Characteristics of wastewater treatment by *chlorella sorokiniana* and comparison with activated sludge

Jie Fan, Liang Cao, Cheng Gao, Yue Chen and Tian C. Zhang

**ABSTRACT**

Characteristics of *Chlorella sorokiniana* treating wastewater with consideration of HRT (6 d, 16 h, 8 h), hydraulic conditions, light or dark culture were evaluated and compared with activated sludge. Results showed that optimal HRT was 8 h; if longer, effluent COD and NH$_4^+$-N in the dark began to rebound. Mixing was beneficial to COD removal of algae, while aeration was suitable for nutrient removal. Growth of *C. sorokiniana* in the light was mixotrophic growth and 1.3–1.7 times more than that of dark heterotrophic growth. The maximum specific growth rate ($\mu_{\text{max}}$), productivity, and biomass yields on COD ($Y_{\text{COD}}$), N ($Y_{\text{N}}$), P ($Y_{\text{P}}$) of algae were higher in the light than that in the dark. COD assimilation capacity of algae was similar to activated sludge but with different dynamics. N and P removal efficiency of algae was 5%–10%, 10%–55% respectively higher than activated sludge. This study confirmed the advantage of algae over activated sludge and reveal why algae could assist the activated sludge process.

**Key words** | activated sludge, *chlorella sorokiniana*, HRT, light and dark, wastewater treatment

**INTRODUCTION**

Microalgae-based technology previously dominated in high rate algal ponds, and recently spread into the photobioreactor for wastewater treatment and biofuel production. For microalgae, wastewater is a low-cost nitrogen and phosphorus source. In order to capture nutrients and reduce CO$_2$ in wastewater treatment plants (WWTPs), integration of microalgae technology into activated sludge process has been widely studied. Algae-bacteria consortium is efficient for nutrient removal. However, the consortium is often compared with activated sludge or algae, and the reported range of N and P removal for the algae process and activated sludge process is scattered (Judd et al. 2013), thus the superiority and robustness of algae to activated sludge was not ascertainable.

The performance of algae closely depends on culture conditions, such as hydraulic retention time (HRT), hydraulic conditions (static, mixing or aeration) and culture modes (autotrophic, heterotrophic, and mixotrophic culture). Generally, pollutant removal ability of algae is estimated with HRT to be as long as 2–14 d (Goncalves et al. 2017). Such a long HRT is not practical in WWTPs, and HRT could be shortened since both the rapid growth of algae and pollutant removal occur in the first few days (Kim et al. 2013; Li et al. 2014; Evans et al. 2017). Mixing by magnetic stirrer or orbital shaker is important for an equal light distribution, gas transfer, and preventing microalgae settling (Goncalves et al. 2017). Aeration is necessary for activated sludge to oxidize organic carbon and release CO$_2$ for algae growth. In order to reduce energy demand, a static culture of algae was introduced and performed well for wastewater treatment (Evans et al. 2017). Despite most of the algae being autotrophic, some heterotrophic algae are found in wastewater; for example, *Chlorella sorokiniana* is special for autotrophic, heterotrophic and mixotrophic growth. Compared with the autotrophic growth of algae obtaining energy from photosynthesis, heterotrophic growth (obtaining energy from organic carbon, which happens in the dark) and mixotrophic growth (energy from photosynthesis and organic carbon) were faster (Kim et al. 2015). Heterotrophic culture in the dark is not limited by light but needs an oxygen supply. Autotrophic and mixotrophic growth relies on light availability and is usually inhibited by self-shading and light saturation (Li et al. 2014).

This study aims to estimate characteristics of *Chlorella sorokiniana* treating wastewater with consideration of...
HRT (6 d, 16 h, 8 h), hydraulic conditions (static, mixing, aeration), and culture modes (light or dark culture). Besides, a comparison between C. sorokiniana and activated sludge was conducted to better understand the advantage of algae over activated sludge.

