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Cultivation of *Chlorella vulgaris* in a Light-Receiving-Plate (LRP)-Enhanced Raceway Pond for Ammonium and Phosphorus Removal from Pretreated Pig Urine

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Received: 15 January 2020; Accepted: 18 March 2020; Published: 2 April 2020



Abstract: Fresh pig urine is unsuitable for microalgae cultivation due to its high concentrations of $\text{NH}_4^+\text{-N}$, high pH and insufficient magnesium. In this study, fresh pig urine was pretreated by dilution, pH adjustment, and magnesium addition in order to polish wastewater and produce microalgae biomass. *Chlorella vulgaris* was cultured in an in-house-designed light-receiving-plate (LRP)-enhanced raceway pond to treat the pretreated pig urine in both batch and continuous mode under outdoor conditions. $\text{NH}_4^+\text{-N}$ and TP in wastewater were detected, and the growth of *C. vulgaris* was evaluated by chlorophyll fluorescence activity as well as biomass production. Results indicated that an 8-fold dilution, pH adjusted to 6.0 and $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ dosage of $0.1 \text{ mg}\cdot\text{L}^{-1}$ would be optimal for the pig urine pretreatment. *C. vulgaris* could stably accumulate biomass in the LRP-enhanced raceway pond when cultured by both BG11 medium and the pretreated pig urine. About $1.72 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ of microalgal biomass could be produced and 98.20% of $\text{NH}_4^+\text{-N}$ and 68.48% of TP could be removed during batch treatment. Hydraulic retention time of 7-9d would be optimal for both efficient nutrient removal and microalgal biomass production during continuous treatment.

Keywords: pig urine; *Chlorella vulgaris*; photosynthetic reactor; nutrient removal; chlorophyll fluorescence; hydraulic retention time

1. Introduction

Intensive pig farming produces a large amount of piggery waste (urine and manure). The National Bureau of Statistics of the People's Republic of China has reported that more than 11 million tons of piggery wastewater were directly discharged into the environment in 2014 [1]. Piggery waste has become a major source of pollution in rural areas of China. It releases greenhouse gases and ammonia, and causes eutrophication, algal blooms, as well as soil acidification [2]. The treatment of piggery waste is a continuing environmental issue in china.

Anaerobic digestion (AD) has been widely acknowledged as an effective method to process piggery waste, around the world [3]. AD converts complex organic matter in pig manure into biogas.

However, the high content of nitrogen (urea, uric acid, and ammonium) in pig urine always leads to high ammonium levels during anaerobic digestion. This causes the inhibition of the growth rates of methanogens, and thereby reduces the yield of methane. Thus, liquid and solid separation of piggery waste is needed to alleviate the ammonium inhibition prior to the anaerobic digestion process. The separated pig urine is rich in pollutants such as nitrogen (N), phosphorus (P), and potassium (K) which are suitable nutrients for algal growth. Microalgae can effectively utilize N and P in aquatic environment as well as light energy and CO₂ for photosynthesis to produce biomass [1]. The generated biomass can also be utilized for the production of a variety of high-value products such as food, bioenergy, pharmaceuticals and nutritional products [4,5]. Furthermore, some companies have used microalgae biomass in functional food and feed industry and obtained some economic benefits [6]. Furthermore, compared to the effluent from anaerobic digestion, the separated pig urine is more suitable as a medium to culture microalgae due to: (1) less contamination by heavy metals and microorganisms, (2) less content of organic acids (fulvic acid, humic acid, etc.), which easily cause flocculation of microalgae, and (3) low turbidity and good light transmission. Thus, using pig urine as culture medium for algal cultivation is a promising method for wastewater treatment, pollutants recovery and algal biomass accumulation [7].

However, pig urine is not frequently used as medium to culture microalgae due to its inhibitive effect on the microalgae metabolism. The high concentration of NH₄⁺-N ($\geq 200 \text{ mg}\cdot\text{L}^{-1}$) in fresh pig urine is toxic to the growth of microalgae due to the formation of free ammonia [8]. This could affect the photosynthetic processes in chloroplasts, electron transport chain, non-photochemical quenching, as well as the dark respiration [9], which can decrease the chlorophyll-A content and cause cell lysis of the microalgae [2,3]. In addition, high pH (commonly higher than 8.5) in pig urine can also induce the flocculation of microalgal cells [10] and decrease its population growth [11]. Moreover, magnesium is an important growth factor for green microalgae, since it is the central atom of chlorophyll [12], but it is insufficient in pig urine. Insufficient magnesium can commonly lead to the decreased chlorophyll content and enzymes activities in microalgal cells [13]. Therefore, these issues need to be sufficiently addressed to improve the successful cultivation of microalgae in pig urine.

An economical microalgae treatment system is another important factor affecting the efficiency of pig urine processing. A raceway pond is believed to be the most convenient and cost-effective facility to treat large amounts of wastewater by microalgae. It has the advantages of low capital and operation costs, ease of scale up, and the ability to handle high treatment volumes [14]. However, the self-shadowing effect of the suspended cells leads to limited light penetration, inefficient photosynthesis, and insufficient nutrient removal in the system [15]. This further leads to low dry mass content, which increases the harvest cost of the biomass.

In this study, the pig urine was pretreated by a simple and efficient method to reduce its toxicity to algal cells. Meanwhile, a light-receiving-plate (LRP)-enhanced raceway pond was designed for pig urine treatment to enhance the photosynthesis of algal for the promoted nutrient removal and the improved biomass output. The thin LRP had a short light pathway and could largely increase the irradiated area of the whole system. The microalgae involved was *Chlorella vulgaris* due to its widely reported robust growth property, high lipid/carbohydrate accumulation potential and large N and P removal capacity from various wastewaters [14,16]. The specific objectives were: (1) to optimize the culture conditions of *C. vulgaris* in pig urine and to explore a simple and efficient method for the pretreatment of pig urine; (2) to establish an efficient pig urine purification system for studying the growth characteristics of microalgae and the nutrient removal efficiency from the wastewater; (3) to explore the effect of hydraulic retention time on the continuous pig urine treatment and algal biomass production under outdoor conditions in the LRP-enhanced raceway pond. It was hoped that the results of this study would provide a scientific basis for large-scale outdoor treatment of pig urine.

2. Materials and Methods

2.1. Pig Urine Collection and Analysis

Pig urine was directly collected from a local piggery farm (Fengcheng city, Jiangxi Province, China) during the excretion of pigs. Samples were collected from pigs of different ages (2 to 8 months), and from a mixture of six different pigsties. The collected pig urine was stored in darkness at 4 °C and the supernatant was directly taken for the experiment after precipitation for 24 hours. The characteristics of the sample were analyzed, as shown in Table 1.

Table 1. Characteristics of the collected pig urine.

