. obliquus* ZYY1 exhibited the most robust heterotrophic growth. In the BG11 medium supplemented with glucose, the growth rate of *T. cf. obliquus* ZYY1 under chemoheterotrophic conditions was superior to its growth under photoheterotrophic conditions, reaching its peak with an optimal glucose concentration of 15 g/L. The biomass concentration of *T. cf. obliquus* ZYY1 in raw wastewater was significantly higher than that in sterilized wastewater, which reached  $1.65 \pm 0.01$  g/L on the 10th day of treatment, with removal efficiencies of  $\text{NH}_4^+-\text{N}$ ,  $\text{PO}_4^{3-}-\text{P}$ , and the chemical oxygen demand reached 71.36%, 96.09%, and 93.13%, respectively. After raw wastewater treatment, the flocculation efficiency of *T. cf. obliquus* ZYY1 reached  $97.71 \pm 5.81\%$ . This was attributed to the bacteria present in the raw wastewater, which induced *T. cf. obliquus* ZYY1 to secrete aromatic proteins. This study emphasizes the potential of microalgae as a green technology for sustainable wastewater treatment, offering a practical pathway for environmental protection and resource conservation.

**Keywords:** microalgae; heterotrophic culture; glucose; sustainable swine wastewater treatment; flocculation; extracellular polymeric substance



**Citation:** Bai, K.; Qu, W.; Song, D.; Li, J.; Ho, S.-H. Sustainable Treatment of Swine Wastewater: Optimizing the Culture Conditions of *Tetrademus cf. obliquus* to Improve Treatment Efficiency. *Sustainability* **2024**, *16*, 4633. <https://doi.org/10.3390/su16114633>

Academic Editor: Agostina Chiavola

Received: 17 April 2024

Revised: 24 May 2024

Accepted: 25 May 2024

Published: 29 May 2024



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## 1. Introduction

In recent years, swine farming in China has developed rapidly, producing much swine wastewater that contains nitrogen, phosphorus, and organic matter, which has potential applicability for the environment. Therefore, developing a simple, effective, and cost-effective method for treating swine wastewater is imperative to promote sustainable development. Microalgae are single-celled microorganisms, with the characteristics of rapid growth and strong environmental adaptability, that can utilize pollutants for cells synthesis and simultaneously purify wastewater [1]. Compared with traditional swine wastewater treatment methods, this method does not induce secondary pollution [2]. Consequently, microalgae wastewater treatment technology has attracted increasing attention.

Microalgae species commonly used in wastewater treatment include *Chlamydomonas* sp., *Scenedesmus* sp., *Chlorella* sp., and *Spirulina* sp. For example, *Chlorella* M-12 can grow in swine manure biogas slurry and can remove 80% of nitrogen and phosphorus, as well as 30–50% of chemical oxygen demand (COD) [3]. However, numerous microorganisms in

wastewater make it challenging for microalgae to establish dominance in the ecological niche [4], leading to algal inversion, which disrupts the wastewater treatment system. As such, pretreatments, such as sterilization and dilution, are necessary to prevent microbial contamination during microalgae wastewater treatment [5]. Tan et al. showed that the growth of microalgae was significantly inhibited in anaerobic digested swine wastewater containing high concentrations of  $\text{NH}_4^+$  and Cu [6]. Similarly, Acebu et al. found that the *C. vulgaris* ESP-31 achieved 54.2% TN removal in 25% dilution swine wastewater [7]. Therefore, screening microalgae with a strong tolerance to swine wastewater is highly significant for enabling the large-scale application of microalgae wastewater treatment technology.

*Tetrademus obliquus*, a species of freshwater microalgae, exhibits remarkable attributes including robust reproductive capacity and environmental adaptability. *T. obliquus* GEEL-11 can remediate saline wastewater. Under different salt stress conditions, *T. obliquus* GEEL-11 can remove (87–99%) of nitrogen and (94–100%) of phosphorus from municipal wastewater [8]. *T. obliquus* was able to remove 77% of TKN, 50% of phosphate, and 84% of COD from poultry wastewater [9]. In undiluted piggery wastewater, the biomass concentration of *T. obliquus* was  $1.46 \pm 0.06$  g/L after photo-Fenton pretreatment [10]. The results of the above studies indicate that *T. cf. obliquus* can grow effectively in a variety of wastewaters and exhibit good performance. However, there are limited studies on the growth performance and pollutant removal ability of *T. cf. obliquus* in untreated raw swine wastewater.

The prevailing method for cultivating microalgae in wastewater involves photosynthetic culture, where microalgae use sunlight and  $\text{CO}_2$  as their energy and carbon sources, respectively [11]. However, it is important to note that many microalgae are physiologically capable of utilizing organic compounds as alternative sources for heterotrophic metabolism [12]. By developing these heterotrophic systems, the issue of insufficient light in phototrophic cultures is overcome, allowing for higher cell densities that are independent of weather or seasonal changes and reducing the requirement for large land areas. Heterotrophic cultivation methods for microalgae are categorized into chemoheterotrophic and photoheterotrophic modes. Research on the heterotrophic culture mode that is specifically suitable for *T. obliquus* is limited. Therefore, it is highly significant to optimize the heterotrophic culture mode suitable for *T. obliquus* in this study.

Another bottleneck in the industrialization and promotion of microalgae-based water treatment is the difficulty in collecting microalgae from wastewater. This difficulty arises from their small cell size (3–30  $\mu\text{m}$ ), which is similar to the density of water, and the negative charges in the water, causing cell rejection and suspension in wastewater [13]. Traditional microalgae collection methods mainly include centrifugation, membrane filtration, air flotation, and flocculation, but these methods have disadvantages, such as complex equipment installation, high costs, and metal ion pollution [14]. To address these issues, bio-flocculation technology has emerged as a potential option due to its characteristics of eco-friendliness and simple operation [15]. The extracellular polymeric substances (EPS) secreted by microalgae can neutralize the negative charge of algal cells, promoting microalgae flocculation and holding prospects for industrial applications.

Therefore, in this study, microalgae with a strong tolerance to swine wastewater were selected, and their growth ability was improved by optimizing the culture conditions of the superior microalgae strains. Additionally, the pollutant removal efficiency of the superior microalgae strains in swine wastewater was investigated under the optimal culture conditions, and their flocculation efficiency and mechanism were analyzed comprehensively. The objective of this study is to provide detailed technical guidance for the treatment of swine wastewater using microalgae and to propose an efficient method for the enhanced harvesting of microalgae after wastewater treatment, thereby facilitating its large-scale applications.

## 2. Materials and Methods

### 2.1. Microalgae Isolation, Screening, and Identification

Microalgae were isolated from swine wastewater at a pig farm in Huyanghe City, Xinjiang Uygur Autonomous Region, China. Single microalgae strains were purified through plate streaking and stored in a BG11 medium [16]. The morphological characteristics of the microalgae were observed using an optical microscope (OLYMPUS, DP22, Olympus Co., Ltd., Tokyo, Japan). Subsequently, a biomass accumulation experiment was performed on the microalgae in a BG11 medium with 10 g/L glucose to select the microalgae strains with strong growth capacities. The experiment was performed at 26–28 °C at a stirring rate of 200 rpm, and the pH was regulated between 7.0 and 8.5 using HCl and NaOH. Finally, the superior microalgae species were identified by gene sequencing. The medium of the 10 strains of microalgae was the BG11 medium, and its components have been added in the Supplementary Material.

### 2.2. Optimization of Heterotrophic Culture Conditions for Superior Microalgae Strains

Heterotrophic culture modes of microalgae are classified into chemoheterotrophy and photoheterotrophy. In this study, the optimal cultivation mode for these strains was determined by comparing the light density and chlorophyll content of superior microalgae strains under chemoautotrophic and photoautotrophic conditions. The superior strains, pre-cultivated to the logarithmic growth stage, were inoculated in 0.05 g/L into the BG11 medium with 10 g/L glucose and were stirred at 200 rpm at 26–28 °C for chemoheterotrophy. Based on this, 2000 lux of continuous light was provided for photoheterotrophy. The pH was maintained between 7.0 and 8.5 using HCl and NaOH. The optical density and chlorophyll content of the microalgae were measured daily to determine the optimal culture mode for subsequent experiments. Then, the effects of glucose concentrations (5 g/L, 10 g/L, 15 g/L, and 20 g/L) on the growth and chlorophyll content of the microalgae were investigated under the same culture conditions in the optimal culture mode to determine the optimal glucose concentration. The above optimal culture conditions of the microalgae were used for pre-culture of the microalgae for swine wastewater treatment, enabling rapid growth to the logarithmic phase and then were inoculated in the wastewater.

### 2.3. Effect of Superior Microalgae Strains on Swine Wastewater Treatment

The swine wastewater was collected from a pig farm in Huyanghe City, Xinjiang Uygur Autonomous Region, China. After allowing the raw wastewater to settle for a duration of 24 h, it was filtered through 10 layers of gauze, and then the supernatant was divided into 500 mL reactors. The pH of the wastewater was adjusted to 7.0. A portion of the wastewater was further sterilized at 121 °C for 20 min using a vertical-pressure steam sterilizer (YXQ-LS-50SII, Boxun Medical & Biological Instrument Co., Shanghai, China), to obtain sterilized wastewater. The superior microalgal strains were cultured to the logarithmic growth stage under optimal conditions. Then, they were inoculated at 0.05 g/L into both raw and sterilized wastewater and maintained under the optimal cultivation mode. The biomass and chlorophyll concentrations of microalgae, pH, and concentrations of  $\text{NH}_4^+$ -N,  $\text{PO}_4^{3-}$ -P, and COD in the wastewater were measured daily until the pollutant concentrations were almost unchanged.

### 2.4. Flocculation Effect and Mechanism of Microalgae

The flocculation efficiencies of microalgae were determined at 0, 2, 4, 8, 12, and 24 h every 2 days during the treatment of swine wastewater by the microalgae, and the EPS components of the microalgae were extracted and analyzed.