MATERIALS AND METHODS

Algae, activated sludge, and wastewater

The microalgae Chlorella sorokiniana was selected for research, as Chlorella sp. is an indigenous and pollution-tolerant algae in the secondary clarifiers of WWTPs. C. sorokiniana was purchased from Institute of Hydrobiology, Chinese Academy of Science (Wuhan, China). C. sorokiniana was enriched for 3 days in 250 mL Erlenmeyer flasks containing 100 mL sterilized synthetic wastewater (see below) with inoculation of 10% (v/v) in an incubator (9052DHP, Labonce, China). The incubator condition was as follows: 6 W fluorescent lamp, under 12 h:12 h light/dark photoperiod, temperature 25 °C, and shaking the flasks 3 times/d by hand.). The flasks were sealed with cotton plugs to prevent bacterial contamination. Algae were harvested by centrifugation at 4,000 rpm for 10 min.

Activated sludge with MLSS of 2,500 mg L⁻¹ was collected from the aeration basin of LBZ WWTP, Wuhan, China. Activated sludge was cultured with the synthetic wastewater under an anaerobic (2 h)-aerobic (4 h)-settling (2 h) cycle.

Synthetic wastewater was autoclaved at 121 °C for 20 min. According to real weak influent of LBZ WWTP, the COD, NH₄-N and PO₄³⁻ concentrations of synthetic wastewater were prepared at approximately 200, 20, and 4 mg L⁻¹, respectively. The ingredients of synthetic wastewater were as follows (mg L⁻¹): glucose 155, sodium acetate 82, NH₄Cl 76, KH₂PO₄ 13, MgSO₄·7H₂O 25, CaCl₂ 28, and 1 mL trace elements. The trace elements consisted of (g·L⁻¹): ZnSO₄·7H₂O 22, H₃BO₃ 11.4, MnCl₂·4H₂O 5.06, CoCl₂·4H₂O 1.61, CuSO₄·5H₂O 1.57, FeSO₄·7H₂O 4.99.

<table>
<thead>
<tr>
<th>No.</th>
<th>Biomass</th>
<th>Light or dark</th>
<th>HRT</th>
<th>Hydraulic condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>40 mg L⁻¹ C. sorokiniana</td>
<td>Light of 2,500 lux</td>
<td>6 d</td>
<td>Continuous mixing</td>
</tr>
<tr>
<td>(2)</td>
<td>200 mg L⁻¹ C. sorokiniana</td>
<td>⊗ Light of 2,500 lux ⊗ dark</td>
<td>16 h → 8 h</td>
<td>⊗ Static, ⊗ mixing, ⊗ aeration, ⊗ M/O</td>
</tr>
<tr>
<td>(3)</td>
<td>200 mg L⁻¹ activated sludge</td>
<td>⊗ Light of 2,500 lux ⊗ dark</td>
<td>8 h</td>
<td>⊗ Aeration, ⊗ M/O</td>
</tr>
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</table>

Static means without mixing and aeration. M/O consisted of mixing (2 h), aeration (4 h), and settling (2 h); at HRT of 16 h, two cycles of M/O were continuously operated.

Table 1 | Experiment conditions

Experiment design

The experiments were conducted in 250 mL Erlenmeyer flasks containing 200 mL synthetic wastewater. The flasks were covered with a cotton plug, which protected from bacterial contamination in the air and allowed air exchange. Experiment conditions are shown in Table 1. The biomass of algae was adjusted to 40 mg L⁻¹ and 200 mg L⁻¹ based on linear regression between dry weight (DW) and OD₆₈₀ (DW = 0.2338*OD₆₈₀ – 0.0038, R² = 0.9968). Activated sludge of 2,500 mg·L⁻¹ was diluted to 200 mg·L⁻¹.

