Mixed Wastewater Coupled with CO₂ for Microalgae Culturing and Nutrient Removal

Lili Yao¹,², Jianye Shi¹,², Xiaoling Miao¹,²*

¹ State Key Laboratory of Microbial Metabolism and School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China, ² Biomass Energy Research Center, Shanghai Jiao Tong University, Shanghai 200240, China

* miaoxiaoling@sjtu.edu.cn

Abstract

Biomass, nutrient removal capacity, lipid productivity and morphological changes of Chlorella sorokiniana and Desmodesmus communis were investigated in mixed wastewaters with different CO₂ concentrations. Under optimal condition, which was 1:3 ratio of swine wastewater to second treated municipal wastewater with 5% CO₂, the maximum biomass concentrations were 1.22 g L⁻¹ and 0.84 g L⁻¹ for C. sorokiniana and D. communis, respectively. Almost all of the ammonia and phosphorus were removed, the removal rates of total nitrogen were 88.05% for C. sorokiniana and 83.18% for D. communis. Lipid content reached 17.04% for C. sorokiniana and 20.37% for D. communis after 10 days culture. CO₂ aeration increased intracellular particle numbers of both microalgae and made D. communis tend to be solitary. The research suggested the aeration of CO₂ improve the tolerance of microalgae to high concentration of NH₄-N, and nutrient excess stress could induce lipid accumulation of microalgae.

Introduction

Concerns about the depletion of petroleum resources reserves [1] coupled with the rise of the global energy demand, and an increasing awareness of the environmental impact of associated CO₂ emissions, have made the development of renewable and environmentally friendly energy sources necessary [2]. In this sense, biodiesel which has properties similar to fossil-fuels production from photosynthetic microorganisms has been recognized as reliable and renewable energy sources for the steady supply of energy. Many studies have demonstrated that microalgae were superior to other raw materials for the production of biodiesel [3–4]. However, the development of microalgae-based biodiesel still faces many challenges. One of the most critical challenges is to establish economical means of supplying water and nutrients for cultivation since microalgae require a huge volume of medium for mass scale growth, which leading to major operating costs associated with the nutritional supply [5]. Meanwhile, the reclamation of wastewater is of pivotal importance to achieving sustainability in our society at the global level. Fortunately, in addition to their high biomass and lipid productivities, some microalgae strains also have potential environmental benefits, such as mitigation of CO₂ through photosynthesis [6] and bioremediating.
wastewater by removing large amounts of nutrients and heavy metals [7]. Therefore, an algae-based wastewater and CO2 treatment system may be the key to solve both problems.

Swine wastewater (SW) often contained high concentrations of nitrogen and phosphorus, and it needed dilution with fresh water before use to achieve a high yield of biomass [5, 8] as well as to increase the transmission of light in microalgal cultures. Meanwhile, secondary treated municipal wastewater (STMW) supported the microalgal growth and lipid production, but the low concentrations of nutritional constituents result in a low biomass yield [9]. Hence, swine wastewater and municipal wastewater mixture might have great potential to provide good substrates for microalgal growth and get a high yield of lipid without the need for dilution with fresh water or nutrients supplementation. Some microalgae also show better growth potential under high CO2 concentrations [10–11] and have potential of mitigating flue gas CO2 through photosynthesis [6]. Thus, coupling mixed wastewater with CO2 might be an efficient mode for microalgae to produce biodiesel feedstock along with wastewater and CO2 treatment.

In this study, different culture systems with a series of wastewater mixed from SW and STMW, together with different CO2 concentrations were established. The biomass production, nutrient removal capacity, lipid yield and morphological change of two selected microalgal strains Chlorella sorokiniana and Desmodesmus communis in these different culture systems were investigated. The better culture conditions were proposed.

**Materials and Methods**

**Pretreatment and preparation of different wastewater media**

To collect the SW and STMW, we obtained permission from a key piggery, Minhang Breeding Stock Farm (Shanghai, China), and the Minhang Municipal Wastewater Treatment Plant.

SW was generated during the barn flush operations and then was passed through a bar screen and an inclined screen to achieve preliminary solid-liquid separation. After these processes, the wastewater was discharged to a primary sedimentation pond to precipitate the remaining solids, before slowly flowing into the storage pond from which SW was obtained. STMW used throughout the experiments was collected from the secondary treatment pond. Both SW and STMW samples were immediately filtered using microfilters (GB/T1914-93) to remove suspended particles after sampling and then were stored in a refrigerator at -20°C to avoid variation in the wastewater composition.