### 2.5. Analysis and Testing Methods

#### 2.5.1. Measurement of Microalgal Growth

The microalgae were screened using the optical density method, and the culture conditions were optimized. The absorbance ( $\text{OD}_{680}$ ) of microalgae was measured at a

wavelength of 680 nm using a UV spectrophotometer (DR6000, HACH, Loveland, CO, USA) [6]. Due to the dark brown color of the swine wastewater, the microalgae cells were counted using the hemocyte plate microscope counting method. Meanwhile, a microalgal solution of 20 mL was taken and centrifuged at 5000 rpm using a high-speed freezing centrifuge (TGL-18000CR, Anting Scientific Instrument Factory, Shanghai, China). The supernatant was then discarded, washed thrice with distilled water, and then dried to a constant weight at 80 °C using an electrothermal constant temperature drying oven (202, Ever Bright Medical Treatment Instrument Co., Ltd., Beijing, China). The weights were determined using an analytical balance (AUY120, Shimadzu, Kyoto, Japan). The relationship between cell number  $J$  and dry weight  $I$  (g/L) was calculated according to Equation (1).

$$I = 1.92 \times 10^{-8} \times J - 0.22, R^2 = 0.9802 \quad (1)$$

Finally, the biomass productivity  $A$  (mg/L/d) and relative growth rate  $B$  of the microalgae were calculated according to Equations (2) and (3), respectively:

$$A = (C_t - C_0)/t \quad (2)$$

where  $C_0$  and  $C_t$  are the biomass concentrations (mg/L) at the beginning and end of microalgae growth, respectively, and  $t$  is the growth time of microalgae (d).

$$B = (\ln C_2 - \ln C_1)/(t_2 - t_1) \quad (3)$$

Here,  $t_1$  and  $t_2$  are the different times (d) of microalgae growth, and  $C_1$  and  $C_2$  are the biomass concentrations (mg/L) of microalgae at  $t_1$  and  $t_2$ , respectively.

#### 2.5.2. Determination of Chlorophyll Content of microalgae

The microalgae cells were crushed using a cell breaker (MM400, Leitch, Wetzlar, Germany), centrifuged at 3000 rpm for 5 min, and 50 µL of the supernatant was mixed with 950 µL of ethanol. The absorbance of the solution was measured at the wavelengths of 649 nm and 664 nm with zeroed by 95% ethanol, and the concentrations (mg/L) of chlorophyll a (Chl a) and chlorophyll b (Chl b) were calculated using Equations (4) and (5), respectively [17].

$$\text{Chl a} = 13.36 \times \text{OD}_{664} - 5.19 \times \text{OD}_{649} \quad (4)$$

$$\text{Chl b} = 27.43 \times \text{OD}_{649} - 5.19 \times \text{OD}_{664} \quad (5)$$

#### 2.5.3. Identification of Microalgae

The microalgal DNA was extracted using a fungal genomic DNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China) following the instructions and subsequently forwarded to Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China) for sequencing of the internal transcribed spacer 2 (ITS2) fragments using primers ITS2-2F and ITS2-3R [18].

#### 2.5.4. Measurement of Water Quality of Swine Wastewater

A microalgal solution of 30 mL was centrifuged at 5000 rpm for 5 min, and the supernatant was taken for water quality detection. The pH was determined by a Rex pH meter (PHS-25, Yidian Scientific Instrument Co., Ltd., Shanghai, China),  $\text{NH}_4^+$ -N was determined using the Nash reagent photometry method [19], and  $\text{PO}_4^{3-}$ -P was determined using molybdenum-antimony resistance spectrophotometry [20].  $\text{K}_2\text{Cr}_2\text{O}_7$  was used as an oxidant, and COD was measured using a COD analyzer (DR1010, HACH, USA) [21]. The pollutant removal efficiency  $D$  (%) and removal rate  $E$  (mg/L/d) were calculated according to Equations (6) and (7), respectively:

$$D = (F_t - F_0)/F_0 \times 100 \quad (6)$$

$$E = (F_t - F_0)/t \quad (7)$$

where  $F_0$  and  $F_t$  are the pollutant concentrations (mg/L) of the influent and wastewater, respectively, and  $t$  is the wastewater treatment time (d).

### 2.5.5. Flocculation Efficiency of Microalgae and Extraction and Analysis of EPS

The microalgae flocculation efficiency  $G$  (%) was calculated according to Equation (8), where  $H_0$  is the initial concentration of microalgae cells, and  $H_t$  is the concentration of residual microalgae cells after flocculation.

$$G = (1 - H_t/H_0) \times 100 \quad (8)$$

Approximately 10 mL of the microalgal liquid was taken and incubated in a 45 °C water bath for 30 min and then centrifuged at 5000 rpm for 4 min at 4 °C. Later, the supernatant was filtered through a 0.45 µm polyether sulfone filter membrane (PES, Jinten, Lafayette, Louisiana, USA) to obtain the EPS of the microalgae. Subsequently, the composition of the EPS was analyzed using a three-dimensional fluorescence spectrometer (FS 920, Edinburgh Instruments Ltd., Livingston, UK) with an excitation wavelength range of 220–500 nm and an emission wavelength range of 220–650 nm (incremental intervals of 5 nm) [22].

## 2.6. Data Analyses

All experiments were conducted in triplicate, and the data were processed and analyzed using Excel 2021. The results were presented as mean  $\pm$  standard deviation. Data comparisons were performed using the least significant difference method in SPSS 25.0 (IBM, Inc., Armonk, NY, USA), where  $p < 0.05$  was considered statistically significant.

## 3. Results and Discussion

### 3.1. Microalgae Isolation, Screening, and Identification

The morphology of the 10 microalgae strains isolated from swine wastewater is shown in Figure 1. The cells of strains BKL1, BKL2, LGY1, LGY2, and RS1 exhibited a spherical shape, with cell diameters of approximately 3–7 µm. The cells of strains RS2 and ZYY2 were hexagonal in shape, and the diagonal of the cells was 5–8 µm with interconnected cells. In contrast, the cells of strain SD1 were mostly pentagonal, with cell sizes of 3–5 µm, typically forming groups of eight or nine cells in two rows. The cells of strain SD2 showed triangular or irregularly quadrilateral shapes, with cell sizes of 3–5 µm, and the cells were usually single or multiple connected. The cells of strain ZYY1 were nearly rhombic, 18–22 µm in length and 4–6 µm in width, and the four cells were arranged in straight lines or slightly interlaced above and below.

Heterotrophic growth in the BG11 medium with 10 g/L glucose showed that the microalgal strain ZYY1 exhibited the strongest growth ability (Figure 2a), followed by ZYY2 and RS1, while the growth of other microalgal strains was not obvious. On days 0–5, ZYY1 and ZYY2 adapted to the medium with less biomass accumulation, and the  $OD_{680}$  values of ZYY1 and ZYY2 were  $0.42 \pm 0.03$  and  $0.25 \pm 0.01$  on the 5<sup>th</sup> day, respectively. ZYY1 entered the logarithmic growth phase on the 5<sup>th</sup> day, while ZYY2 exhibited a lower growth capacity. At the end of the cultivation period, the maximum  $OD_{680}$  values of ZYY1, ZYY2, and RS1 were  $3.15 \pm 0.03$ ,  $0.81 \pm 0.04$ , and  $0.50 \pm 0.01$ , respectively, indicating that ZYY1 was the superior microalgal strain in terms of growth capacity.

The pH is associated with the osmotic pressure of the culture medium and the utilization of organic carbon through microalgae respiration, playing a crucial role in microalgae growth. The pH of the culture medium for all microalgal strains showed an increasing trend, increasing from approximately 7 to 9, which might be attributed to the release of microalgae metabolites into the medium, leading to a pH rise to alkaline (Figure 2b).