Under light conditions, two fluorescent lamps were placed 20 cm above the top of the flasks and continuously illuminated with light intensity measured at the top of the reactors by illuminometer (TA8124, Tasi, China). Under dark condition, flasks were wrapped with aluminum foil to avoid light. The mixing was conducted by magnetic stirrer at 150 rpm. Aeration was provided by air compressor with porous stones, and dissolved oxygen (DO) concentration kept to be 2–4 mg·L⁻¹. In the mixing/aeration (M/O) mode of 16 h, mixing for 2 h, aeration for 4 h, settling for 2 h, and repeated another cycle. Under static condition, it was possible to appear non-homogeneous distribution of algae which means a lower concentration in the upper-layer and higher concentration in the bottom-layer, so the sample for OD₆₈₀ was taken from the mid-layer of mixed liquid in order to avoid non-homogeneous distribution of algae. Moreover, microalgae were too small to settle; in this study, algae were suspended in wastewater due to exponential growth during 16 h culture and algal adhesion to the wall was not observed. Samples taken in static, mixing or aeration phases were measured with OD₆₈₀, which was converted to DW of algae by linear regression. Samples taken at the end of reaction were used to determined pH, COD, NH₄-N, NO₂-N, NO₃-N, PO₄³⁻-P.

Corrected Proof

Analytical methods

Samples were filtered through 0.45 μm Whatman membrane, then COD, NH₄⁺-N, NO₂⁻-N, NO₃⁻-N, PO₄³⁻-P, and MLSS were measured according to Standard Methods (APHA 2022). pH was determined by a pH meter (Thermo Orion, USA). Microalgal biomass was measured as OD₆₈₀ and converted to DW by a linear regression between DW and OD₆₈₀. The specific growth rate at exponential phase (μmax) and productivity (Pr) were calculated according to Equations (1) and (2).

$$\mu_{\text{max}} = \frac{\ln w - \ln w_0}{t - t_0}$$

$$P_r = \frac{w - w_0}{t - t_0}$$

where, w₀ and w refer to weight at t₀ and t.

The biomass yield coefficient on substrate (YS) was calculated as:

$$Y_S = \frac{\Delta DW}{\Delta S}$$

where, ΔDW refers to change of dry weight (mgL⁻¹); ΔS refers to change of substrate concentration; S represents COD, or NH₄⁺-N, or PO₄³⁻-P (mgL⁻¹).

Statistical analysis

Paired t-test was applied to analyze the difference between two groups. p < 0.05 was considered statistically different at the confidence level of 95%. The optimal condition was confirmed by orthogonal test L₈ (4 × 2⁴), consisting of factors of hydraulic conditions (static, mixing, aeration, and M/O) and light conditions (light, dark).

RESULTS AND DISCUSSION

Wastewater treatment by C. sorokiniana at HRT of 6d

During 6 days operation in the light, degradation of COD, P, (Figure 1(a)) and NO₃⁻-N (Figure 1(b)) mainly occurred during the first 2d, after which the degradation rates rapidly decreased. Although HRT shorter than 2d was efficient for algae to treat wastewater, the effluent of NH₄⁺-N did not meet discharge standards of WWTPs in China. So the biomass of algae in operation with HRT of 16 h and 8 h should be increased as to guarantee the effluent. In Figure 1(b), NH₄⁺-N and NO₃⁻-N were absorbed by algae, while NO₂⁻-N was not utilized by algae and slightly accumulated. NH₄⁺-N could be assimilated directly into microalgal cells. The reduction of NO₃⁻⁻→NO₂⁻⁻→NH₄⁺⁻ was need to assimilate NO₃⁻⁻ and NO₂⁻⁻ into algae (Goncalves et al. 2017), and the different reaction rate between NO₃⁻⁻→NO₂⁻⁻ and NO₂⁻⁻→NH₄⁺⁻ may result in slight accumulation of NO₂⁻⁻ (van der Steen et al. 2015).