The different wastewater media were prepared using different proportions of two types of wastewater. The different proportions (v:v) of SW to STMW were as follows: SW 4:0 STMW (4:0); SW 3:1 STMW (3:1); SW 2:2 STMW (2:2); SW 1:3 STMW (1:3); an SW 0:4 STMW (0:4). The media were characterized in terms of ammonium nitrogen (NH4+–N), nitrate (NO3–N), total nitrogen (TN), total phosphorus (TP) and pH, and the results are shown in Table 1. For ease of Compared with the modified BG-11 medium [12], we considered media 4:0 and 3:1 as media containing high levels of nitrogen and phosphorus. Correspondingly, media 2:2 and 1:3 were considered to have intermediate levels of nitrogen and phosphorus, and medium 0:4 was considered to have low levels of nitrogen and phosphorus.

**Microalgae cultivation**

The two microalgae Chlorella sorokiniana and Desmodesmus communis were screened from acid swege and selected based on the tolerance and performance under high concentration of CO2 by the Biomass Energy Research Center of Shanghai Jiao Tong University, China. These two strains could achieve relatively high biomass concentration when cultivated in wastewater medium with high level of CO2, and have potential to form the wastewater and CO2 treatment system. They were preserved in the modified BG-11 medium containing (g L−1) NaNO3, 1.5;
K₂HPO₄, 0.03; MgSO₄·7H₂O, 0.075; CaCl₂·2H₂O, 0.036; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA, 0.001; Na₂CO₃, 0.020 and 1 mL of micronutrient solution containing (g L⁻¹) H₃BO₃, 2.86; MnCl₂·4H₂O, 1.81; ZnSO₄·7H₂O, 0.222; NaMoO₄·5H₂O, 0.390; CuSO₄·5H₂O, 0.0790; Co(NO₃)₂·6H₂O, 0.0494 [12]. The two strains were individually cultured in 250 mL Erlenmeyer flasks containing 120 mL sterilized modified BG-11 before inoculation to formulated wastewater media.

In different cultivation experiments, C. sorokiniana and D. communis were cultivated in 1 L Erlenmeyer flask (20 cm length, 10 cm diameter) with 600 mL working volume of different wastewater media at 28±2°C under 126 μmol m⁻² s⁻¹ light intensity on a light/dark cycle of 12 h/12 h for 10 days. The light intensity was measured by a light meter. A gas distributor provided with different flow rates of CO₂ mixed with ambient air was used to prepare CO₂ concentrations of 0.03% (air), 5% and 10%. Cultures were aerated continuously with CO₂-enriched air via bubbling from the bottom of modified Erlenmeyer flask with an aeration rate of 0.2 vvm (volume gas per volume media per minute). The control (wastewater without algal inoculums) was conducted under the same conditions.

Morphological analysis

The images of C. sorokiniana and D. communis were observed every other day during the cultivation by an optical microscope (OLYMPUS, CX41, magnification up to 1,000X), and the morphology was documented using a Mshot Digital Imaging System (MC50, Mshot, China).

Cell growth measurement

Biomass concentrations (x, g L⁻¹) were determined directly by dry cell weight. 10 mL sample was taken from culture to measure the dry cell weight daily. Microalgae were harvested by centrifugation (5804R, Eppendorf, Germany) at 8000 rpm for 10 min and washed twice with distilled water. The pellet was lyophilized drying in a freeze drier (FD-1-50, Boyikang, China) for dry weight measurement.

The biomass productivity P (g L⁻¹ d⁻¹) and specific growth rate μ (d⁻¹) were calculated according to the following Eqs (1) and (2):

\[ P = \frac{(X_t - X_0)}{(t_1 - t_0)} \]
\[ \mu = \frac{\ln \left( \frac{X_t}{X_0} \right)}{(t_1 - t_0)} \]

where \( X_t \) and \( X_0 \) were the dry cell weight concentration (g L⁻¹) at time \( t_1 \) and \( t_0 \), respectively.

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**Table 1. Characters of total nitrogen (TN), ammonium nitrogen (NH₄⁺-N), nitrate (NO₃⁻-N), total phosphorus (TP) and pH in BG-11 and different wastewater media.**

| Medium(SW: STMW) | TN(mg L⁻¹) | NH₄⁺-N(mg L⁻¹) | NO₃⁻-N(mg L⁻¹) | TP(mg L⁻¹) | pH  |
|------------------|------------|----------------|----------------|------------|-----|
| BG-11            | 247.059    | 0.000          | 247.059        | 5.345      | 8.02|
| 0:4              | 39.851±0.495 | 9.189±0.105 | 21.042±0.059 | 0.424±0.002 | 7.39|
| 1:3              | 188.611±1.003 | 104.392±0.860 | 61.026±0.919 | 15.768±0.204 | 8.04|
| 2:2              | 337.320±4.869 | 199.963±0.360 | 104.905±1.503 | 30.877±0.034 | 8.12|
| 3:1              | 477.179±8.283 | 293.032±1.942 | 146.531±1.771 | 46.094±0.007 | 8.16|
| 4:0              | 632.986±2.643 | 387.226±2.662 | 188.496±3.163 | 61.534±0.568 | 8.16|

SW: swine wastewater, STMW: secondary treated municipal wastewater.