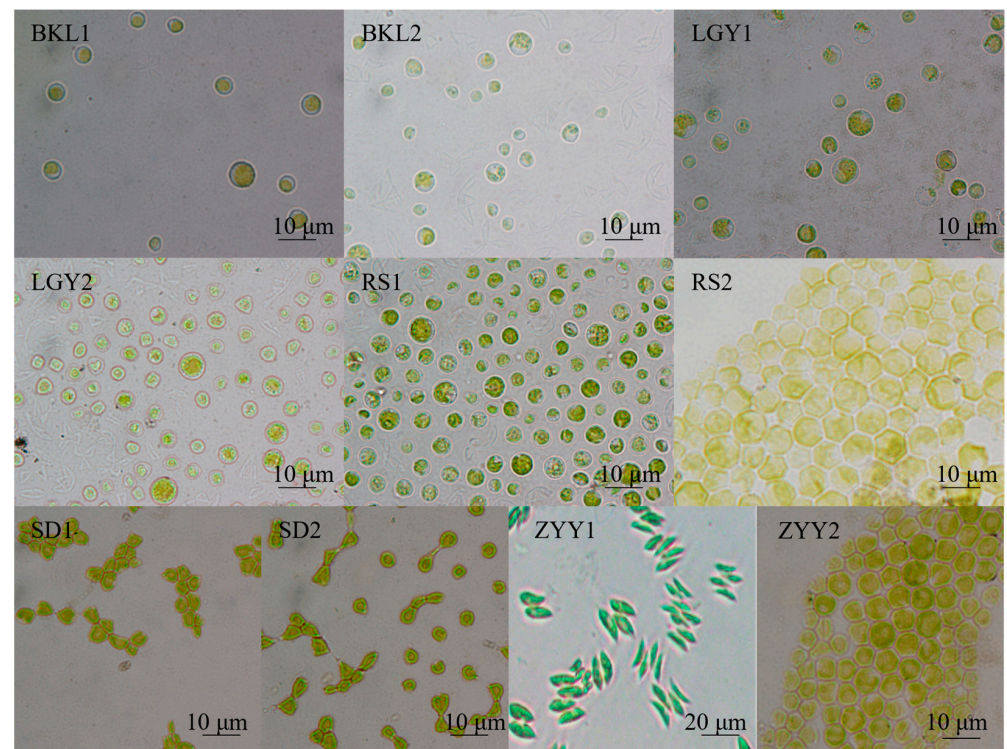


**Figure 1.** Cell morphology of microalgae isolated from swine wastewater.

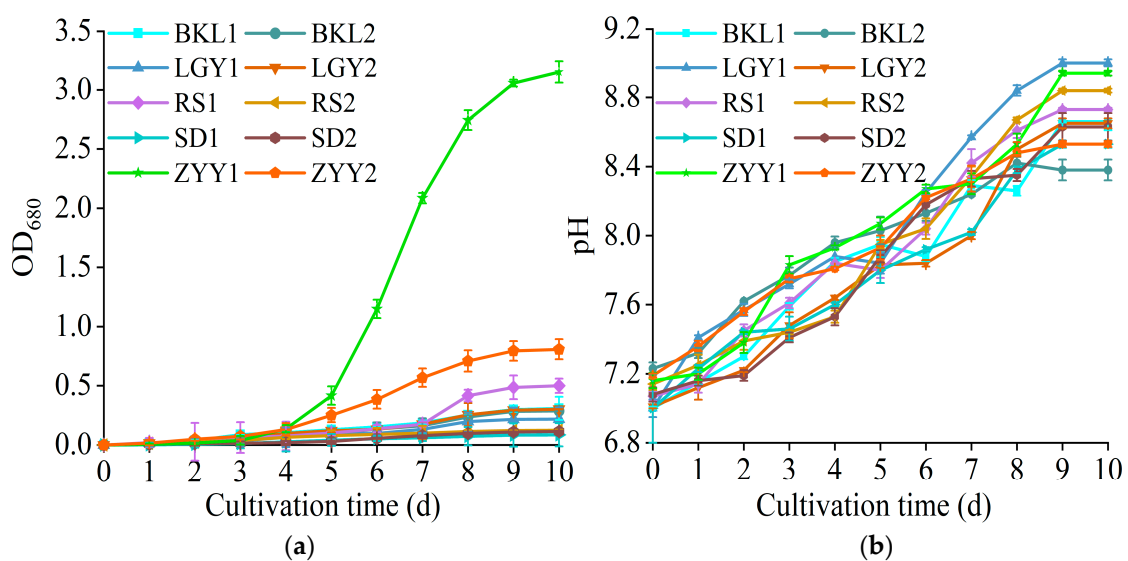


**Figure 2.** (a) Heterotrophic growth and (b) pH changes of microalgae in BG11 medium with 10 g/L glucose. Data are the mean  $\pm$  SD ( $n = 3$ ).

The internal transcribed spacer 2 (ITS2) non-coding region sequence of the microalgal strain ZYY1 was identified, and the obtained sequences were compared with the published ITS2 sequence of microalgae using BLAST at the National Center for Biotechnology Information. The results showed that the similarity of the ITS gene sequences of the microalgal strain ZYY1 and *Tetradismus obliquus* reached 99.57%. The cells of *T. obliquus* are rhombic, in coenobia of 2, 4, and 8 cells. The aggregated cells of *T. obliquus* are generally found as four-celled coenobia, with a length of 10–21  $\mu\text{m}$  and a width of 3–9  $\mu\text{m}$  [23,24]. The cellular morphology of strain ZYY1 was similar to that of *T. obliquus*. Therefore, the strain ZYY1 was named *T. cf. obliquus* based on the ITS gene sequences, cellular morphology,

and the phylogenetic tree. The phylogenetic tree of *T. cf. obliquus* can be found in the Supplementary Materials.

### 3.2. Optimization of the Heterotrophic Culture Model of *T. cf. obliquus* ZYY1

In the first 4 d of growth under chemoheterotrophic and photoheterotrophic conditions, the biomass concentration of *T. cf. obliquus* ZYY1 increased slowly, then rapidly increased from the 5<sup>th</sup> to 7<sup>th</sup> day and stabilized, reaching a peak on the 10<sup>th</sup> day. This result was consistent with the typical S-shaped growth curve of microorganisms (Figure 3a). Both cultivation modes exhibited high biomass productivity, with chemoheterotrophy displaying higher growth kinetic parameters compared to photoheterotrophy (Table 1). The maximum biomass concentration, daily average biomass productivity, and maximum specific growth rate under chemoheterotrophy were  $1.94 \pm 0.01$  g/L, 189 mg/L/d, and  $1.26 \text{ d}^{-1}$ , respectively; the above three growth kinetic parameters of photoheterotrophy were  $1.58 \pm 0.04$  g/L, 153 mg/L/d, and  $0.67 \text{ d}^{-1}$ , respectively. These results indicated that chemoheterotrophic culture promoted the rapid growth of *T. cf. obliquus* ZYY1 more effectively. This may be due to the fact that in chemoheterotrophic cultures, *T. cf. obliquus* ZYY1 can utilize carbon sources from swine wastewater more efficiently for growth as it does not need to invest significant energy in maintaining the complex structures and mechanisms necessary for photosynthesis. Thus, the direct utilization of organic matter may enhance the overall metabolic efficiency and growth rate of the microalgae [25]. As shown in Figure 3b, the photoheterotrophy exhibited a higher chlorophyll concentration than the chemoheterotrophy, suggesting that darkness is not conducive to chlorophyll synthesis [26].

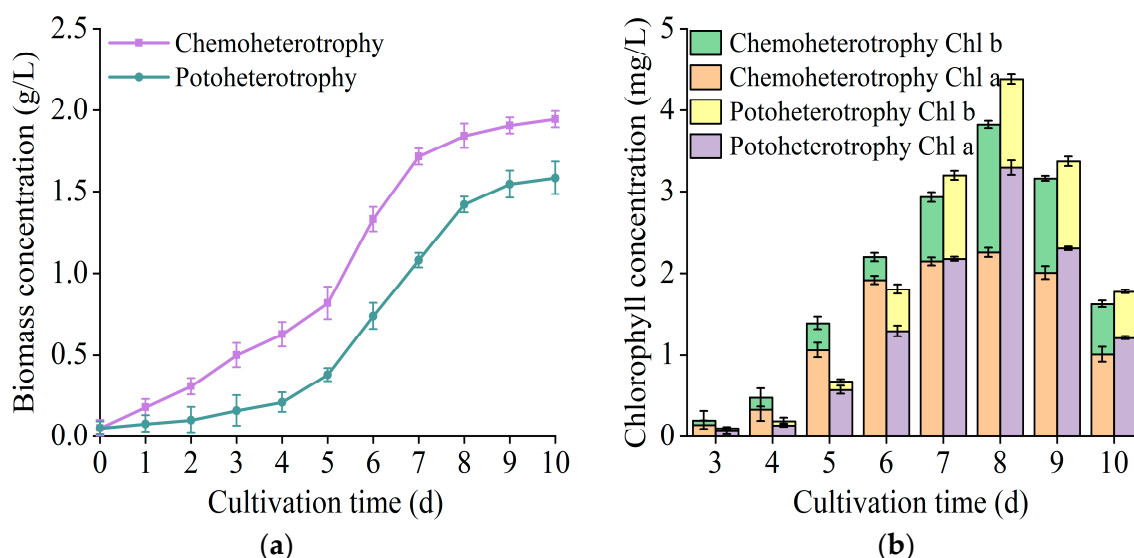


**Figure 3.** (a) Changes in biomass concentration and (b) chlorophyll concentration of *T. cf. obliquus* ZYY1 under different heterotrophic modes. Data are the mean  $\pm$  SD ( $n = 3$ ).

**Table 1.** Growth kinetic parameters of *T. cf. obliquus* ZYY1 under different heterotrophic modes and glucose concentrations.

| Cultivation Mode  | Biomass Concentration (g/L) | Biomass Productivity (mg/L/d) | Maximum Specific Growth Rate ( $\text{d}^{-1}$ ) |
|-------------------|-----------------------------|-------------------------------|--------------------------------------------------|
| photoheterotrophy | $1.58 \pm 0.04$             | 153                           | 0.67                                             |
| chemoheterotrophy | $1.94 \pm 0.01$             | 189                           | 1.26                                             |

Data are the mean  $\pm$  SD ( $n = 3$ ).

The *T. cf. obliquus* ZYY1 was inoculated in a BG11 medium with glucose concentrations of 5 g/L, 10 g/L, 15 g/L, and 20 g/L and cultured under chemoheterotrophic conditions

(Figure 4a). The corresponding biomass concentration of ZYY1 gradually increased from the initial 0.05 g/L to  $1.64 \pm 0.01$ ,  $1.78 \pm 0.04$ ,  $2.16 \pm 0.03$ , and  $1.97 \pm 0.02$  g/L, respectively. The average daily biomass productivity reached 164 mg/L/d, 178 mg/L/d, 216 mg/L/d, and 197 mg/L/d, respectively, and the corresponding maximum specific growth was  $0.82 \text{ d}^{-1}$ ,  $0.82 \text{ d}^{-1}$ ,  $0.96 \text{ d}^{-1}$ , and  $0.82 \text{ d}^{-1}$ , respectively (Table 2). The growth of *T. cf. obliquus* ZYY1 was optimal at a glucose concentration of 15 g/L, but increasing the concentration to 20 g/L decreased its growth rate. This was consistent with previous research on *C. protothecoides*, reporting that excessive glucose concentrations were not conducive to its growth [27]. This might be due to the elevated energy demand for the fundamental metabolism of microalgae when glucose concentration exceeds a specific threshold, leading to insufficient energy for biomass accumulation [28].

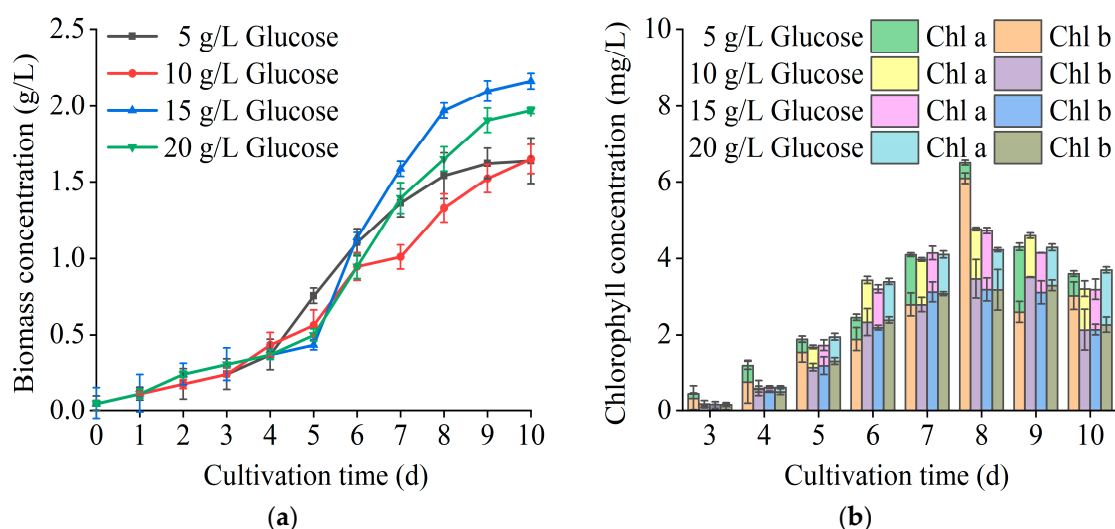


**Figure 4.** (a) Changes in biomass concentration and (b) chlorophyll concentration of *T. cf. obliquus* ZYY1 under different glucose concentrations. Data are the mean  $\pm$  SD ( $n = 3$ ).