Parameter	Value
pH	9.20 ± 0.10
Total carbon (TC) (mg·L ⁻¹)	3103.10 ± 7.20
Total organic carbon (TOC) (mg·L ⁻¹)	2146.30 ± 14.30
Total nitrogen (TN) (mg·L ⁻¹)	3159.70 ± 22.10
Ammonium (NH ₄ ⁺ -N) (mg·L ⁻¹)	1155.50 ± 10.50
Total phosphorus (TP) (mg·L ⁻¹)	82.20 ± 2.10
Chemical oxygen demand (COD) (mg·L ⁻¹)	4496.10 ± 20.80
Potassium (K ⁺) (mg·L ⁻¹)	1150.90 ± 23.20
Magnesium (Mg ²⁺) (mg·L ⁻¹)	0.62 ± 0.04
Cadmium (Cd ²⁺) (mg·L ⁻¹)	<0.01
Copper (Cu ²⁺) (mg·L ⁻¹)	0.11 ± 0.01
Zinc (Zn ²⁺) (mg·L ⁻¹)	0.17 ± 0.02

2.2. Microalgae Strain and Subculture

The *Chlorella vulgaris* FACHB-31, purchased from the Institute of Hydrobiology, Chinese Academy of Sciences, PR China, was used for this study. The strain was preserved in 250 mL autoclaved (121 °C, 30 min) BG11 medium [17] at a temperature of 26 ± 1 °C, and light intensity of 2500 Lux. Prior to inoculation into wastewaters, microalgae cells were recovered from the culture media through centrifugation at 5000 rpm for 15 min at room temperature and washed with distilled water three times to remove the residual nutrients.

2.3. Optimization of *C. vulgaris* Growth Conditions in Pig Urine

A single-factor experiment was conducted to evaluate the effects of pig urine dilution rate, pH value, light intensity, and MgSO₄·7H₂O dosage on microalgal growth. The effect of pig urine dilution rate was studied by culturing *C. vulgaris* in 2, 4 and 8 fold diluted pig urine under light intensity of 2500 Lux. The effect of pig urine pH value was studied by culturing *C. vulgaris*, after the pig urine was diluted 8 to 1, with different pH values (4, 5, 6 and 7) under light intensity of 2500 Lux. The effect of light intensity was studied by culturing *C. vulgaris* in pig urine, diluted 8 to 1, with pH adjusted to 6, under light intensities of 500 Lux, 1500 Lux, 2500 Lux and 3000 Lux. The effect of MgSO₄·7H₂O dosage was studied by culturing *C. vulgaris* pig urine, diluted 8 to 1, pH adjusted to 6, with different MgSO₄·7H₂O dosages (0.0 g·L⁻¹, 0.1 g·L⁻¹ and 1.0 g·L⁻¹) under light intensity of 2500 Lux. At least three replicate experiments were conducted for each treatment. Pig urine was diluted using deionized water. The pH value of the pig urine was adjusted by adding 1 mol·L⁻¹ HCl or NaOH. Fresh *C. vulgaris* slurry, harvested from Section 2.2, was inoculated into 300 mL of pretreated pig urine in a 500 mL Erlenmeyer flask, and the OD₆₈₀ (optical density at 680 nm) values of the medium were controlled between 0.20 and 0.40. All the cultures were maintained at 26 ± 1 °C for 15 days. The contents were shaken by hand twice a day and their OD₆₈₀ values were also daily measured. The water evaporation loss was balanced daily by adding deionized water.

2.4. Design of the Algal Cultivation System

The design of the LRP-enhanced raceway pond is shown in Figure 1. In general, the system consisted of two parts, a traditional raceway pond and an LRP. The raceway pond (length*width*height: 80*20*15 cm) was made of polymethyl methacrylate (PMMA) and its work volume was 8 L. A cover plate was utilized to enclose the raceway pond to avoid contamination and evaporation. An air compressor and a stirring device were set inside the raceway pond to aerate and stir the algal culture. The temperature of the medium was monitored by a thermometer. The LRP (length*width*height: 60*0.6*30 cm) was a flat panel photobioreactor which was made of polycarbonate for increasing the light-receiving area of the whole system. It had thirty internal passages (length*width*height: 60*0.6*1 cm) and the microalgae culture inside the raceway pond could be circulated in it, to receive light by the submersible pump. In addition, pig urine was pumped into the system from a container for nutrients supplement by a peristaltic pump and the medium flow rate was controlled by a controller. The medium was simultaneously pumped out of the system at the same flow rate by another peristaltic pump. The water evaporation loss was daily balanced by deionized water. After each batch experiment, the whole system was washed twice by deionized water and the 10 g·L⁻¹ NaClO was used to clean the system twice for disinfection. Afterwards, sterilized cold water was applied to clean up the residual NaClO [17]. The outdoor experiments were conducted during July 10, 2017- August 20, 2017 at 115°94' E, 28°69' N.

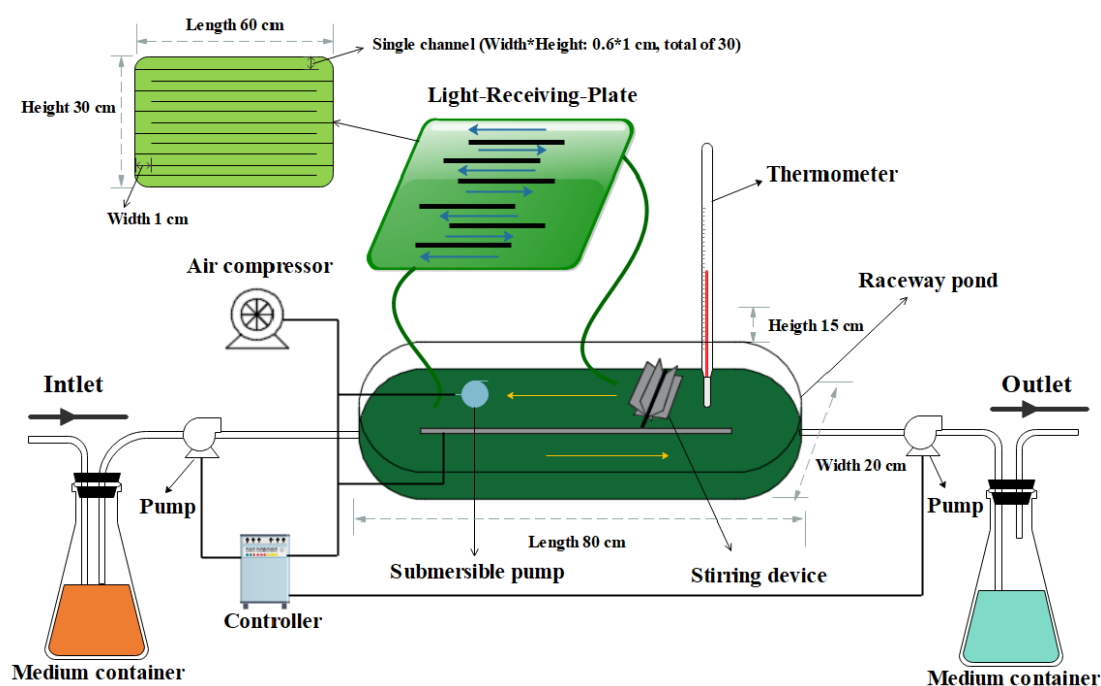


Figure 1. Schematic diagram of the light-receiving-plate (LRP)-enhanced raceway pond.