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Lipid extraction and quantification

The chloroform/methanol method was used for total lipid content measurement [13]. Dry microalgal cells (0.2 g) added with 6 mL distilled water and ultrasonicated by a sonicator (JY, 92-II, China) for 8 min, then mixed with solvent of chloroform:methanol (2:1, V/V) and left overnight, the cell debris were removed by centrifugation at 8000 rpm for 10 min, the chloroform layer was extracted and transferred to a new screw-cap tube. Chloroform was added again to give a constant solvent ratio, and the residual cell debris were extracted three times by above solvent extraction procedure to ensure that lipids were almost extracted. The chloroform layer in the new screw-cap tube was washed with the same volume of 0.1% NaCl solution to wash out soluble impurities and the purified chloroform layer was evaporated to a constant weight in a fuming hood under vacuum at 60°C. The total lipid content (Lc, % of biomass dry weight) was calculated using the following equation:

\[ L_c = \frac{(m_2 - m_0)}{m_1} \times 100\% \]  

where \( m_1 \) was the weight of the dry microalgal cells, \( m_0 \) was the weight of the empty new screw-cap tube, \( m_2 \) was the weight of the new screw-cap tube with the dried lipids.

Nitrate and phosphate concentration analysis

The nutrients (\( \text{NH}_4^+\text{-N} \), TN and TP) uptake rate was measured every other day during the cultivation. A 10 mL liquid culture sample was centrifuged at 8000 rpm for 10 min, and the supernatant was filtered through a 0.45 μm syringe filter. Total nitrogen (TN) and ammonium nitrogen (\( \text{NH}_4^+\text{-N} \)) were measured using an automatic chemistry analyzer (Smartchem 200, Alliance, France), total phosphorus (TP) in the medium was measured using acid potassium persulfate digestion by molybdenum antimony-colorimetric method [14].

Results and Discussion

Growth of microalgae in different wastewater media and the CO\(_2\) concentration

As previous studies have reported, the concentrations of nitrogen and phosphorus from media significantly affected microalgae growth [15–17]. The growth of \( C. \) sorokiniana and \( D. \) communis cultured in different mixing ratios of wastewater under different CO\(_2\) concentrations are shown in Fig 1. Under 0.03% CO\(_2\) (without extra CO\(_2\) aeration), \( C. \) sorokiniana showed better growth in modified BG-11 and medium 0:4 (Fig 1a). The maximum biomass concentrations were 0.57 g L\(^{-1}\) and 0.31 g L\(^{-1}\) in BG-11 and medium 0:4 after 10 days cultivation, respectively (Table 2). The growth of \( C. \) sorokiniana in media 1:3, 2:2, 3:1 and 4:0 were nearly inhibited under 0.03% CO\(_2\) (Fig 1a, Table 2). Ruangsomboon reported, within the range of 22 to 444 mg L\(^{-1}\), that the increase in the phosphorus concentration was beneficial to microalgal growth [16]. Because the concentration of phosphorus was within this range (Table 1), we inferred that \( \text{NH}_4^+\text{-N} \) might be the main factor inhibiting the growth of \( C. \) sorokiniana. It is well known that ammonia nitrogen above a particular concentration, which is microalgal species and culture pH dependent, would inhibit microalgal growth and reduce the utilization of wastewaters [18–19]. The main mechanism by which ammonia inhibits microalgae is by poisoning their photosynthetic system [20–21].

The growth of \( C. \) sorokiniana in media containing SW was significantly increased when extra CO\(_2\) was aerated (Fig 1b and 1c). Under 5% CO\(_2\), \( C. \) sorokiniana achieved the highest maximum biomass concentration (1.31 g L\(^{-1}\)) and maximum biomass productivity (0.247 g L\(^{-1}\) d\(^{-1}\)) in medium 2:2, followed by 1.22 g L\(^{-1}\) and 0.193 g L\(^{-1}\) d\(^{-1}\) in medium 1:3 (Table 2).
Fig 1. Biomass concentrations of *Chlorella sorokiniana* (a,b,c) and *Desmodesmus communis* (d,e,f) in different media under 0.03%, 5% and 10% CO₂ concentrations, respectively. Media without microalgae inoculation was marked as Blank. Error bars represent ± SD of three replicates.