**Table 2.** Growth kinetic parameters of *T. cf. obliquus* ZYY1 in different glucose concentrations under chemoheterotrophy mode.

| Glucose Concentration (g/L) | Biomass Concentration (g/L) | Biomass Productivity (mg/L/d) | Maximum Specific Growth Rate ( $\text{d}^{-1}$ ) |
|-----------------------------|-----------------------------|-------------------------------|--------------------------------------------------|
| 5                           | $1.64 \pm 0.01$             | 164                           | 0.82                                             |
| 10                          | $1.65 \pm 0.01$             | 178                           | 0.82                                             |
| 15                          | $2.16 \pm 0.01$             | 216                           | 0.96                                             |
| 20                          | $1.97 \pm 0.02$             | 197                           | 0.82                                             |

Data are the mean  $\pm$  SD ( $n = 3$ ).

### 3.3. The Effect of *T. cf. obliquus* ZYY1 on Treating Swine Wastewater

Despite the advantage of highly efficient nutrient removal, studies on the bioremediation of raw swine wastewater using *T. cf. obliquus* are scarce. The growth and removal of pollutants during the treatment of sterilized and raw wastewater using *T. cf. obliquus* ZYY1 were monitored, and characteristics of the wastewater are shown in Table 3.

**Table 3.** Physical and chemical properties of different swine wastewater.

| Wastewater Type       | pH              | $\text{NH}_4^+-\text{N}$ (mg/L) | $\text{PO}_4^{3-}-\text{P}$ (mg/L) | COD (mg/L)           |
|-----------------------|-----------------|---------------------------------|------------------------------------|----------------------|
| Raw wastewater        | $7.00 \pm 0.01$ | $404.40 \pm 13.04$              | $52.10 \pm 1.00$                   | $6005.84 \pm 422.24$ |
| Sterilized wastewater | $7.02 \pm 0.08$ | $386.20 \pm 9.63$               | $53.70 \pm 0.20$                   | $5849.65 \pm 162.40$ |

Data are the mean  $\pm$  SD ( $n = 3$ ).

As shown in Figure 5a, *T. cf. obliquus* ZYY1 could grow normally in both types of wastewater. The biomass accumulation ( $1.65 \pm 0.01$  g/L), mean daily biomass productivity ( $165$  mg/L/d), and maximum specific growth rate ( $1.36$  d<sup>−1</sup>) of *T. cf. obliquus* ZYY1 in raw wastewater were higher than that in sterilized wastewater, which were  $1.45 \pm 0.01$  g/L,  $145$  mg/L/d, and  $0.88$  d<sup>−1</sup>, respectively. A previous study has shown that the presence of bacteria, reduced by high temperatures and pressures, does not promote the growth of microalgae [29]. However, raw wastewater may contain microalgae-promoting bacteria, which can enhance the growth rate of microalgae. Previous studies have demonstrated that the microalgae bacteria consortia of *Chlorella vulgaris* and *Rhodobacter sphaeroides* removed 100% NH<sub>4</sub><sup>+</sup>-N, 96% TP, 97% COD and 97% COD in sterilized piggery-starch mixed wastewaters (the PW/SW ratio was 5:1) [30].

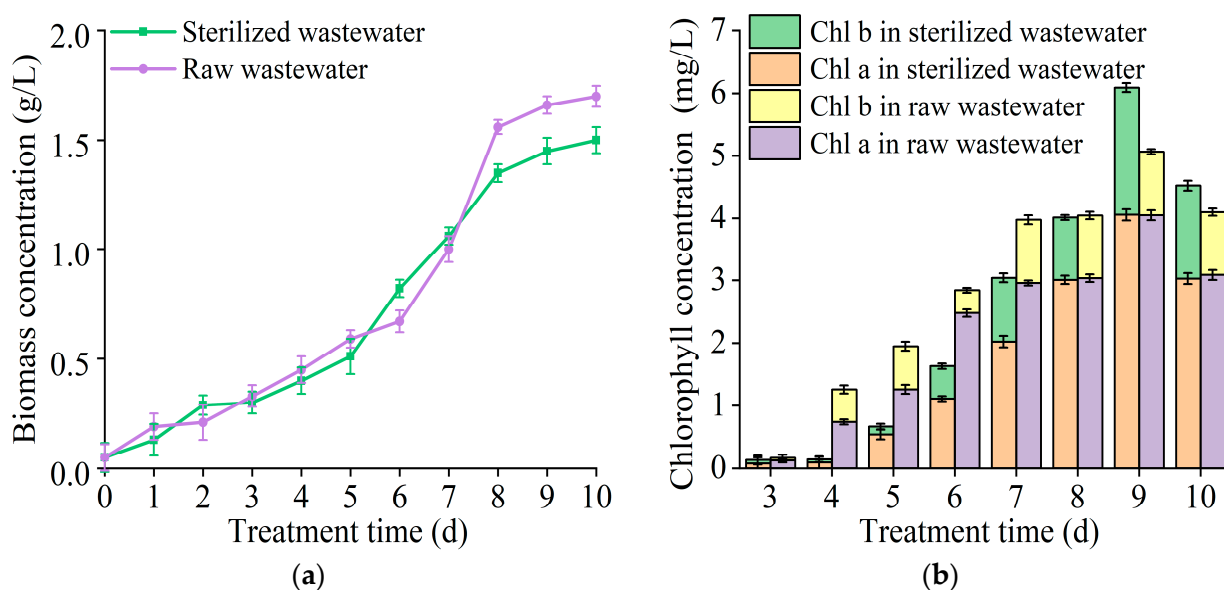


**Figure 5.** (a) Biomass concentration and (b) chlorophyll concentration of *T. cf. obliquus* ZYY1 in different swine wastewater. Data are the mean  $\pm$  SD ( $n = 3$ ).

As shown in Figure 5b, the chlorophyll concentration of *T. cf. obliquus* ZYY1 initially increased and then decreased with prolonged wastewater treatment. The chlorophyll concentration of *T. cf. obliquus* ZYY1 in raw wastewater was higher than that in the first 8 days but lower than that in sterilized wastewater from the 9<sup>th</sup> day. This might be because the nitrogen concentration in the raw wastewater was significantly lower than that in the sterilized wastewater from the 9<sup>th</sup> day, indicating that ZYY1 did not have sufficient nitrogen for chlorophyll synthesis. It has been found that the levels of chlorophyll a and b decrease sharply with the reduction in nitrogen levels [31].

Subsequently, the removal of pollutants from the two types of wastewater was compared. The results showed that *T. cf. obliquus* ZYY1 was more effective in treating the raw wastewater. After a 10-day treatment period, the NH<sub>4</sub><sup>+</sup>-N concentration in raw wastewater decreased from  $404.40 \pm 13.04$  mg/L to  $115.84 \pm 11.04$  mg/L, achieving a removal efficiency of 71.36%. Meanwhile, the removal efficiency of NH<sub>4</sub><sup>+</sup>-N in sterilized wastewater was poor, which decreased from  $386.20 \pm 9.63$  mg/L to  $168.48 \pm 10.84$  mg/L, with a removal efficiency of 56.37% (Figure 6a and Table 3). Microalgae can utilize NH<sub>4</sub><sup>+</sup>-N to synthesize cellular substances through glutamine synthetase, which has been widely proven to have the ability to remove NH<sub>4</sub><sup>+</sup>-N [32]. Qu et al. found that *Coelastrella* sp. KE4 showed remarkable nutrient removal efficiency with 99.52% NH<sub>4</sub><sup>+</sup>-N in real swine wastewater ( $529.72$  mg/L NH<sub>4</sub><sup>+</sup>-N) [33]. The removal efficiency of NH<sub>4</sub><sup>+</sup>-N in both types of wastewater was relatively low, probably because the optimal carbon/nitrogen ratio of

microalgae was 6.63:1, while the carbon/nitrogen ratio of wastewater was unbalanced (14.85–15.15:1).