2.5. *C. vulgaris* Cultivation and Pig Urine Treatment in LRP-enhanced Raceway Pond

2.5.1. Cultivation of *C. vulgaris* in LRP-Enhanced Raceway Pond

8 Liters of autoclaved BG11 medium were added into the raceway pond and the system was inoculated with *C. vulgaris* slurry (Section 2.2). Then, the air compressor, the stirrer, and the submersible pump were turned on to uniformly disperse the algal cells into the medium, and the OD₆₈₀ value of the medium was controlled at 0.15. The system was run continuously for 10 days under outdoor conditions. The aeration rate was set at 0.7 L·min⁻¹; the flow rate of the algae culture inside the internal passages of the LRP was controlled as 0.2 L·min⁻¹ (5.56 cm·s⁻¹). The stirring speed of the stirrer was set at 30 rpm. During the process, all of the on-off switches for the air compressor, the stirrer, and the submersible

pump, were controlled by a controller, in order to balance the energy consumption and photosynthetic efficiency of the system (Figure 1). The system was controlled to run for an hour and powered off for an hour with a total of 6 cycles during the daylight hours (07:00-18:00). The temperature and light intensity were measured every 2 hours during daylight (07:00-18:00). In total, 20 mL of algal culture was sampled daily for further analysis at 18:00.

2.5.2. Pig Urine Treatment by LRP-Enhanced Raceway Pond in Batch Mode

During this experiment, the autoclaved BG11 medium (8 L) was first introduced into the raceway pond, and then the system was run under the same conditions of Section 2.5.1. After the OD_{680} value of the algal culture reached about 1.2, half of the medium was replaced with the pretreated pig urine. Pig urine was first diluted 4 to 1 with deionized water, and then treated by the addition of $MgSO_4 \cdot 7H_2O$ ($0.1 \text{ g} \cdot \text{L}^{-1}$) and the adjustment of pH to 6.0. Finally, the dilution rate of the pig urine was adjusted to 8 to 1, after 1:1 mixed with algal culture. The treatment of the pig urine by *C. vulgaris* was conducted under the same conditions of Section 2.5.1 for 5 days and 20 mL algal culture was sampled at 08:00 and 20:00 every day for further analysis.

2.5.3. Pig Urine Treatment by LRP-Enhanced Raceway Pond in Continuous Mode

To evaluate the effect of hydraulic retention time on the pig urine, treatment efficiency in the LRP-enhanced raceway pond, the pretreated pig urine (8 to 1 dilution, $0.1 \text{ g} \cdot \text{L}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{pH}=6.0$) was continuously pumped into the system with different flow rates. First, algal biomass in the system was accumulated using the same method of Section 2.5.2. After the OD_{680} value of the algal culture reached about 0.75, the pretreated pig urine was continuously pumped into the raceway pond and the medium with algal cells was also continuously pumped out of the system at a same flow rate as the influent. This was the initiation of a continuous process. Then the continuous treatment experiment was started, and the system was run for 20 days under the same conditions of Section 2.5.1. The inflow rate as well as the outflow rate of pig urine was adjusted every 5 days to generate four different hydraulic retention times (HRT), e.g., 9-days, 7-days, 5-days and 3-days, respectively. The corresponding medium inflow rates were $0.89 \text{ L} \cdot \text{day}^{-1}$, $1.14 \text{ L} \cdot \text{day}^{-1}$, $1.60 \text{ L} \cdot \text{day}^{-1}$, $2.67 \text{ L} \cdot \text{day}^{-1}$, respectively. Furthermore, the corresponding rates of urine exchange were 11.1%, 14.3%, 20%, 33.3%, respectively. In total, 20 mL of algal culture was daily sampled for further analysis at 18:00.

2.6. Analytical Method

2.6.1. Microalgal Growth Analysis

The optical density of the samples at wavelength of 680 nm were determined daily by using a spectrophotometer (Metash, UV-9000, China); the microalgal biomass density ($\text{mg} \cdot \text{L}^{-1}$) was calculated based on a calibration curve as follows:

$$\text{Biomass density } (\text{mg} \cdot \text{L}^{-1}) = 327.42 \cdot OD_{680} - 25.16; R^2 = 0.99 \quad (1)$$

To plot the calibration curve (Equation (1)), *C. vulgaris* was first cultured in pretreated pig urine (the same in Section 2.5.3) under the same conditions as Section 2.2. After the OD_{680} of the culture reached about 1.64, the suspended cells were harvested and rinsed following the same method in Section 2.2. Then the algal slurry was extensively diluted using BG11 medium to obtain samples with an absorbance (OD_{680}) in the range of 0.2–1.5. After this, 50 mL of each diluted sample were harvested by filtering using the pre-weighed $0.45 \mu\text{m}$ GF/C filter membrane (Whatman, Little Chalfont, England), and their cell dry mass contents ($\text{mg} \cdot \text{L}^{-1}$) were determined by oven drying at $105 \text{ }^\circ\text{C}$ for 12 h. Finally, the sample cell dry mass content was plotted against the value of OD_{680} .

During the pig urine treatment in batch mode, the microalgal biomass production and productivity were calculated using Equations (2) and (3):

$$\text{Biomass production (g} \cdot \text{m}^{-2}) = 8 \cdot (D_5 - D_0) / (1000 \cdot 0.16) \quad (2)$$

$$\text{Biomass productivity (g} \cdot \text{m}^{-2} \cdot \text{day}^{-1}) = 8 \cdot (D_5 - D_0) / (1000 \cdot 0.16 \cdot 5) \quad (3)$$

where D_0 and D_5 are the algal biomass density at day 0 and day 5; 8 (L) and 0.16 (m^2) are the volume of the medium in the raceway pond and the footprint area of the raceway pond, respectively; 5 is the duration time of the treatment.

During the pig urine treatment in continuous mode, the microalgal biomass production and productivity during each HRT were calculated using Equations (4) and (5):

$$\text{Biomass production (g} \cdot \text{m}^{-2}) = \frac{8 \cdot [A(i+5) - A(i)] + \int_i^{i+5} [A(t) \cdot \mu] dt}{1000 \cdot 0.16} \quad (4)$$

$(i = 0, 5, 10, 15; \mu = 0.89, 1.14, 1.60, 2.67)$

$$\text{Biomass productivity (g} \cdot \text{m}^{-2} \cdot \text{day}^{-1}) = \frac{8 \cdot [A(i+5) - A(i)] + \int_i^{i+5} [A(t) \cdot \mu] dt}{1000 \cdot 0.16 \cdot 5} \quad (5)$$

$(i = 0, 5, 10, 15; \mu = 0.89, 1.14, 1.60, 2.67)$

where $A(t)$ is the function of microalgal biomass density (A) vs culture time (t); i is the day that a new HRT was applied; μ is the flow rate under each HRT; 5 is the duration time of each HRT.