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Table 2. The maximum biomass concentration ($X_{max}$), maximum biomass productivity ($P_{max}$) and maximum specific growth rate ($\mu_{max}$) of Chlorella sorokiniana and Desmodesmus communis cultivated in different media under 0.03%, 5% and 10% CO2 concentrations, respectively.

| Medium(SW: STMW) | 0.03% CO2 | 5% CO2 | 10% CO2 |
|------------------|-----------|--------|--------|
|                  | $X_{max}$ (g L$^{-1}$) | $P_{max}$ (g L$^{-1}$ d$^{-1}$) | $\mu_{max}$ (d$^{-1}$) | $X_{max}$ (g L$^{-1}$) | $P_{max}$ (g L$^{-1}$ d$^{-1}$) | $\mu_{max}$ (d$^{-1}$) | $X_{max}$ (g L$^{-1}$) | $P_{max}$ (g L$^{-1}$ d$^{-1}$) | $\mu_{max}$ (d$^{-1}$) |
| C. sorokiniana   |           |        |        |           |        |        |           |        |        |
| BG-11            | 0.57 ± 0.01 | 0.99 ± 0.02 | 0.465 ± 0.012 | 0.86 ± 0.07 | 0.136 ± 0.004 | 0.425 ± 0.064 | 0.34 ± 0.04 | 0.079 ± 0.001 | 0.519 ± 0.002 |
| 0:4              | 0.31 ± 0.05 | 0.088 ± 0.016 | 0.663 ± 0.016 | 0.53 ± 0.01 | 0.116 ± 0.013 | 0.637 ± 0.067 | 0.54 ± 0.01 | 0.133 ± 0.008 | 1.305 ± 0.016 |
| 1:3              | 0.57 ± 0.00 | 0.044 ± 0.006 | 0.402 ± 0.019 | 1.22 ± 0.05 | 0.193 ± 0.023 | 0.519 ± 0.017 | 0.98 ± 0.07 | 0.211 ± 0.010 | 0.554 ± 0.003 |
| 2:2              | 0.13 ± 0.00 | 0.037 ± 0.011 | 0.400 ± 0.050 | 1.31 ± 0.02 | 0.247 ± 0.005 | 0.511 ± 0.021 | 1.16 ± 0.05 | 0.271 ± 0.020 | 0.586 ± 0.006 |
| 3:1              | 0.26 ± 0.03 | 0.050 ± 0.001 | 0.273 ± 0.029 | 0.99 ± 0.06 | 0.193 ± 0.008 | 0.495 ± 0.005 | 1.02 ± 0.00 | 0.168 ± 0.003 | 0.514 ± 0.015 |
| 4:0              | 0.07 ± 0.01 | 0.020 ± 0.002 | 0.099 ± 0.002 | 0.30 ± 0.01 | 0.080 ± 0.001 | 0.397 ± 0.017 | 0.71 ± 0.03 | 0.109 ± 0.000 | 0.434 ± 0.012 |
| D. communis      |           |        |        |           |        |        |           |        |        |
| BG-11            | 0.64 ± 0.01 | 0.071 ± 0.007 | 0.208 ± 0.003 | 0.65 ± 0.07 | 0.155 ± 0.002 | 1.779 ± 0.002 | 0.40 ± 0.05 | 0.181 ± 0.001 | 0.163 ± 0.000 |
| 0:4              | 0.80 ± 0.05 | 0.172 ± 0.025 | 1.514 ± 0.038 | 0.83 ± 0.06 | 0.205 ± 0.012 | 0.408 ± 0.051 | 0.61 ± 0.01 | 0.128 ± 0.008 | 1.110 ± 0.035 |
| 1:3              | 0.72 ± 0.01 | 0.113 ± 0.006 | 0.577 ± 0.057 | 0.84 ± 0.05 | 0.174 ± 0.032 | 0.629 ± 0.053 | 1.02 ± 0.07 | 0.163 ± 0.021 | 0.724 ± 0.026 |
| 2:2              | 0.73 ± 0.01 | 0.120 ± 0.006 | 0.636 ± 0.058 | 0.80 ± 0.03 | 0.148 ± 0.005 | 0.554 ± 0.009 | 0.91 ± 0.01 | 0.151 ± 0.022 | 0.818 ± 0.024 |
| 3:1              | 0.39 ± 0.02 | 0.078 ± 0.005 | 0.573 ± 0.015 | 0.52 ± 0.02 | 0.078 ± 0.013 | 0.655 ± 0.006 | 0.74 ± 0.05 | 0.117 ± 0.014 | 0.628 ± 0.033 |
| 4:0              | 0.31 ± 0.04 | 0.090 ± 0.003 | 0.343 ± 0.004 | 0.39 ± 0.02 | 0.055 ± 0.001 | 0.323 ± 0.035 | 0.54 ± 0.01 | 0.086 ± 0.011 | 0.367 ± 0.001 |