Effect of culture conditions on performance of C. sorokiniana at HRT of 16 h

As the high removal efficiency was confirmed at HRT of shorter than 2d, the HRT was shortened to be 16 h while biomass of C. sorokiniana was increased to 200 mg L⁻¹. C. sorokiniana was special for autotrophic, heterotrophic,
and mixotrophic growth (Kim et al. 2013). Heterotrophic Chlorella sp. possessed hexose/H⁺ symport system which was responsible for the uptake of glucose.

In Figure 2(a), biomass of algae in the light was 1.3–1.7 times of that in the dark. The result was in accordance with previous research (Zhang et al. 2017). At the start of the

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**Figure 2** Effect of culture conditions on performance of *C. sorokiniana*. (a) biomass of dry weight, (b) pH, (c) COD removal, (d) COD removal under dark-aeration and dark-mixing conditions, (e) NH₄⁺-N removal, (f) P removal.
experiment, pH was adjusted to 7.0 and then uncontrolled over time. Through glucose metabolism, CO$_2$ was generated and pH came down. pH under dark static conditions even reduced to 6.5, while under other dark conditions pH firstly decreased and then raised due to CO$_2$ stripping by mixing and aeration. Furthermore, pH in the light was always higher than that in the dark (Figure 2(b)). In our study no additional CO$_2$ was supplied, the source of CO$_2$ for photosynthesis was 0.03–0.06% CO$_2$ in the atmosphere (Wang et al. 2008) and CO$_2$ was generated from organic carbon metabolism (Li et al. 2014) (glucose and acetate in this study), and then photosynthesis of algae caused elevation of pH. Thus pH elevation in the light confirmed that mixotrophic (heterotrophic and autotrophic) growth occurred in the light.

COD was effectively removed whether under the light or dark conditions (Figure 2(c)). Organic carbon (e.g. glucose, acetate, ethanol and glycerol) could be metabolized by heterotrophic algae. Previously, a mutant colorless Chlorella sp. was found to use glucose very slowly under dark anaerobic condition, due to lack of energy and lactate dehydrogenase enzyme (Komor & Tanner 1974). But in our study, when DO was exhausted in the initial 4 h under dark-mixing condition (Figure 2(d)), COD continued to degrade until the end of 8 h, and aeration showed no improvement for COD degradation. This result is possibly because mixing supplemented atmospheric O$_2$ for algae; DO in the initial 4 h reserved some energy for algae; algae could degrade glucose without DO, since Chlorella minutissima was found to utilize glucose in anaerobic-dark condition (Bhatnagar et al. 2010) and Chlorella sorokiniana could tolerate the deoxygenation-dark condition (Attalah et al. 2009).

Rebound of effluent COD (Figure 2(c)) and NH$_4^+$-N (Figure 2(e)) in the dark was observed at the end of 8 h, while effluent P continued to decrease (Figure 2(f)). It was noted that under all conditions pH did not exceed 7.5, and chemical phosphorus precipitation could be excluded as it occurred at pH higher than 9.0 (Evans et al. 2017), thus the effluent P was assimilated by algae. That is, the algae were alive and the rebound of effluent COD and NH$_4^+$-N was not due to cell lysis. Nevertheless, the rebound of effluent COD and NH$_4^+$-N was not observed in the light. It was likely that heterotrophic growth in the dark reached the stationary phase earlier compared to mixotrophic growth in the light (Figure 2(a)), and algae excreted algal organic matters (AOM) when their growth reached the stationary phase (Evans et al. 2017). Accumulation of AOM and protein liberation in the dark (heterotrophic) was more than that in the light (mixotrophic) (Bhatnagar et al. 2010), resulting in rebound of effluent COD and NH$_4^+$-N.

**Optimal condition of C. sorokiniana treating wastewater at HRT of 8 h**

In consideration of the release of COD and NH$_4^+$-N by algae in the dark, COD, NH$_4^+$-N, and P removal was evaluated based on HRT of 8 h. The optimal condition was determined from pollutant removal efficiency and confirmed by orthogonal test L$_{23}$ (4$^1 \times 2^3$, data not shown).