2.6.2. Wastewater Nutrient Analysis

The samples for nutrient analysis were first centrifuged at $5000 \times g$ for 10 min. Then, the supernatants were collected and filtered using $0.45 \mu\text{m}$ membrane filters (Millipore Corporation, USA). After which, the filtrates were properly diluted and the Chemical Oxygen Demand (COD), Ammonium ($\text{NH}_4^+ \text{-N}$), Total Nitrogen (TN) and Total Phosphorus (TP) were analyzed by a Hach DR 5000 Spectrophotometer following the method reported by Zheng et al. [2]. The Total Organic Carbon (TOC) and Inorganic Carbon (IC) were determined by a multi N/C3100 Analyzer (Analytik Jena AG, Jena, Germany). The concentrations of the metal ions (Zn^{2+} , Cu^{2+} , Cd^{2+} , K^+ , and Mg^{2+}) were measured using a C200 Multi-parameter Ion Specific Meter (Hanna Instruments, Italy) following the method reported by Cao et al. [18]. The nutrient removal efficiency and removal rate during pig urine treatment in batch mode were calculated according to Equations (6) and (7):

$$\text{Nutrient removal efficiency (\%)} = \left[(C_0^* - C_5) / C_0^* \right] * 100\% \quad (6)$$

$$\text{Nutrient removal rate (mg} \cdot \text{m}^{-2} \cdot \text{day}^{-1}) = 8 \cdot (C_0^* - C_5) / (0.16 \cdot 5) \quad (7)$$

where C_0^* ($\text{mg} \cdot \text{L}^{-1}$) and C_5 ($\text{mg} \cdot \text{L}^{-1}$) are the nutrient concentrations at day 0 and day 5, respectively. For the nutrient removal efficiency and removal rate during pig urine treatment in continuous mode, results were calculated following Equations (8) and (9):

$$\text{Nutrient removal efficiency (\%)} = 100 \cdot \frac{8 \cdot [C(i) - C(i+5)] + 5 \cdot C_0^* \cdot \mu - \int_i^{i+5} [C(t) \cdot \mu] dt}{5 \cdot C_0^* \cdot \mu + 8 \cdot C(i)} \quad (8)$$

$(i = 0, 5, 10, 15; \mu = 0.89, 1.14, 1.60, 2.67)$

$$\text{Nutrient removal rate (mg} \cdot \text{m}^{-2} \cdot \text{day}^{-1}) = \frac{8 \cdot [C(i) - C(i+5)] + 5 \cdot C_0^* \cdot \mu - \int_i^{i+5} [C(t) \cdot \mu] dt}{5 \cdot 0.16} \quad (9)$$

$(i = 0, 5, 10, 15; \mu = 0.89, 1.14, 1.60, 2.67)$

where $C(t)$ is the function of nutrient concentration (C) vs culture time (t).

2.6.3. Chlorophyll Fluorescence Analysis

The chlorophyll fluorescence parameters of *C. vulgaris* were measured using Water-PAM (WALZ, Effeltrich, Germany). The samples were first diluted by deionized water to obtain the same biomass concentration (OD_{680}) before measuring the light response curves of photosynthesis. Then each sample of 3 mL microalgae suspension was placed in darkness for 15 min. After which, the values of maximum photochemical efficiency (F_v/F_m) of PS II and the actual conversion efficiency of light energy (Φ_{PSII}) were recorded, details are reported elsewhere [2].

2.7. Statistical Analysis

All of the analytical experiments were conducted in triplicate, and the results are presented as mean values \pm standard deviation. Analysis of variance (ANOVA) was carried out wherever applicable.

3. Results and Discussion

3.1. Optimization of *C. vulgaris* Growth Conditions in Pig Urine

The characterization of pig urine is listed in Table 1. The *C. vulgaris* growth could be inhibited by the high concentration of NH_4^+-N ($1155.5 \text{ mg}\cdot\text{L}^{-1}$) in unpretreated pig urine due to the uncoupling effect of free ammonia on the photosynthetic processes in chloroplasts [7]. Meanwhile, the K ($1150.9 \text{ mg}\cdot\text{L}^{-1}$) was sufficient but the Mg ($0.62 \text{ mg}\cdot\text{L}^{-1}$) was insufficient for microalgal growth when compared to BG11 medium. Furthermore, the pH value of 9.2 also showed strong alkalinity in unpretreated pig urine. Therefore, the pig urine needed to be pretreated for the optimized cultivation of microalgae. In this study, pig urine was diluted, and its pH as well as the Mg content were both adjusted. The effects of dilution rate, pH value, light intensity, and Mg dosage on the growth of *C. vulgaris* were studied. Results are illustrated in Figure 2.

As can be seen from Figure 2a, *C. vulgaris* in 8:1 diluted pig urine witnessed a rapid growth after 4 days of lag phase, and the biomass density reached to $220.08 \text{ mg}\cdot\text{L}^{-1}$ at day 13. *C. vulgaris* grew more slowly in 4:1 diluted pig urine, and only a 10% increase of the biomass was obtained after 15 days cultivation. For the 2:1 diluted pig urine, *C. vulgaris* failed to grow, and plenty of cells were dead and formed as sedimentation at the bottom of the flask. Possibly the high concentration of NH_4^+-N and the salts contribute to the growth inhibition or even the death of *C. vulgaris* in pig urine with low dilution rate. In this study, the NH_4^+-N concentration of the 2- and 4- diluted pig urine reached $577.5 \text{ mg}\cdot\text{L}^{-1}$ and $288.75 \text{ mg}\cdot\text{L}^{-1}$, respectively. The high concentration of NH_4^+-N was detrimental to algal cells due to the free ammonia [8]. In addition, high levels of salts (Table 1) could also affect the nutrient assimilation and metabolism of microalgae, and even threaten its growth, due to high osmotic pressure [3]. Thus, the pig urine needed to be diluted; a dilution rate of 8:1 was found to be optimal, according to this study. This was in consistent with the findings of Deng et al [19]. They found that *C. vulgaris* grew well by properly diluted wastewater.

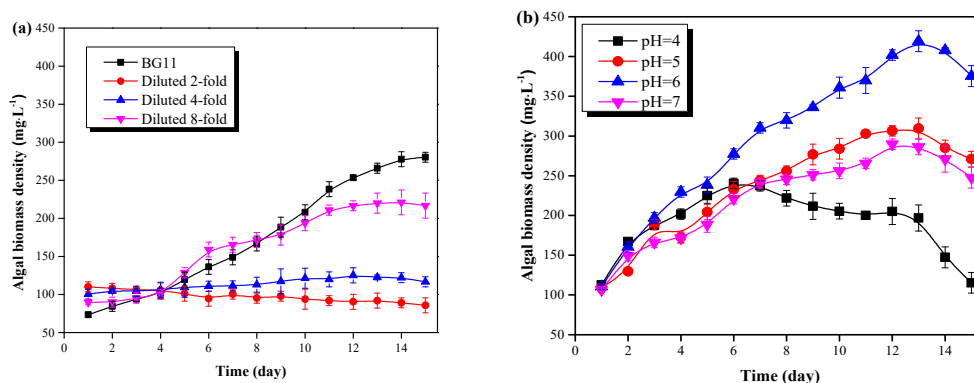


Figure 2. Cont.