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10% CO2 was aerated, the highest maximum biomass concentration (1.16 g L$^{-1}$) and maximum biomass productivity (0.271 g L$^{-1}$ d$^{-1}$) were also achieved in medium 2:2 (Table 2). Additionally, when cultivated in 1:3 and 2:2 and modified BG-11, C. sorokiniana achieved higher biomass concentration under 5% CO2 than under 10% CO2, a finding that was consistent with previous studies that the growth of microalga would be inhibited if the aerated CO2 was above a particular concentration [11, 21]. However, C. sorokiniana in media 3:1 and 4:0, which contain a relatively high concentration of nitrogen and phosphorus, grew better under 10% CO2 than 5% CO2 (Fig 1b, 1c and Table 2). These results suggested that the aeration of CO2 could improve the tolerance of C. sorokiniana with a high initial concentration of NH$_4^+$-N, and medium with a higher NH$_4^+$-N concentration might prefer a higher concentration of CO2 aeration. The aeration of CO2 probably changed the pH of the medium, leading to the change in the uptake pattern for nutrimental elements [22–23].

Although growing relatively better than C. sorokiniana at 0.03% CO2, D. communis showed a similar trend when extra CO2 was aerated (Fig 1d, 1e and 1f). D. communis obtained the highest maximum biomass concentrations of 0.84 g L$^{-1}$ and 1.02 g L$^{-1}$ in medium 1:3 with 5% and 10% CO2, respectively (Table 2). It was interesting to note that D. communis grew better under 5% CO2 than under 10% CO2 when it was cultivated in medium 0:4 and modified BG-11 (Fig 1e, 1f and Table 2). However, when D. communis was grown in other media, it grew better under 10% CO2 than 5% CO2 (Fig 1d, 1e, 1f and Table 2). These results suggested that the optimal CO2 concentration for microalgal growth was medium and strain dependent.
Finding the proper wastewater composition coupled with the optimal CO₂ concentration is a feasible way to promote the production of microalgal biomass.

**Nutrient removal during cultivation**

Microalgaebased nutrient removal in wastewater is a much accepted concept worldwide. Nutrient-rich wastewater has been considered to be more appropriate for microalgal growth because it enables an increment in biomass concentration along with nutrient removal [24]. As shown in Table 1, medium 4:0 (SW:STMW) contained the highest levels of TN, NH₄⁺-N and TP, while medium 0:4 (SW:STMW) had the lowest content of these nutrients. The analysis of NH₄⁺-N showed that nearly all ammonia in the different media was removed by *C. sorokiniana* and *D. communis* under 0.03% CO₂ (Fig 2a, 2b and Table 3). A similar phenomenon was also found by other scientists [22–23, 25–26]. The removal of ammonia was not only due to its uptake by *C. sorokiniana* and *D. communis* but also due to stripping and loss to the atmosphere. It has been noted that ammonia stripping and loss to the atmosphere may be the most important mechanisms of ammonia removal when microalgae or cyanobacteria are used for nutrient removal from wastewater [27]. Previous researchers have found that when media contained a high initial concentration of ammonia, the intensified growth inhibition would cause a decrease in ammonia uptake; consequently, ammonia was more susceptible to be stripped and lost, particularly in alkaline medium [26]. In the present study, a net increase in pH values without extra CO₂ aeration was observed (Fig 3a and 3b), increasing the removal rate of ammonia.

When extra CO₂ was aerated, the removal rate of ammonia showed a decrease in media 2:2, 3:1 and 4:0 (Fig 2c, 2d, 2e, 2f and Table 3). This result was probably due to the dissolution and ionization of CO₂ and acidification of the media. As shown in Fig 3d, 3e, 3f, 3g, 3h and 3i, the pH value of the media decreased with extra CO₂ aeration. A low-pH environment could reduce the stripping and loss of ammonia because it promoted the equilibrium concentration of ammonium and suppressed the generation of free ammonia [26, 28]. Thus, although the removal rate of ammonia was decreased under 5% and 10% CO₂, the ammonia uptake by *C. sorokiniana* and *D. communis* was not necessarily decreased.

Except for the case of *C. sorokiniana* at 0.03% CO₂, the total nitrogen was reduced to half of the original level after two days for both microalgae (Fig 4). In addition, both *C. sorokiniana* and *D. communis* reduced more TN in wastewater medium 1:3 than in other media (Table 3). Thus, 1:3 may be an excellent choice for good nutrient-removal capacity and high biomass productivity. The highest TN removal rates achieved by *C. sorokiniana* and *D. communis* were 88.05% and 88.68%, respectively (Table 3). This result indicated that there were still some organic compounds that could not be assimilated by microalgae, which in consistence with that in a previous report [29].