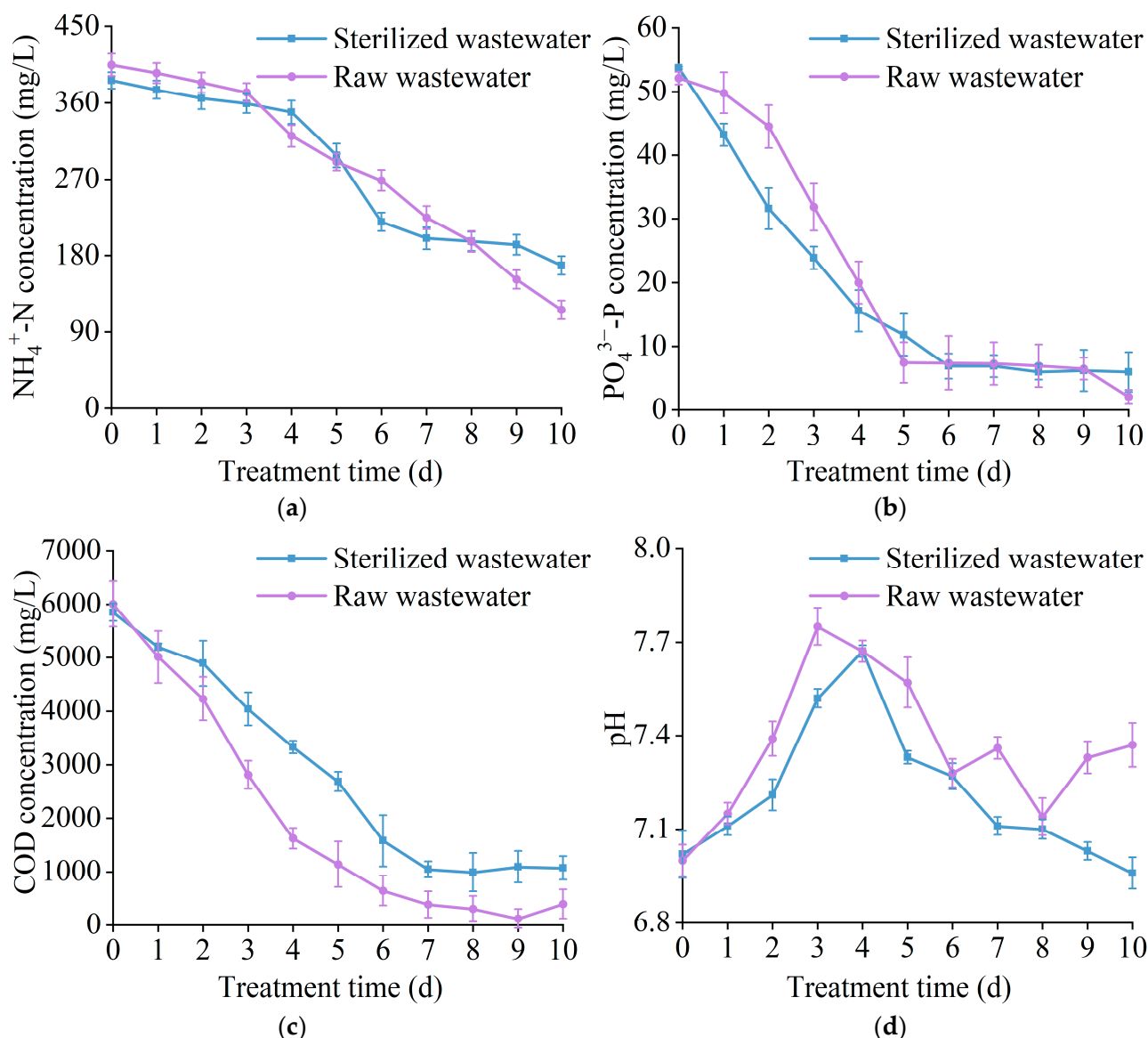


**Figure 6.** (a) Changes in  $\text{NH}_4^+\text{-N}$  concentration, (b)  $\text{PO}_4^{3-}\text{-P}$  concentration, (c) COD concentration, and (d) pH during the treatment of different swine wastewater by *T. cf. obliquus* ZYY1. Data are the mean  $\pm$  SD ( $n = 3$ ).

*T. cf. obliquus* ZYY1 demonstrated higher efficiency in removing  $\text{PO}_4^{3-}\text{-P}$  from both types of wastewater. The initial  $\text{PO}_4^{3-}\text{-P}$  concentration was  $52.10 \pm 1.00$  mg/L and  $53.70 \pm 0.20$  mg/L in raw and sterilized wastewater, respectively, which was lower than 10 mg/L on the 5<sup>th</sup> and 6<sup>th</sup> days and the decrease was small (Figure 6b). This phenomenon can be explained by the mechanism of phosphorus over-absorption by the microalgae, where phosphorus is stored in phosphate polysomes under phosphorus-rich conditions to be utilized in phosphorus-poor environments [34]. After treatment, the removal efficiency of  $\text{PO}_4^{3-}\text{-P}$  in raw and sterilized wastewater reached 96.09% and 88.88%, respectively (Table 4).

**Table 4.** The effect of *T. cf. obliquus* ZYY1 on treating different swine wastewater.

|                               | Parameter                   | Raw Wastewater      | Sterilized Wastewater |
|-------------------------------|-----------------------------|---------------------|-----------------------|
| effluent concentration (mg/L) | $\text{NH}_4^+-\text{N}$    | $115.84 \pm 11.04$  | $168.48 \pm 10.84$    |
|                               | $\text{PO}_4^{3-}-\text{P}$ | $2.03 \pm 0.13$     | $5.97 \pm 0.13$       |
|                               | COD                         | $392.32 \pm 276.08$ | $1070.96 \pm 16.24$   |
| removal efficiency (%)        | $\text{NH}_4^+-\text{N}$    | $71.36 \pm 3.57$    | $56.37 \pm 2.82$      |
|                               | $\text{PO}_4^{3-}-\text{P}$ | $96.09 \pm 4.81$    | $88.88 \pm 4.44$      |
|                               | COD                         | $93.13 \pm 4.67$    | $81.69 \pm 4.08$      |

Data are the mean  $\pm$  SD ( $n = 3$ ).

As shown in Figure 6c, the COD concentrations in raw and sterilized wastewater decreased from  $6005.84 \pm 422.24$  mg/L and  $5849.65 \pm 162.40$  mg/L to  $392.32 \pm 276.08$  mg/L and  $1070.96 \pm 16.24$  mg/L, respectively, with removal efficiencies of 93.13% and 81.69% (Table 4). Previous studies have shown that bacteria–algae systems offer improved COD removal efficiency [35]. In this study, the raw swine wastewater treatment system formed a bacteria–algae system, which together accelerated the degradation of organic matter, resulting in higher COD removal efficiency. Additionally, an increase in COD concentration from  $119.04 \pm 178.64$  mg/L to  $392.32 \pm 276.08$  mg/L was observed on the 9<sup>th</sup> day of raw wastewater treatment by *T. cf. obliquus* ZYY1, which might be related to the effect of bacteria on the species and content of organic matter released by the microalgae. Similarly, an increase in COD concentration was observed in the study of *Chlorella vulgaris* MBFJNU-1 treating unsterilized swine biogas slurry [36]. The effluent concentrations of  $\text{PO}_4^{3-}-\text{P}$  and COD in the raw wastewater were  $2.03 \pm 0.13$  mg/L and  $392.32 \pm 276.08$  mg/L, respectively (Table 3), which met the pollutant discharge standards for livestock and poultry farming (GB18596-2001) [37].

The pH of the wastewater was monitored during the treatment process to analyze the possible reasons for the purification of the wastewater (Figure 6d). The pH of both types of wastewater was slightly alkaline and showed a trend of first increasing and then decreasing. Meanwhile, the pH of raw wastewater and sterilized wastewater was maintained between 7.00–7.75 and 6.96–7.67, respectively. Therefore, the volatilization of  $\text{NH}_4^+-\text{N}$  and phosphate precipitation at  $\text{pH} > 9$  were not involved [38], indicating that the pollutant removal by *T. cf. obliquus* ZYY1 was mainly through assimilation. In raw wastewater, 5.69 g of microalgae were produced per 1 g  $\text{NH}_4^+-\text{N}$  removal.

In summary, *T. cf. obliquus* ZYY1 was able to tolerate the raw swine wastewater, and the nutrients in the wastewater were suitable for the growth of microalgae without being inhibited by the microorganisms. Compared with the sterilized wastewater, *T. cf. obliquus* ZYY1 grew more vigorously in the raw wastewater without needing additional nutrients. Additionally, it exhibited a strong nutrient assimilation ability and thus can save the energy cost of sterilization, which is suitable for industrial applications [39].

### 3.4. Flocculation Effect and Mechanism of *T. cf. obliquus* ZYY1

To enhance production guidance, the flocculation efficiency of *T. cf. obliquus* ZYY1 was further evaluated. As shown in Figure 7, *T. cf. obliquus* ZYY1 exhibited high flocculation efficiency, and the raw wastewater was conducive to its flocculation. Additionally, the flocculation efficiency of *T. cf. obliquus* ZYY1 in the raw wastewater was as follows: on the 8<sup>th</sup> day ( $97.71\% \pm 5.81\%$ )  $>$  6<sup>th</sup> day ( $96.76 \pm 5.92\%$ )  $>$  4<sup>th</sup> day ( $96.25 \pm 4.99\%$ )  $>$  2<sup>nd</sup> day ( $91.53 \pm 8.02\%$ )  $>$  10<sup>th</sup> day ( $91.45 \pm 5.24\%$ ). As for the sterilized wastewater, the flocculation efficiency of *T. cf. obliquus* ZYY1 was as follows: on the 6<sup>th</sup> day ( $87.31 \pm 9.9\%$ )  $>$  2<sup>nd</sup> day ( $86.77 \pm 8.28\%$ )  $>$  4<sup>th</sup> day ( $86.62 \pm 3.85\%$ )  $>$  8<sup>th</sup> day ( $86.46 \pm 6.73\%$ )  $>$  10<sup>th</sup> day ( $83.66 \pm 6.73\%$ ). The flocculation efficiency of *T. cf. obliquus* ZYY1 was significantly different between the raw and sterilized wastewater, with the highest flocculation efficiency of *T. cf. obliquus* ZYY1 in sterilized wastewater being 10.64% lower than that in raw wastewater. This suggests that bacteria are suitable for the microalgae flocculation, which was consistent

with the research results of He et al. It has been found that the flocculation efficiency of bacteria on microalgae reached  $87.37 \pm 2.96\%$  when the cell number ratio of bacteria (*Citrobacter* W4) to microalgae (*Chlorella pyrenoidosa*) was 1:4 [40].