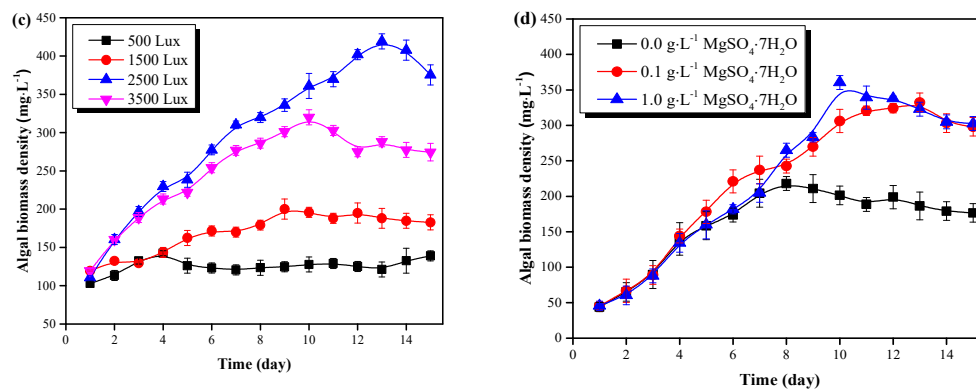


Figure 2. *C. vulgaris* growth results in the pretreated pig urine. (a) The effect of pig urine dilution rate on the microalgal growth. (b) The effect of pig urine pH value on the microalgal growth. (c) The effect of light intensity on the microalgal growth. (d) The effect of pig urine MgSO₄·7H₂O dosage on the microalgal growth.

The effect of pH on the growth of *C. vulgaris* in 8-fold diluted pig urine is shown in Figure 2b. *C. vulgaris* could grow well in a pH range of 5.0–7.0 without a lag phase. The optimal pH value of pig urine for *C. vulgaris* growth was found to be 6.0, with the largest amount of biomass (419.15 mg·L⁻¹) being accumulated at this pH value, after 13 days cultivation. Such density was significantly ($P < 0.05$) larger than that of pH of 5.0 (309.46 mg·L⁻¹) and 7.0 (286.22 mg·L⁻¹) as well as the values in Figure 2a (≤ 221 mg·L⁻¹). This signified that acidification of the pig urine enhanced the growth of microalgae, which was in consistent with the findings of Qiu et al. [11]. They found that biomass productivity increased with decreasing pH. This was mainly because the acidic condition could: (1) inhibit the formation of free ammonia, (2) promote the absorption and utilization of macromolecular nutrients by *C. vulgaris* via acidolysis, and (3) cause metabolic disturbance of other microbial cells [2,18]. However, *C. vulgaris* could not grow well at a pH of 4.0, under which the biomass density increased only for 6 days and then sharply decreased to 115.30 mg·L⁻¹ at day 15. Lower pH (4.0) could have a negative effect on cell division and enzyme activity, and thus inhibit cell growth [7].

The growth results of *C. vulgaris* in pretreated pig urine (8-fold dilution, pH=6.0) under different light intensity, is shown in Figure 2c. Results demonstrate that light intensity of 2500 Lux to 3500 Lux would be optimal for *C. vulgaris* growth in pretreated pig urine. *C. vulgaris* could grow well under light intensity of 1500 Lux, 2500 Lux and 3500 Lux, and increased light intensity could enhance its growth. In contrast, *C. vulgaris* could hardly accumulate biomass under light intensity of 500 Lux. This possibly indicates that the light intensity of 500 Lux would be the light compensation point of *C. vulgaris*, where the microalgae photosynthetic accumulation rate equilibrates with the respiration rate [20]. Proper light supplement is important for the efficient accumulation of microalgal biomass; too high or too low light intensity would inhibit the *C. vulgaris* growth due to light inhibition or light starvation [21].

The growth of *C. vulgaris* under different MgSO₄·7H₂O dosage rates (0.0 mg·L⁻¹, 0.1 mg·L⁻¹ and 1.0 mg·L⁻¹) in the pretreated pig urine (8-fold dilution, pH=6.0) is shown in Figure 2d. In the first 7 days of cultivation, *C. vulgaris* witnessed a rapid growth with no significant difference between the treatments. After day 7, *C. vulgaris* continued with its increase in the treatments with the addition of MgSO₄·7H₂O (0.1 g·L⁻¹ and 1.0 g·L⁻¹). However, the microalgae without MgSO₄·7H₂O addition adhered to the wall of the flasks, and the cells gradually turned yellow. By the end of the experiment, the biomass densities of *C. vulgaris* were similar (298.00 mg·L⁻¹ and 302.59 mg·L⁻¹) in the pretreated pig urine with the addition of MgSO₄·7H₂O (0.1 g·L⁻¹ and 1.0 g·L⁻¹), which were significantly higher than the treatment without MgSO₄·7H₂O addition ($P < 0.05$). The supplement of Mg in the Mg deficient pig urine (0.62 mg·L⁻¹) enhanced the growth of *C. vulgaris*; these results agree with the findings of Tuantet et al. [22]. Mg is essential for microalgal photosynthesis since it is the fundamental element for chlorophyll synthesis [12]. However, Mg in pig urine in this study was insufficient to sustain

satisfactory phototrophic growth. Excessive concentration of Mg might cause flocculation of *C. vulgaris* and high cost [10], but a $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ dosage of $0.1 \text{ g}\cdot\text{L}^{-1}$ was found to be optimal for *C. vulgaris* growth in pretreated pig urine.