Phosphorus can be found in lipids, proteins, nucleic acids and the intermediates of carbohydrate metabolism and is also an essential macro-nutrient for microalgal growth. Fig 5 and Table 3 showed the removal of TP from five wastewater media. It should be noted that the removal of phosphorus in wastewater was not only affected by microalgal cell uptake but also by external conditions such as pH and dissolved oxygen. When the pH is elevated close to 10, phosphate will precipitate from wastewater [30], explaining the high phosphorus removal rate under 0.03% CO₂ (Fig 5a and 5b and Table 3). In medium 1:3, *C. sorokiniana* and *D. communis* removed more than 99.5% TP, except for *C. sorokiniana* under 0.03% CO₂ (90.79%). This result showed again that medium 1:3 was suitable for *C. sorokiniana* and *D. communis* to remove nutrients from wastewater.
Fig 2. Time course of ammonium nitrogen (NH$_4^+$-N) evolution for *Chlorella sorokiniana* and *Desmodesmus communis* cultivated in different media under 0.03% (a and b), 5% (c and d) and 10% (e and f) CO$_2$ concentrations, respectively. Media without microalgae inoculation was marked as Blank, Error bars represent ±SD of three replicates.

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Table 3. The removal rate of total nitrogen (TN), ammonium nitrogen (NH4+-N) and total phosphorus (TP) by C. sorokiniana and D. communis cultivated in different media under 0.03%, 5% and 10% CO2 concentrations, respectively. Media without microalgae inoculation was marked as Blank.

| Medium(SW: STMW) | 0.03% CO2 | | 5% CO2 | | 10% CO2 |
|------------------|-----------|-----------|-----------|-----------|
|                  | TN        | NH4+-N    | TP        | TN        | NH4+-N    | TP        | TN        | NH4+-N    | TP        |
| C. sorokiniana   |           |           |           |           |           |           |           |           |           |
| 0:4              | 50.59 ± 3.95 | 100.00 ± 0.00 | 100.00 ± 0.00 | 50.38 ± 1.79 | 93.60 ± 0.57 | 89.99 ± 0.56 | 55.71 ± 5.65 | 97.50 ± 1.57 | 95.00 ± 7.06 |
| 1:3              | 74.34 ± 1.82 | 99.19 ± 0.44 | 90.79 ± 0.81 | 88.05 ± 1.62 | 100.00 ± 0.00 | 99.63 ± 0.22 | 87.22 ± 1.58 | 99.19 ± 0.56 | 99.73 ± 0.00 |
| 2:2              | 74.63 ± 7.72 | 99.74 ± 0.12 | 80.90 ± 0.90 | 63.90 ± 1.24 | 97.51 ± 1.84 | 93.02 ± 0.10 | 75.61 ± 0.37 | 87.20 ± 3.90 | 91.43 ± 0.10 |
| 3:1              | 74.48 ± 0.74 | 99.88 ± 0.13 | 90.80 ± 0.15 | 57.74 ± 3.39 | 90.63 ± 4.25 | 76.15 ± 12.83 | 77.64 ± 0.84 | 73.05 ± 4.36 | 83.35 ± 0.00 |
| 4:0              | 40.43 ± 1.05 | 98.04 ± 2.14 | 90.64 ± 3.32 | 56.34 ± 1.16 | 83.60 ± 4.57 | 75.98 ± 5.86 | 74.45 ± 4.96 | 63.18 ± 3.75 | 76.69 ± 0.91 |
| D. communis      |           |           |           |           |           |           |           |           |           |
| 0:4              | 50.35 ± 4.35 | 100.00 ± 0.00 | 100.00 ± 0.00 | 41.25 ± 0.77 | 97.43 ± 3.36 | 100.00 ± 0.00 | 56.09 ± 3.41 | 96.60 ± 0.37 | 90.00 ± 0.01 |
| 1:3              | 88.68 ± 0.71 | 100.00 ± 0.00 | 100.00 ± 0.00 | 83.18 ± 3.38 | 95.36 ± 5.37 | 100.00 ± 0.00 | 88.02 ± 0.37 | 100.00 ± 0.00 | 99.73 ± 0.00 |
| 2:2              | 78.05 ± 6.22 | 100.00 ± 0.00 | 97.93 ± 0.27 | 58.24 ± 7.82 | 88.39 ± 1.49 | 93.14 ± 0.83 | 78.12 ± 1.99 | 80.27 ± 5.90 | 98.86 ± 0.00 |
| 3:1              | 75.75 ± 0.84 | 99.98 ± 0.08 | 92.53 ± 3.26 | 51.37 ± 1.14 | 78.42 ± 0.68 | 82.30 ± 1.12 | 76.95 ± 0.58 | 67.58 ± 4.07 | 92.88 ± 0.00 |
| 4:0              | 74.46 ± 2.64 | 99.42 ± 0.16 | 89.52 ± 3.03 | 62.04 ± 1.66 | 64.97 ± 8.75 | 81.15 ± 0.46 | 67.34 ± 0.41 | 57.01 ± 4.16 | 82.53 ± 4.16 |
| Blank            |           |           |           |           |           |           |           |           |           |
| 0:4              | 16.96 ± 4.09 | 10.00 ± 1.38 | 2.82±0.03 | 19.47 ± 0.02 | 26.33 ± 6.13 | 8.98±7.32 | 28.25 ± 4.64 | 17.86 ± 5.75 | 8.98 ± 3.39 |
| 1:3              | 11.25 ± 0.97 | 12.99 ± 0.26 | 15.61 ± 4.42 | 19.21 ± 4.68 | 14.89 ± 2.93 | 18.98 ± 0.56 | 19.47 ± 4.30 | 18.13 ± 1.68 | 31.66 ± 2.22 |
| 2:2              | 10.65 ± 0.28 | 12.21 ± 2.37 | 17.79 ± 5.95 | 10.65 ± 0.28 | 15.03 ± 1.62 | 22.64 ± 0.91 | 15.10 ± 1.88 | 18.14 ± 3.63 | 25.88 ± 3.67 |
| 3:1              | 12.62 ± 1.23 | 5.92±1.49 | 8.66±2.16 | 13.67 ± 2.70 | 8.11±1.62 | 16.20 ± 6.85 | 11.68 ± 2.29 | 14.10 ± 0.33 | 19.45 ± 0.08 |
| 4:0              | 12.90 ± 2.87 | 9.32±0.40 | 9.11±0.88 | 10.53 ± 0.49 | 11.77 ± 3.04 | 13.98 ± 0.92 | 10.69 ± 0.26 | 14.05 ± 4.02 | 19.67 ± 3.53 |
efficiently packed in cells and used under stressed conditions for cell survival [31]. When microalgae were cultivated under nutrient-limiting conditions, the photosynthetic carbon flow changes into metabolic pathways that may generate energy-rich compounds, such as lipids [33].