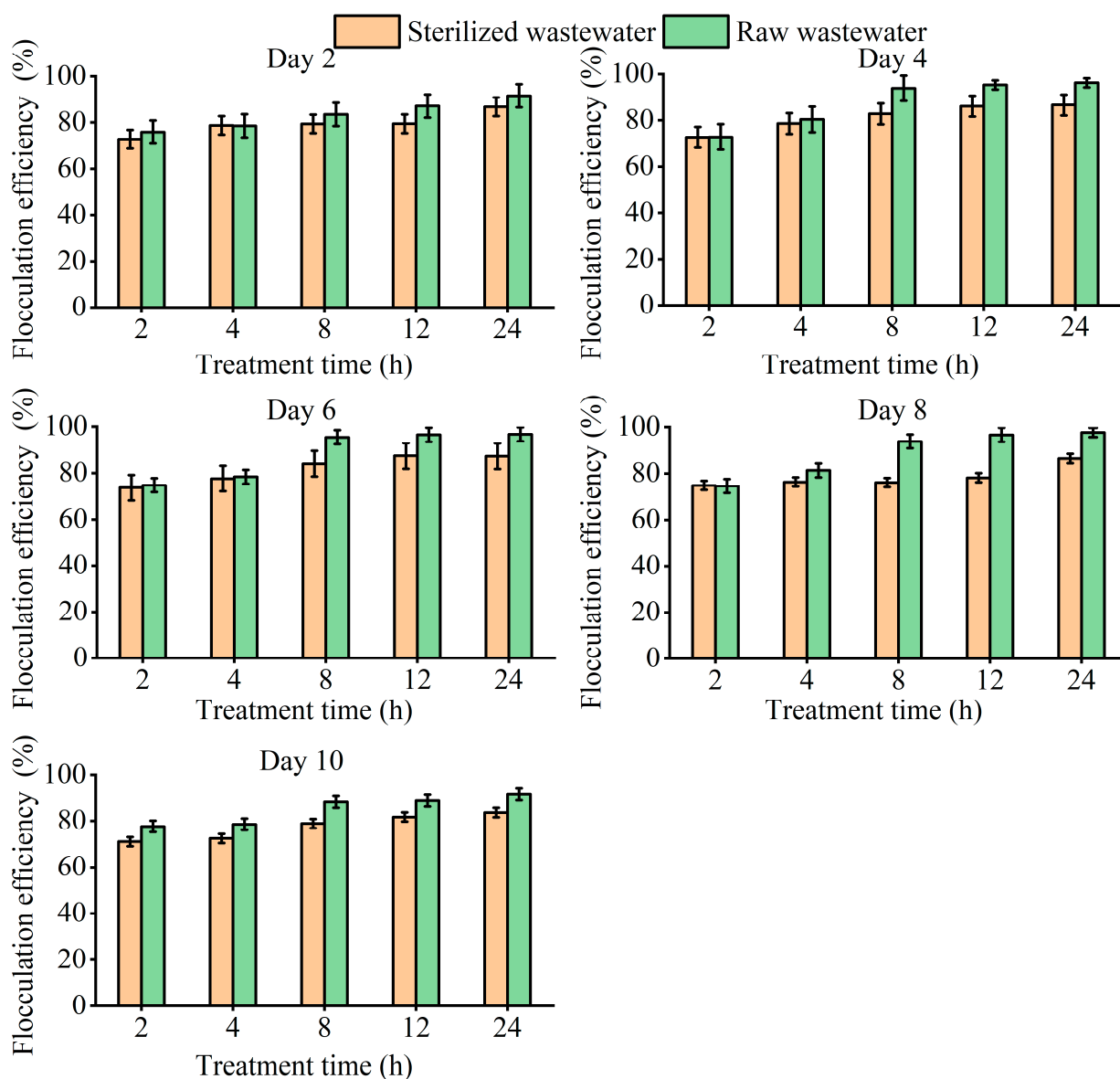


**Figure 7.** The flocculation efficiency of *T. cf. obliquus* ZYY1 in the treatment of different swine wastewater. Data are the mean  $\pm$  SD ( $n = 3$ ).

The solution suitable for the growth of microalgae is negatively charged due to its isoelectric point, and the repulsive effect causes the cells to remain in a dispersed and suspended state [41]. Bacteria in wastewater can induce microalgae to release substances such as proteins and polysaccharides which carry a positive charge in wastewater and lead to the aggregation and settling of microalgal cells. The composition of EPS was analyzed using a three-dimensional fluorescence spectrometer to explore the flocculation mechanism of *T. cf. obliquus* ZYY1. As shown in Figure 8, fluorescence was observed at the excitation/emission wavelengths of 280/350 nm, suggesting the existence of tryptophan in the EPS [42]. The focus is on fluorescence observed at the excitation/emission wavelengths of 225/340–350 nm on the 4<sup>th</sup>–10<sup>th</sup> day during raw wastewater treatment by *T. cf. obliquus* ZYY1, which indicates the presence of aromatic proteins in EPS. The flocculation efficiency of *T. cf. obliquus* ZYY1 increased with an increase in fluorescence intensity of the aromatic

proteins and reached its peak on the 8<sup>th</sup> day. It has been found that aromatic proteins are important components involved in the formation of microbial cell aggregates [43]. The results showed that flocculation by *T. cf. obliquus* ZYY1 might be related to the production of aromatic proteins in EPS. Comparably, *T. cf. obliquus* ZYY1 did not produce aromatic proteins during the treatment of sterilized wastewater, indicating that its flocculation mechanism might be triggered by bacteria in the raw wastewater to secrete aromatic proteins. Zhou et al. [44] proved that *Chlorophyta* sp. released aromatic proteins through their reaction with the bacterial quorum sensing molecule N-acyl-homoserine lactone, promoting the aggregation of microalgae.

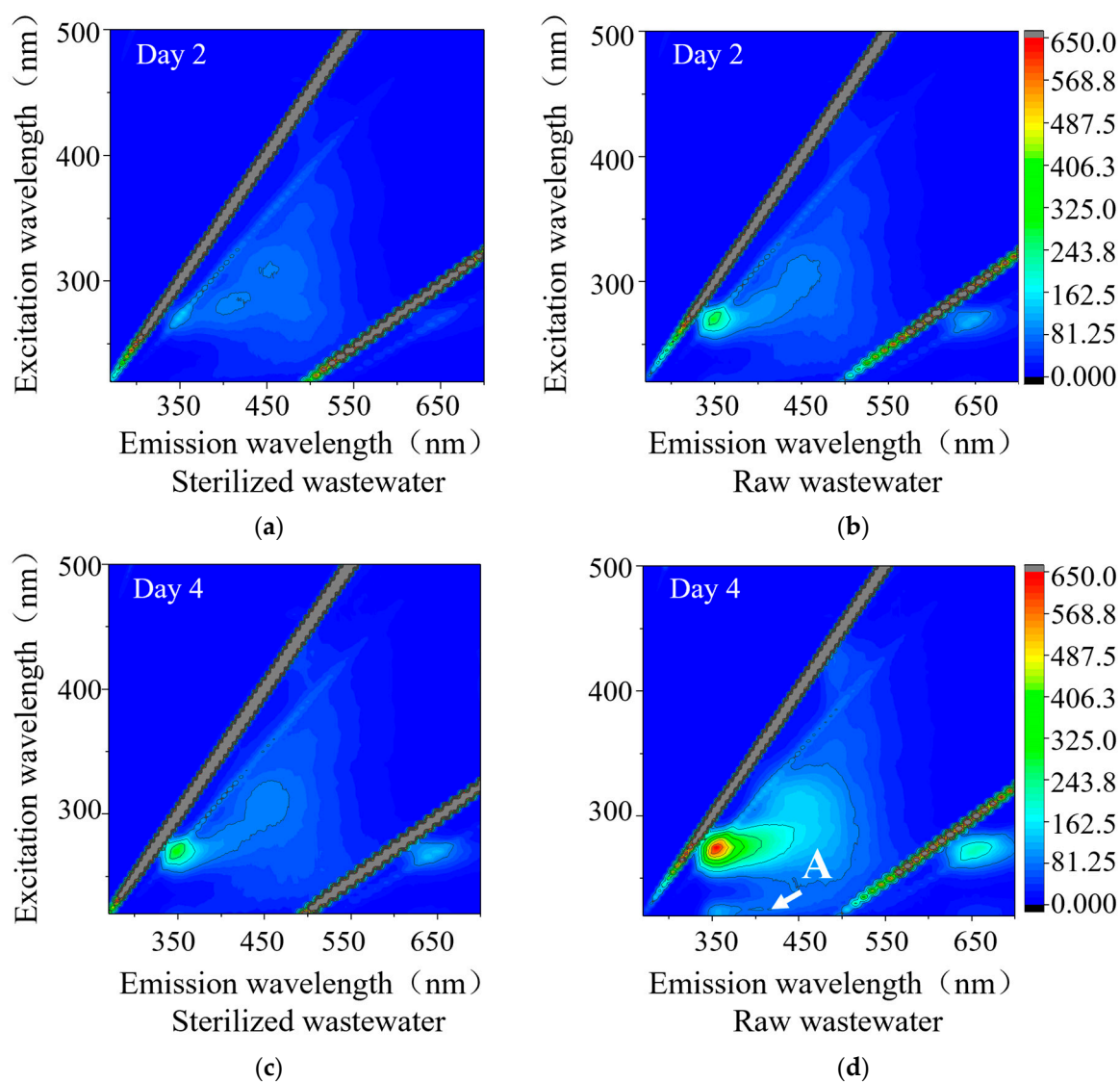


Figure 8. Cont.