3.2. Growth of *C. vulgaris* in the Reactor

To explore the growth performance of *C. vulgaris* in LRP-enhanced raceway pond under outdoor conditions, the whole system was put outdoors, and sunlight was applied for the microalgae growth with no temperature control. As shown in Figure 3a, *C. vulgaris* could grow rapidly in LRP-enhanced raceway pond under outdoor conditions with only a short lag phase (1 day). The biomass density continuously increased and the maximum value of OD_{680} reached $199.78 \text{ mg}\cdot\text{L}^{-1}$ at day 10 and the algal biomass productivity was $0.89 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$. F_v/F_m and Φ_{PSII} are the maximum quantum yield of PSII and the actual quantum yield of PSII, respectively. They are important parameters of microalgal photosynthesis and are commonly used to evaluate photosynthetic performance under stress [23]. It can be seen from Figure 3b that both F_v/F_m and Φ_{PSII} were first decreased and then gradually increased toward stability. After two days of cultivation, the values of F_v/F_m and Φ_{PSII} reached 0.70 and 0.50, respectively, which were significantly higher than the initial values ($P < 0.05$). The adaption (lag phase, Figure 3a) of *C. vulgaris* from indoor to outdoor conditions might contribute to the decrease of both F_v/F_m and Φ_{PSII} on day 1. After adaption, the growth of *C. vulgaris* moved into the exponential phase due to the sufficient light and nutrients supplement and the suitable temperature. The values of F_v/F_m and Φ_{PSII} only varied with the temperature and light intensity. During the experiment, the temperature and light intensity ranged from 27°C to 33°C and 700 Lux to 5000 Lux during the day, respectively. Their mean values were 30°C and 2444 Lux (Figure 3c), respectively, which were suitable conditions for the enhanced growth of *C. vulgaris* [14]. Moreover, efficient photosynthesis in this system would be another reason for the rapid growth of *C. vulgaris*. By enhancing the raceway pond with LRP, algal cells could be alternately pumped into the plate to receive additional light for photosynthesis. Thus, the LRP increased the total irradiated area of the whole system, leading more cells to be illuminated.

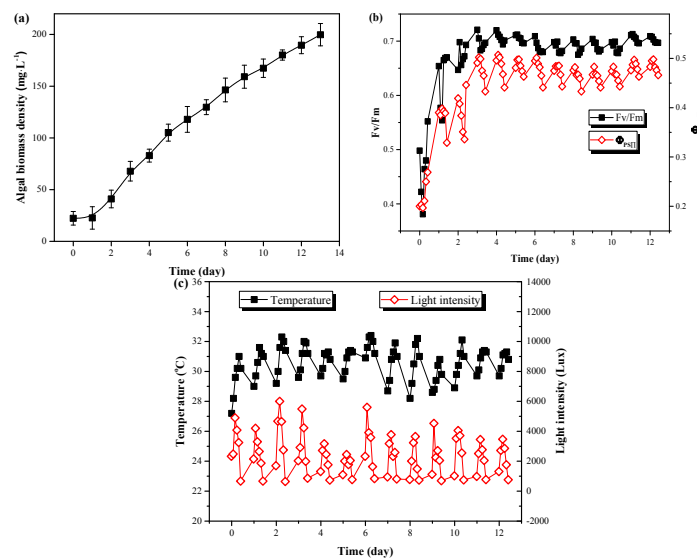


Figure 3. The changes of microalgal biomass density (a) and chlorophyll fluorescence parameters (b) as well as environmental parameters (c) during outdoor cultivation of *C. vulgaris* in LRP-enhanced raceway pond.

3.3. Pig Urine Treatment by LRP-Enhanced Raceway Pond in Batch Mode

The pretreated pig urine was treated by *C. vulgaris* in the LRP-enhanced raceway pond under outdoor conditions (same as Section 3.2) to evaluate the wastewater treatment performance of this

system. In the pretreated pig urine (8-fold of dilution, $0.1 \text{ g}\cdot\text{L}^{-1} \text{ MgSO}_4\cdot 7\text{H}_2\text{O}$, $\text{pH}=6.0$), *C. vulgaris* could rapidly accumulate biomass from biomass density of $199.78 \text{ mg}\cdot\text{L}^{-1}$ to $371.35 \text{ mg}\cdot\text{L}^{-1}$ in 5 days (Figure 4a), corresponding to biomass productivity of $1.72 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$. Meanwhile, the biomass both increased during day and night (Figure 4a and Table 2), with the biomass accumulated during day ($1.39 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$) significantly larger than during night ($0.33 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$) ($P < 0.05$). Compared to the results of Section 3.2, *C. vulgaris* accumulated more biomass in a short time in fresh pig urine medium than in BG11 medium. This is possibly due to the following reasons: (1) the nitrogen source in the pretreated pig urine (acidized) was mainly $\text{NH}_4^+\text{-N}$ which is the preferred form by *C. vulgaris* when compared to $\text{NO}_3^-\text{-N}$ in BG11, because $\text{NH}_4^+\text{-N}$ is already in reduced form and it can be used directly for the synthesis of proteins with less energy consumed [14,24]; (2) The organics (TOC, $2146 \text{ mg}\cdot\text{L}^{-1}$) in pig urine might have induced the mixotrophic/heterotrophic growth of *C. vulgaris* during day/night [15]. Pig urine contains certain amounts of simple organics like amino acids, which could be directly utilized by *C. vulgaris*. This might also be the reason why *C. vulgaris* accumulated biomass during the night. (3) Lower inoculation (199.78 vs. 22.32) of the BG11 medium might be another reason for its lower biomass productivity since lower cell density commonly led to photo-inhibition due to the weak shadowing effect between cells [25]. (4) Bacteria might also contribute to the robust growth of *C. vulgaris* in the un-sterilized pig urine [26]. The changes of chlorophyll fluorescence parameters during the treatment of the pretreated pig urine are shown in Figure 4b. As can be seen from Figure 4b, F_v/F_m did not decrease with the addition of pig urine, and Φ_{PSII} increased first and then stabilized at 0.6. Both of F_v/F_m and Φ_{PSII} were higher in the pig urine than in the BG11 medium (Section 3.2). Thus, no negative effect of the pretreated pig urine on *C. vulgaris* photosynthesis existed. Furthermore, the pretreated pig urine could be used as a good medium to enhance the growth of *C. vulgaris* when compared to BG11 medium.

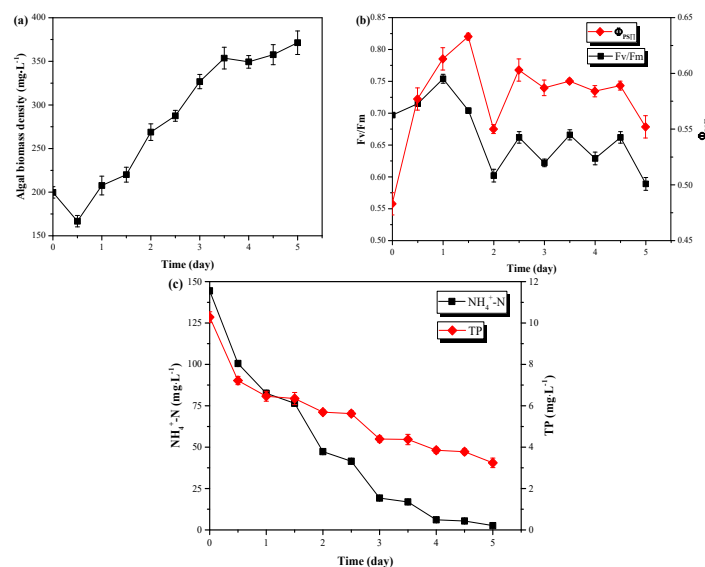


Figure 4. The changes of *C. vulgaris* biomass density (a) and chlorophyll fluorescence parameters (b) as well as nutrients concentrations (c) during outdoor treatment of pretreated pig urine in LRP-enhanced raceway pond.