When 5% and 10% of CO₂ were aerated, the lipid contents of *C. sorokiniana* in medium 4:0 reached 25.10% and 15.09%, respectively, values that were higher than those in other media (Table 4). *D. communis* had a similar trend in lipid accumulation to *C. sorokiniana*—the lipid content peaked at 30.33% and 22.75% in medium 4:0 under 5% and 10% CO₂, respectively.

![Fig 3. Time course of pH evolution for *Chlorella sorokiniana*, *Desmodesmus communis* and control (Blank) cultivated in different media under 0.03% (a, b and c), 5% (d, e and f) and 10% (g, h and i) CO₂ concentrations, respectively. Error bars represent ± SD of three replicates.](doi:10.1371/journal.pone.0139117.g003)
Figure 4. Time course of total nitrogen (TN) evolution for *Chlorella sorokiniana* and *Desmodesmus communis* cultivated in different media under 0.03% (a and b), 5% (c and d) and 10% (e and f) CO$_2$ concentrations, respectively. Media without microalgae inoculation was marked as Blank. Error bars represent ±SD of three replicates.

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Fig 5. Time course of total phosphorus (TP) evolution for *Chlorella sorokiniana* and *Desmodesmus scommunis* cultivated in different media under 0.03% (a and b), 5% (c and d) and 10% (e and f) CO2 concentrations, respectively. Media without microalgae inoculation was marked as Blank. Error bars represent ±SD of three replicates.

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These results suggested that nutrient excess could also induce the accumulation of intracellular lipids under one type of environmental stress such as nutrient limitation.

**Morphology change in microalgae during cultivation**

The morphology of microalgal cells was closely related to their culture conditions, indicating that nutrients and gas aeration could significantly affect the form of microalgal cells [15, 34–35]. The morphological features of *C. sorokiniana* and *D. communis* cultivated in different wastewater media and modified BG-11 under an aeration of 0.03% and 5% CO₂ are shown in Fig 6. The morphology of the two microalgae under 10% CO₂ in photographs was similar with 5%, and images are shown in figure in S1 Fig and will not be discussed here. The cells of *C. sorokiniana* cultivated in medium 0:4 generated more intracellular particles than cells in other media after 3 days of culture under 0.03% CO₂ (Fig 6a). When microalgae were cultivated in medium 0:4, a nitrogen limitation condition in this study, photosynthetic carbon flow changes into metabolic mechanisms that may generate energy-rich compounds, such as carbohydrates and lipids [33]. When aerated with CO₂, both 5% and 10%, a significantly increase of intracellular particles number in *C. sorokiniana* cells cultivated in medium 1:3 and BG-11 was observed by an optical microscope after 6 days of cultivation (Fig 6b).