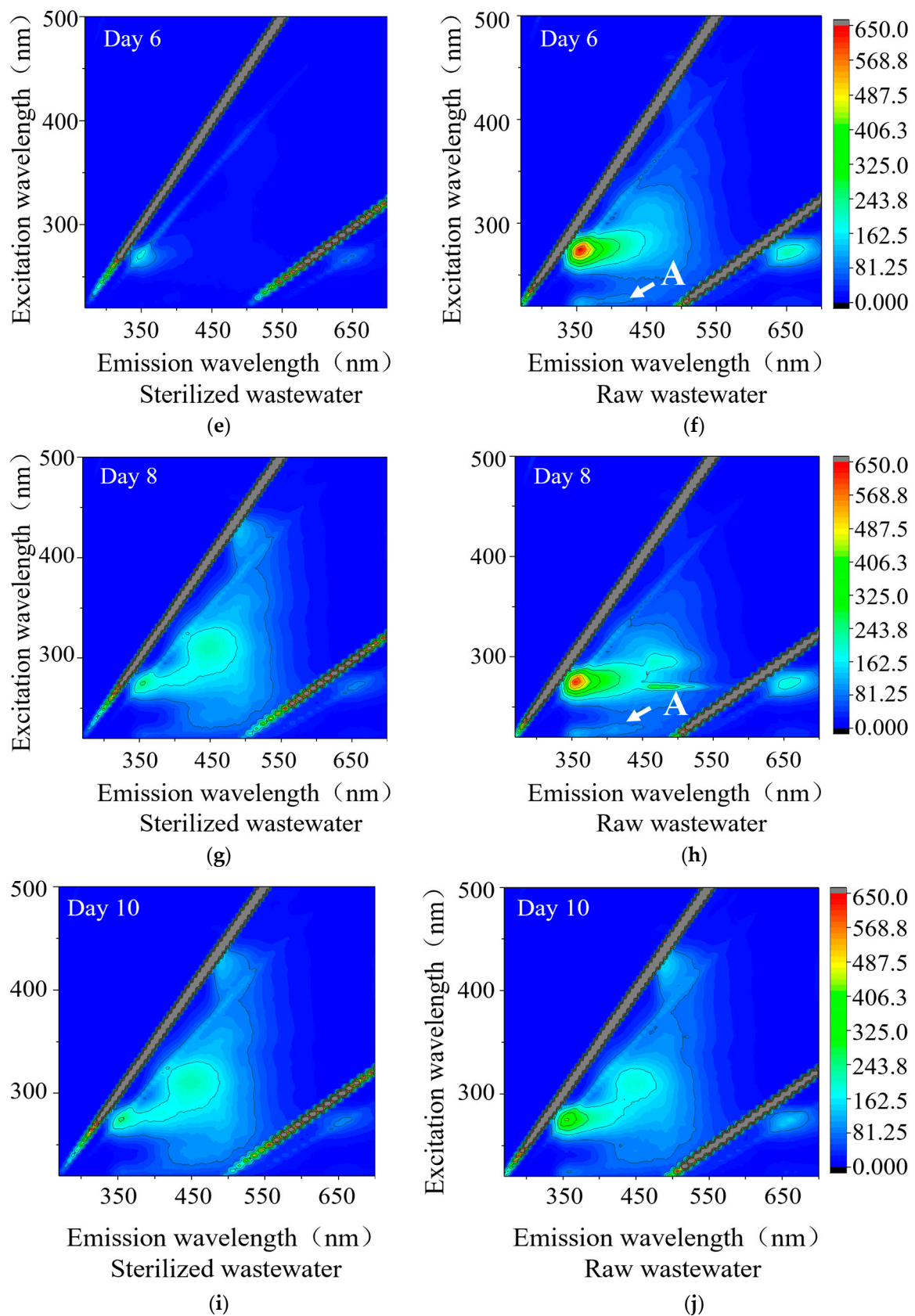


**Figure 8.** The three-dimensional excitation and emission matrix (3D-EEM) fluorescence spectra of EPS from *T. cf. obliquus* ZZY1 in the sterilized and raw wastewater treated on the 2nd day (a,b), 4th day (c,d), 6th day (e,f), 8th day (g,h), and 10th day (i,j). A represents the aromatic proteins in the EPS.

#### 4. Conclusions

In summary, *T. cf. obliquus* ZYY1 exhibited a strong heterotrophic growth capacity among the top 10 microalgae species. The results showed that chemoheterotrophy enhanced the growth rate of microalgae compared to photoheterotrophy. By inoculating *T. cf. obliquus* ZYY1 in a BG11 medium with 15 g/L glucose under chemoheterotrophic conditions, the highest average daily productivity of 216 mg/L/d and a specific growth rate of 0.96 d<sup>−1</sup> were achieved. Moreover, *T. cf. obliquus* ZYY1 is suitable for the treatment of raw swine wastewater, achieving a maximum biomass concentration of 1.65 ± 0.01 g/L and exhibiting remarkable removal efficiencies for NH<sub>4</sub><sup>+</sup>-N (71.36%), PO<sub>4</sub><sup>3−</sup>-P (96.09%), and COD (93.13%). The removal efficiencies were higher than that of sterilized swine wastewater. The higher flocculation efficiency of microalgae in raw swine wastewater was attributed to the presence of bacteria in the raw wastewater inducing microalgae to secrete aromatic proteins. This study can serve as a valuable reference for the application of microalgae cultivation technology in wastewater treatment and microalgal cell harvesting.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/su16114633/s1>, Figure S1: The phylogenetic tree of the *T. cf. obliquus* ZYY1.

**Author Contributions:** Writing—original draft preparation, K.B.; Writing—review and editing, W.Q.; Data curation, D.S.; Conceptualization, J.L.; Supervision, S.-H.H. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Innovation and Entrepreneurship Training Program for College Students, grant number 202310759045; the Research Initiation Program for High-level Talents of Shihezi University, grant number RCZK202316; the Xinjiang Uygur Autonomous Region “Tianchi Talents” Introduction Program, grant number CZ002302; the 2023 Guiding Scientific and Technological Planning Projects for the Xinjiang Production and Construction Corps, grant number 2023ZD044; the Youth Innovation and Cultivation Talent Project of Shihezi University, grant number CXPY202201; and the Open Sharing Fund for the Large-scale Instruments and Equipments of Shihezi University.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** No new data were created or analyzed in this study. Data sharing is not applicable to this article.

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

#### References

- Li, K.; Liu, Q.; Fang, F.; Luo, R.H.; Lu, Q.; Zhou, W.G.; Huo, S.H.; Cheng, P.F.; Liu, J.Z.; Addy, M.; et al. Microalgae-based wastewater treatment for nutrients recovery: A review. *Bioresour. Technol.* **2019**, *291*, 121934. [CrossRef]
- Huang, K.X.; Vadiveloo, A.; Zhou, J.L.; Yang, L.; Chen, D.Z.; Gao, F. Integrated culture and harvest systems for improved microalgal biomass production and wastewater treatment. *Bioresour. Technol.* **2023**, *376*, 128941. [CrossRef] [PubMed]
- Chen, C.R.; Tao, F.; Han, T.; Gao, F.Z.; Dong, T.L.; Jiang, W.Z.; Lu, H.F.; Zhang, Y.H.; Li, B.M. Photoacclimation caused by high frequency flashing light assists *Chlorella* sp. M-12 wastewater treatment and biomass accumulation in dark color biogas slurry. *J. Appl. Phycol.* **2022**, *34*, 2929–2940. [CrossRef]
- Monlau, F.; Sambusiti, C.; Ficara, E.; Aboulkas, A.; Barakat, A.; Carrère, H. New opportunities for agricultural digestate valorization: Current situation and perspectives. *Energy Environ. Sci.* **2015**, *8*, 2600–2621. [CrossRef]
- Chen, Z.P.; Xie, Y.; Qiu, S.; Li, M.T.; Yuan, W.Q.; Ge, S.J. Granular indigenous microalgal-bacterial consortium for wastewater treatment: Establishment strategy, functional microorganism, nutrient removal, and influencing factor. *Bioresour. Technol.* **2022**, *353*, 127130. [CrossRef]
- Tan, X.-B.; Zhao, Z.-Y.; Gong, H.; Jiang, T.; Liu, X.-P.; Liao, J.-Y.; Zhang, Y.-L. Growth of *Scenedesmus obliquus* in anaerobically digested swine wastewater from different cleaning processes for pollutants removal and biomass production. *Chemosphere* **2024**, *352*, 141515. [CrossRef]
- Acebu, P.I.G.; de Luna, M.D.G.; Chen, C.Y.; Abarca, R.R.M.; Chen, J.H.; Chang, J.S. Bioethanol production from *Chlorella vulgaris* ESP-31 grown in unsterilized swine wastewater. *Bioresour. Technol.* **2022**, *352*, 127086. [CrossRef] [PubMed]