Microalgae can utilize N, P and other elements in the wastewater to form cells through photosynthesis. It has been reported that during the growth of *C. vulgaris*, phosphorus (P) is used to synthesize nucleic acids, phospholipids, ATP, lipids, proteins, carbohydrates, etc. [16], while nitrogen is used to synthesize various biological substances such as peptides, proteins, enzymes, chlorophyll, energy transfer molecules, genetic materials, etc. [15,27]. Figure 4c shows the changes of $\text{NH}_4^+\text{-N}$ and TP in the pretreated pig urine. *C. vulgaris* could sharply decrease the $\text{NH}_4^+\text{-N}$ and TP concentrations from $100.5 \text{ mg}\cdot\text{L}^{-1}$ to $2.6 \text{ mg}\cdot\text{L}^{-1}$ and from $7.22 \text{ mg}\cdot\text{L}^{-1}$ to $3.24 \text{ mg}\cdot\text{L}^{-1}$ within 5 days, respectively.

These results effectively met the discharge standard of these pollutants for piggery wastewater (GB18596-2001, $\text{NH}_4^+\text{-N} \leq 80 \text{ mg}\cdot\text{L}^{-1}$, $\text{TP} \leq 8 \text{ mg}\cdot\text{L}^{-1}$), given by Ministry of environmental protection of People's Republic of China. Furthermore, most of this removal occurred during the day, but not at night (excluding the removal in day 1) (Figure 4c and Table 2). The removal efficiencies of $\text{NH}_4^+\text{-N}$ and TP by *C. vulgaris* were 98.20% and 68.48%, respectively. Possibly the unusable phosphorus in organic forms resulted in this low TP removal efficiency since *C. vulgaris* could not utilize them for growth [28]. The removal rates of $\text{NH}_4^+\text{-N}$ and TP in this system was $1418.40 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ and $70.40 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$, respectively, which were significantly higher than the results of a vertical-algae-biofilm reactor (N: $130 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$, P: $23 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$) [29] and a pilot scale revolving algae biofilm reactor (N: $< 800 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$, P: $30 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$) [30]. The results from this study might be due to the efficient photosynthesis of LRP enhanced system.

Table 2. Pig urine treatment results in batch mode.

Parameter	Culture Period		
	Total	Day ^a	Night ^b
Algal biomass production ($\text{g}\cdot\text{m}^{-2}$)	8.58	6.92	1.65
Algal biomass productivity ($\text{g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$)	1.72	1.39	0.33
Nutrient removal efficiency (%)	$\text{NH}_4^+\text{-N}$	98.20	57.53
	TP	68.48	36.09
Nutrient removal rate ($\text{mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$)	$\text{NH}_4^+\text{-N}$	1418.40	816.04
	TP	70.40	25.41

^a 8:00-20:00. ^b 20:00-8:00.

3.4. Pig Urine Treatment by LRP-Enhanced Raceway Pond in Continuous Mode

To further evaluate the applicability and feasibility of pig urine treatment by LRP-enhanced raceway pond, the pretreated pig urine (8-fold dilution, $0.1 \text{ g}\cdot\text{L}^{-1} \text{ MgSO}_4\cdot 7\text{H}_2\text{O}$, $\text{pH}=6.0$) was treated by *C. vulgaris* under outdoor condition (same as Section 3.2) with different hydraulic retention times (HRT: 9d, 7d, 5d, 3d). Figure 5a shows the trend of *C. vulgaris* biomass density with different HRT. It can be seen from the figure that the density of *C. vulgaris* rapidly increased with a HRT of 9 days and 7 days, with the value increasing from $236.78 \text{ mg}\cdot\text{L}^{-1}$ to $262.31 \text{ mg}\cdot\text{L}^{-1}$ and from $262.31 \text{ mg}\cdot\text{L}^{-1}$ to $302.91 \text{ mg}\cdot\text{L}^{-1}$, respectively. However, when the HRT was adjusted to 5 days, the density of *C. vulgaris* decreased, from $302.91 \text{ mg}\cdot\text{L}^{-1}$ to $257.40 \text{ mg}\cdot\text{L}^{-1}$ after 5 days operation. As the HRT was further shortened, the density of *C. vulgaris* decreased sharply. When the HRT was adjusted to 3 days, the biomass density decreased from $257.40 \text{ mg}\cdot\text{L}^{-1}$ to $167.36 \text{ mg}\cdot\text{L}^{-1}$. The microalgal biomass productivity under HRT of 9d, 7d, 5d and 3d was $1.56 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$, $2.39 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$, $2.32 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ and $2.78 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$, respectively. Clearly, biomass productivity increased with the increase of HRT. The obtained biomass productivity was slightly higher than a Twin-Layer photobioreactor ($1.3 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$) [31] and a revolving algae biofilm reactor ($0.3 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ – $1.0 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$) [30] but significantly lower than other systems, e.g., $6.30 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ [31] and $19.01 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ – $27.18 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ [14].

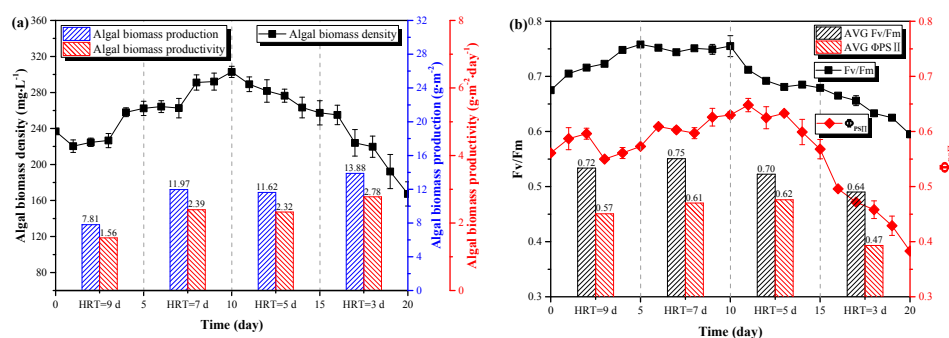


Figure 5. Cont.

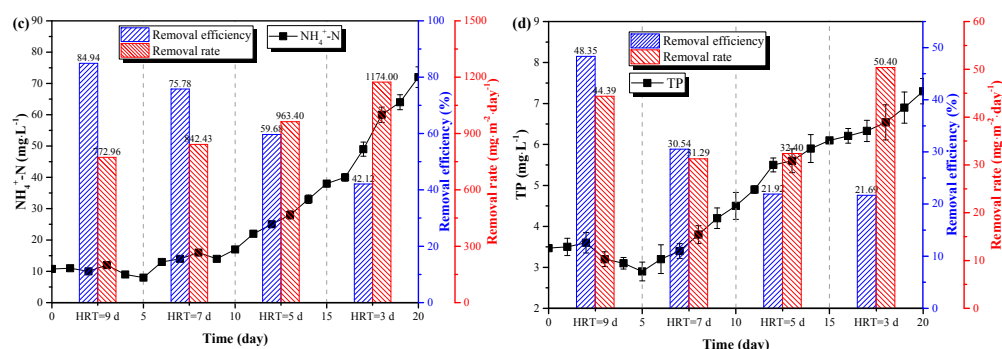


Figure 5. The results of pretreated pig urine treatment under different HRT. (a) The variations of *C. vulgaris* biomass density and production. (b) the variations of *C. vulgaris* chlorophyll fluorescence parameters. The variations of $\text{NH}_4^+\text{-N}$ (c) and TP (d) concentrations, removal efficiencies and removal rates.