The morphological changes in *D. communis* had a similar tendency to *C. sorokiniana*. In addition, *D. communis* has other features because it is a strain belonging to the Scenedesmaceae family. The cells are displayed as a 4-celled cenobium before inoculation (Fig 6f). However, we can only observed dispersed solitary cells when *D. communis* was cultivated in media 1:3, 2:2, 3:1 and 4:0 on the 6th day under a 0.03% CO₂ concentration (Fig 6c). However, on the 10th day, most cells were grouped into 2- or 4-celled cenobium again and only a small number of cells remained solitary (Fig 6c). This phenomenon was also found in *Scenedesmus* sp. CCNM 1077 [15]. When additional CO₂ was aerated, it appeared earlier, and no 2- or 4-celled cenobium could be found on the 3rd day (Fig 6d). Solitary cells showed a remarkable morphological difference compared with cenobium. They presented with a more regular ellipsoid with a larger size, and the spines became unobtrusive. *D. communis* cells all remained solitary except in

| Medium(SW: STMW) | 0.03% CO₂ | 5% CO₂ | 10% CO₂ |
|------------------|-----------|--------|---------|
|                  | Lc (%)    | Lc (%) | Lc (%)  |
| *C. sorokiniana* | BG-11     |        |         |
| 0:4              | 14.15±2.52| 17.91±0.74| 13.16±0.74|
| 1:3              | 25.15±2.13| 18.08±2.90| 14.94±1.32|
| 2:2              | 18.47±0.97| 17.04±0.47| 10.44±1.11|
| 3:1              | 21.89±1.55| 17.37±1.86| 12.64±3.34|
| 4:0              | 19.56±1.80| 15.78±1.58| 10.09±5.56|
| *D. communis*    | BG-11     |        |         |
| 0:4              | 21.51±0.56| 22.81±0.23| 15.81±5.66|
| 1:3              | 23.05±2.80| 21.89±2.43| 17.28±0.27|
| 2:2              | 17.20±0.41| 20.37±0.53| 14.87±0.56|
| 3:1              | 22.21±2.17| 22.69±4.66| 16.96±2.05|
| 4:0              | 23.81±1.21| 16.60±2.31| 20.65±1.16|
|                  | 22.16±1.74| 30.33±1.58| 22.75±4.01|

Table 4. The lipid content (Lc) and lipid productivity (Lp) of *Chlorella sorokiniana* and *Desmodesmus communis* in different media under 0.03%, 5% and 10% CO₂ concentrations after 10 days culture, respectively. Error bars represent ± SD of three replicates. Since the lipid productivity was calculated as the average value of lipid content multiplied by the average biomass concentration and divided by 10, it is reported as a single value without standard deviation.
media 0:4 and 1:3 under 5% CO₂ (Fig 6d), a finding that was different from that under 0.03% CO₂. Combined with the result of growth (Fig 1), we hypothesized that 4-celled cenobium was not conducive to cell division and growth because *D. communis* tended to be solitary or 2-celled during the logarithmic phase. Because CO₂ aeration promoted growth, *D. communis* had a stronger tendency to be solitary.

**Conclusions**

In summary, the present study showed that it is feasible to increase biomass and total lipid productivity by mixing SW and STMW coupled with a proper CO₂ concentration. Both *Chlorella sorokiniana* and *Desmodesmus communis* cultivated in 1:3 (SW:STMW) medium achieved the highest nutrient removal rate with or without extra CO₂ aeration. *C. sorokiniana* obtained the maximum biomass concentration (1.31g L⁻¹) and maximum lipid productivity (0.023g L⁻¹ d⁻¹) in medium 2:2 (SW:STMW) under 5% CO₂ concentration. The results suggested that SW and STMW have great potential to become sources of nutrition for microalgae by mixing them at a suitable ratio.
Supporting Information

S1 Fig. Morphology pictures of *Chlorella sorokiniana* (a) and *Desmodesmus communis* (b) in different media under 10% CO$_2$ concentrations after 3, 6 and 10 days culture, respectively. Pictures of *Chlorella sorokiniana* and *Desmodesmus communis* before inoculation (Day 0) were shown in c and d, respectively.

Author Contributions

Conceived and designed the experiments: LY. Performed the experiments: LY JS. Analyzed the data: LY JS XM. Contributed reagents/materials/analysis tools: XM. Wrote the paper: LY JS XM.

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