8. Alalawy, A.I.; Yang, Q.; Zhang, M.; Almutairi, F.M.; Al-Jahani, G.M.; Almasoudi, F.M.; Alotaibi, M.A.; Salama, E. Removal of nutrients from salt-rich wastewater via freshwater microalga *Tetrademus obliquus*. *Biomass Convers. Biorefin.* **2023**, *12*, 1152. [\[CrossRef\]](#)
9. Vladoic, J.; Jazic, J.M.; Ferreira, A.; Maletic, S.; Cvetkovic, D.; Agbaba, J.; Vidovic, S.; Gouveia, L. Application of green technology to extract clean and safe bioactive compounds from *Tetrademus obliquus* biomass grown in poultry wastewater. *Molecules* **2023**, *28*, 2397. [\[CrossRef\]](#) [\[PubMed\]](#)
10. Ferreira, A.; Figueiredo, D.; Cardeiras, R.; Nabais, R.; Ferreira, F.; Ribeiro, B.; Cordovil, C.; Acién, F.G.; Gouveia, L. Exploring different pretreatment methodologies for allowing microalgae growth in undiluted piggery wastewater. *Agronomy* **2022**, *12*, 580. [\[CrossRef\]](#)
11. Abreu, A.P.; Morais, R.C.; Teixeira, J.A.; Nunes, J. A comparison between microalgal autotrophic growth and metabolite accumulation with heterotrophic, mixotrophic and photoheterotrophic cultivation modes. *Renew. Sustain. Energy Rev.* **2022**, *159*, 112247. [\[CrossRef\]](#)
12. Guidhe, A.; Ansari, F.A.; Singh, P.; Bux, F. Heterotrophic cultivation of microalgae using aquaculture wastewater: A biorefinery concept for biomass production and nutrient remediation. *Ecol. Eng.* **2017**, *99*, 47–53.
13. Christenson, L.; Sims, R. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. *Biotechnol. Adv.* **2011**, *29*, 686–702. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Huntley, M.E.; Johnson, Z.I.; Brown, S.L.; Sills, D.L.; Gerber, L.; Archibald, I.; Machesky, S.C.; Granados, J.; Beal, C.; Greene, C.H. Demonstrated large-scale production of marine microalgae for fuels and feed. *Algal Res.* **2015**, *10*, 249–265. [\[CrossRef\]](#)
15. Shitanaka, T.; Fujioka, H.; Khan, M.; Kaur, M.; Du, Z.Y.; Khanal, S.K. Recent advances in microalgal production, harvesting, prediction, optimization, and control strategies. *Bioresour. Technol.* **2024**, *391*, 129924. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Sero, E.T.; Siziba, N.; Bunhu, T.; Shoko, R. Isolation and screening of microalgal species, native to Zimbabwe, with potential use in biodiesel production. *All Life* **2021**, *14*, 256–264. [\[CrossRef\]](#)
17. Xie, Y.P.; Ho, S.H.; Chen, C.N.N.; Chen, C.Y.; Jing, K.J.; Ng, I.S.; Chen, J.F.; Chang, J.S.; Lu, Y.H. Disruption of thermo-tolerant *Desmodesmus* sp F51 in high pressure homogenization as a prelude to carotenoids extraction. *Biochem. Eng. J.* **2016**, *109*, 243–251. [\[CrossRef\]](#)
18. Yu, N.; Wei, Y.L.; Zhang, X.; Zhu, N.; Wang, Y.L.; Zhu, Y.; Zhang, H.P.; Li, F.M.; Yang, L.; Sun, J.Q.; et al. Barcode ITS2: A useful tool for identifying *Trachelospermum jasminoides* and a good monitor for medicine market. *Sci. Rep.* **2017**, *7*, 5037. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Wang, Y.; Guo, W.Q.; Yen, H.W.; Ho, S.H.; Lo, Y.C.; Cheng, C.L.; Ren, N.Q.; Chang, J.S. Cultivation of *Chlorella vulgaris* JSC-6 with swine wastewater for simultaneous nutrient/COD removal and carbohydrate production. *Bioresour. Technol.* **2015**, *198*, 619–625. [\[CrossRef\]](#)
20. Cheng, P.F.; Wang, Y.Z.; Liu, T.H.; Liu, D.F. Biofilm attached cultivation of *Chlorella pyrenoidosa* is a developed system for swine wastewater treatment and lipid production. *Front. Plant Sci.* **2017**, *8*, 1594. [\[CrossRef\]](#)
21. Chen, X.; Yuan, C.B.; Zhu, Y.A.; Liu, H.; Chen, W.; Zhang, Q. Bioaugmentation with *Acinetobacter* sp. TAC-1 to enhance nitrogen removal in swine wastewater by moving bed biofilm reactor inoculated with bacteria. *Bioresour. Technol.* **2022**, *359*, 127506. [\[CrossRef\]](#)
22. Yang, L.M.; Li, H.K.; Wang, Q. A novel one-step method for oil-rich biomass production and harvesting by co-cultivating microalgae with filamentous fungi in molasses wastewater. *Bioresour. Technol.* **2019**, *275*, 35–43. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Cesário, C.D.; Soares, J.; Cossolin, J.F.S.; Almeida, A.V.M.; Sierra, J.J.B.; Leite, M.D.; Nunes, M.C.; Serrao, J.E.; Martins, M.A.; Coimbra, J.S.D. Biochemical and morphological characterization of freshwater microalga *Tetrademus obliquus* (Chlorophyta: Chlorophyceae). *Protoplasma* **2022**, *259*, 937–948. [\[CrossRef\]](#)
24. Valchev, D.; Ribarova, I.; Uzunov, B.; Stoyneva-Gaertner, M. Reclamation potential of onsite wastewater post-treatment with microalgae: Chemical elements perspective. *Processes* **2023**, *11*, 1819. [\[CrossRef\]](#)
25. San Agustin, D.M.; Ledesma, M.T.O.; Ramírez, I.M.; Noguez, I.Y.; Pabello, V.M.L.; Velasquez-Orta, S.B. A non-sterile heterotrophic microalgal process for dual biomass production and carbon removal from swine wastewater. *Renew. Energy* **2022**, *181*, 592–603. [\[CrossRef\]](#)
26. Hooper, J.K.; Maloney, M.A.; Asbury, L.R.; Marks, D.B. Accumulation of chlorophyll a/b-binding polypeptides in *Chlamydomonas reinhardtii* y-1 in the light or dark at 38 °C. *Plant Physiol.* **1990**, *92*, 419–426. [\[CrossRef\]](#)
27. Xiong, W.; Li, X.F.; Xiang, J.Y.; Wu, Q.Y. High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 29–36. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Chen, F.; Johns, M.R. Relationship between substrate inhibition and maintenance energy of *Chlamydomonas reinhardtii* in heterotrophic culture. *J. Appl. Phycol.* **1996**, *8*, 15–19. [\[CrossRef\]](#)
29. Zhao, G.; Wang, X.; Hong, Y.; Liu, X.; Wang, Q.; Zhai, Q.; Zhang, H. Attached cultivation of microalgae on rational carriers for swine wastewater treatment and biomass harvesting. *Bioresour. Technol.* **2022**, *351*, 127014. [\[CrossRef\]](#)
30. You, K.; Ge, F.R.; Wu, X.D.; Song, K.Y.; Yang, Z.X.; Zhang, Q.; Liu, Y.H.; Ruan, R.G.; Zheng, H.L. Nutrients recovery from piggery wastewater and starch wastewater via microalgae-bacteria consortia. *Algal Res.* **2021**, *60*, 102551. [\[CrossRef\]](#)
31. Sosa-Hernández, J.E.; Rodas-Zuluaga, L.I.; Castillo-Zacarias, C.; Rostro-Alanis, M.; de la Cruz, R.; Carrillo-Nieves, D.; Salinas-Salazar, C.; Grunewald, C.F.; Llewellyn, C.A.; Olguín, E.J.; et al. Light intensity and nitrogen concentration impact on the biomass and phycoerythrin production by *Porphyridium purpureum*. *Mar. Drugs* **2019**, *17*, 460. [\[CrossRef\]](#) [\[PubMed\]](#)

32. Cheng, D.L.; Ngo, H.H.; Guo, W.S.; Chang, S.W.; Nguyen, D.D.; Kumar, S.M. Microalgae biomass from swine wastewater and its conversion to bioenergy. *Bioresour. Technol.* **2019**, *275*, 109–122. [[CrossRef](#)] [[PubMed](#)]
33. Qu, W.Y.; Zhang, C.F.; Chen, X.; Ho, S.H. New concept in swine wastewater treatment: Development of a self-sustaining synergetic microalgae-bacteria symbiosis (ABS) system to achieve environmental sustainability. *J. Hazard. Mater.* **2021**, *418*, 126264. [[CrossRef](#)] [[PubMed](#)]
34. Xu, Y.P.; Duan, P.G.; Wang, F.; Guan, Q.Q. Liquid fuel generation from algal biomass via a two-step process: Effect of feedstocks. *Biotechnol. Biofuels* **2018**, *11*, 83. [[CrossRef](#)] [[PubMed](#)]
35. Li, D.; Liu, R.Q.; Cui, X.Y.; He, M.L.; Zheng, S.Y.; Du, W.J.; Gao, M.; Wang, C. Co-culture of bacteria and microalgae for treatment of high concentration biogas slurry. *J. Water Process Eng.* **2021**, *41*, 102014. [[CrossRef](#)]
36. Xie, D.; Ji, X.W.; Zhou, Y.C.; Dai, J.X.; He, Y.J.; Sun, H.; Guo, Z.; Yang, Y.; Zheng, X.; Chen, B.L. *Chlorella vulgaris* cultivation in pilot-scale to treat real swine wastewater and mitigate carbon dioxide for sustainable biodiesel production by direct enzymatic transesterification. *Bioresour. Technol.* **2022**, *349*, 126886. [[CrossRef](#)] [[PubMed](#)]
37. GB 18596-2001; Discharge Standard of Pollutants for Livestock and Poultry Breeding. Standardization Administration of the People's Republic of China (SAC): Beijing, China, 2001.
38. Su, M.; Dell'Orto, M.; Scaglia, B.; D'Imporzano, G.; Bani, A.; Adani, F. Growth performance, biochemical composition and nutrient recovery ability of twelve microalgae consortia isolated from various local organic wastes grown on nano-filtered pig slurry. *Molecules* **2022**, *27*, 422. [[CrossRef](#)]
39. Zhu, S.N.; Feng, S.R.; Xu, Z.B.; Qin, L.; Shang, C.H.; Feng, P.Z.; Wang, Z.M.; Yuan, Z.H. Cultivation of *Chlorella vulgaris* on unsterilized dairy-derived liquid digestate for simultaneous biofuels feedstock production and pollutant removal. *Bioresour. Technol.* **2019**, *285*, 121353. [[CrossRef](#)] [[PubMed](#)]
40. He, J.W.; Ding, W.Q.; Han, W.; Chen, Y.D.; Jin, W.B.; Zhou, X. A bacterial strain *Citrobacter* W4 facilitates the bio-flocculation of wastewater cultured microalgae *Chlorella pyrenoidosa*. *Sci. Total Environ.* **2022**, *806*, 151336. [[CrossRef](#)]
41. Barros, A.I.; Gonçalves, A.L.; Simoes, M.; Pires, J.C.M. Harvesting techniques applied to microalgae: A review. *Renew. Sustain. Energy Rev.* **2015**, *41*, 1489–1500. [[CrossRef](#)]
42. Liu, Y.B.; Zhang, G.Y.; Li, W.R.; Ding, Y.; You, H.; Zhu, J.; Leng, H.R.; Xu, C.; Xing, X.D.; Xu, J.Y.; et al. The characteristic evolution and formation mechanism of hybrid microalgae biofilm and its application in mariculture wastewater treatment. *J. Environ. Chem. Eng.* **2023**, *11*, 109645. [[CrossRef](#)]
43. Meng, F.G.; Zhou, Z.B.; Ni, B.J.; Zheng, X.; Huang, G.C.; Jia, X.S.; Li, S.Y.; Xiong, Y.; Kraume, M. Characterization of the size-fractionated biomacromolecules: Tracking their role and fate in a membrane bioreactor. *Water Res.* **2011**, *45*, 4661–4671. [[CrossRef](#)] [[PubMed](#)]
44. Zhou, D.D.; Zhang, C.F.; Liang, F.; Liang, X.; Cui, X.C.; Li, Q.C.; Crittenden, J.C. Responses of the microalga *Chlorophyta* sp. to bacterial quorum sensing molecules (N-acylhomoserine lactones): Aromatic protein induced self-aggregation. *Environ. Sci. Technol.* **2017**, *51*, 3490–3498. [[CrossRef](#)]

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