Figure 5b shows the changes in chlorophyll fluorescence parameters. As the HRT shortened, the F_v/F_m and Φ_{PSII} values gradually decreased, and the photosynthesis of *C. vulgaris* was inhibited. When HRT was adjusted to 9 days and 7 days, the average values of F_v/F_m and Φ_{PSII} were relatively stable at 0.72 and 0.57, respectively. This was consistent with the increase of biomass density in Figure 5a. However, with shorter HRTs of 5 days and 3 days, the F_v/F_m and Φ_{PSII} decreased significantly and photosynthesis of *C. vulgaris* was inhibited even though its productivity was increased, which possibly contributed to the decrease of biomass density in Figure 5a.

Figure 5c,d show the changes of $\text{NH}_4^+\text{-N}$ and TP in the reactor under different HRT. It can be seen from the figures that $\text{NH}_4^+\text{-N}$ and TP contents in the wastewater were low when the HRT was adjusted to 9d and 7d, e.g., $8 \text{ mg}\cdot\text{L}^{-1}$ to $17 \text{ mg}\cdot\text{L}^{-1}$ and $2.9 \text{ mg}\cdot\text{L}^{-1}$ to $4.5 \text{ mg}\cdot\text{L}^{-1}$, respectively. The corresponding $\text{NH}_4^+\text{-N}$ and TP removal rates were 84.94% ($772.96 \text{ mg}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) and 48.35% ($44.39 \text{ mg}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) for HRT=9d, and 75.78% ($842.43 \text{ mg}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) and 30.54% ($31.29 \text{ mg}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) for HRT=7d, respectively. When the HRT was adjusted to 5d and 3d, the contents of $\text{NH}_4^+\text{-N}$ and TP in the medium increased rapidly, and the purification effect of *C. vulgaris* on the wastewater decreased. The corresponding $\text{NH}_4^+\text{-N}$ and TP removal rates were 59.68% ($963.40 \text{ mg}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) and 21.92% ($32.40 \text{ mg}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) for HRT=5d, and 42.12% ($1174.00 \text{ mg}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) and 21.69% ($50.40 \text{ mg}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) for HRT=3d, respectively. As the HRT decreased, the nutrients removal efficiencies also decreased but the nutrient removal rates increased. However, during the four HRTs, the effluent $\text{NH}_4^+\text{-N}$ and TP levels could always effectively meet the discharge standard of pollutants for piggery wastewater (GB18596-2001).

The shorter HRT (5 days and 3 days) would lead to the large dilution rate of the medium, under which the growth rate of the cells was lower than the dilution rate. Thus, the biomass density decreased. Meanwhile, the decrease of the HRT could lead to the increased nutrient loading rate of the system. Furthermore, increased nutrient loading rates could further enhance the nutrients uptake of microalgal cell [15,32], which might contribute to the increased nutrients removal rates of the system (Figure 5c,d). However, increased nutrient loading rate also increased the nutrients concentrations in the system, leading to a higher salinity as well as inhibitor ($\text{NH}_4^+\text{-N}$, NH_3 , etc.) contents, which further increased to inhibit effects of pig urine on *C. vulgaris*. This might also contribute to the decrease of both biomass density and chlorophyll fluorescence parameters of *C. vulgaris* when the HRT is 5 days and 3 days. In addition, decreased biomass density might also lead to the enhanced light irradiation, which might cause light inhibition and further decrease chlorophyll fluorescence parameters of *C. vulgaris* under HRT of 5 days and 3 days. This could be effectively confirmed by the results in Figure 2c, where the biomass density significantly decreased as the light intensity increased from 2500 Lux to 3500 Lux. Therefore, HRT for 7 days to 9 days would be optimal for efficient nutrient removal and microalgal biomass production based on the above discussion.

In this study, the LRP-enhanced raceway pond was designed and used for the treatment of pig urine for the first time, and high nutrient removal efficiency was obtained with this system under energy saving operating conditions (Section 2.5.1). Furthermore, the obtained results fully indicated the excellent scale-up potential of this technology. It is a cost-effective way by using LRP to enhance the raceway ponds for pig urine treatment and microalgal biomass production. The capital cost of the system could be largely reduced compared to other new technologies, because the current, existed commercial raceway ponds could be sufficiently upgraded and better utilized [15]. Meanwhile, the system biomass productivity could be significantly improved by increasing the pond depth. Furthermore, the treated wastewater could also be utilized for the dilution of the fresh pig urine to save freshwater resources. The produced microalgal biomass could also be utilized for the production of feed, fertilizer, or biodiesel, due to its rich contents in proteins, lipids and carbohydrates [7]. However, further studies should be conducted, regarding the pilot scale operation of the LRP-enhanced raceway pond and the corresponding life cycle assessment, chemical composition analysis of the produced microalgal biomass, and nutrient removal of other pollutants (TOC, COD, Cu^{2+} , Zn^{2+} , etc.) prior to the scale-up of this technology.

4. Conclusions

C. vulgaris could grow very well in pretreated fresh pig urine (at 8-fold dilution, pH=6, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ dosage of $0.1 \text{ mg} \cdot \text{L}^{-1}$). The LRP-enhanced raceway pond has been shown to support the robust growth of *C. vulgaris*. Furthermore, the pretreated pig urine could be efficiently polished by *C. vulgaris* in this system in both batch and continuous mode, under outdoor conditions. About $1.72 \text{ g} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ of microalgal biomass could be produced, and 98.20% of NH_4^+ -N and 68.48% of TP could be removed during the batch mode, respectively. A hydraulic retention time of 7 days to 9 days would be optimal for efficient nutrient removal and microalgal biomass production during continuous mode.

Author Contributions: G.Z. designed the experiments, conducted the study, performed the statistics analysis and drafted the manuscript. T.Z., S.X., Z.G., Q.H., H.Y. and M.L. participated in the pig urine treatment and help to revise the manuscript. H.Z., Q.Z., X.W., Y.W., R.R. and Y.L. participated in the design of the study and helped to draft the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (No. 21466022, 21706087, 21766019, 21878139); The “Post-doctoral Innovative Talents Support Program” (BX20190147); The Funding for postdoctoral research projects in Jiangxi Province (2019KY04); The Key Project of Jiangxi Provincial Department of Science and Technology (20161BBF60057, 20181BBF60026).

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

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