For COD removal efficiency (Figure 5(a)), mixing-dark was best and mixing was better than aeration. Although COD removal of algae in the dark (static, mixing, M/O) was 6% higher than that in the light, the difference between light and dark was not statistically significant ($p > 0.05$), indicating light inhibition on COD removal of algae was not obvious. Our result was consistent with the findings of Patel et al. (2019). Compared with other modes (static, mixing, M/O), aeration with DO of 5.0–5.5 mg L$^{-1}$ showed the lowest COD removal whether in the light or in the dark, possibly due to more algal organic matter being secreted in aeration mode. Algae secreted high molecular and hydrophobic organic matter, which could reduce COD removal efficiency (Zhu et al. 2015).

Referring to NH$_4^+$-N removal (Figure 5(b)), aeration-dark was best; the performance in the light was similar to that in the dark ($p > 0.05$) except for M/O condition. M/O behaved better in the dark (NH$_4^+$-N removal of 61%) than in the light (50%). Aeration promoted NH$_4^+$-N assimilation of algae, since the NH$_4^+$-N removal under the condition of aeration or M/O (50%–61%) was higher than that under the static or mixing conditions (46%–52%).

For P removal (Figure 5(c)), aeration-light achieved the highest P removal of 90%. The hydraulic conditions (static, mix, aeration, and M/O) had an apparent impact on P removal in the light rather than in the dark. P removal in the light was 13%–23% higher than that in the dark. Considering slower algae growth in the dark (Figure 2(a)), P removal varied simultaneously with algae biomass (Figure 3(d)), demonstrating that the inferior P removal in the dark was due to lower biomass. Although assimilation into algae was the main mechanism for C, N, and P removal, only P removal was directly related to algae biomass while C and N removal were not linearly correlated with biomass. The reason for this is explained as follows: (i) the requirement for C and N in the biomass composition always changed with culture condition (Kumar et al. 2014), (ii) carbon and nitrogen metabolism were linked, as assimilation of ammonia to form amino acids required carbon skeletons (Evans et al. 2017), (iii) compared to C and N content, the P content in the algae was relatively stable.
(Judd et al. 2015). In this study, dark culture caused the N content of algae to double while P content just increased a little.

**Kinetics and stoichiometry of *C. sorokiniana* at HRT of 8 h**

The maximum specific growth rate ($\mu_{\text{max}}$), productivity ($P_r$), and biomass yield on substrate ($Y$) under different culture conditions are compared in Table 2. Kinetics and stoichiometry were mainly influenced by dark and light culture, while hydraulic conditions had little impact. Under the light condition, algae grew on wastewater at $\mu_{\text{max}}$ of 1.37–1.46 d$^{-1}$ and 1.3–1.5 fold higher than that in the dark. Meanwhile, productivity in the light was 1.6–2.8 fold higher than that in the dark. The results indicated growth potential in the light was much better and supported the biomass difference between light and dark (Figure 2(a)). Apart from energy from organic carbon oxidation, light may provide additive energy for growth (Zhang et al. 2017).

Biomass yield on glucose ($Y_{\text{COD}}$) and ammonia nitrogen ($Y_{\text{NH}_4\text{-N}}$) in Table 2 were similar to values reported by Singhasuwan et al. (2015). Nevertheless, $Y_{\text{COD}}$ of 0.69–0.87 gDW$^{-1}$COD in the dark and 1.38–1.75 gDW$^{-1}$COD were higher than 0.45 obtained with 10 g/L glucose (Chen et al. 2018), the reason being that $Y_{\text{COD}}$ decreased with the increase of the initial concentration of
glucose (Chojnacka & Zielinska 2012). $Y_{\text{COD}}$ in the dark accounted for 50% of that in the light. Also, the decline of $Y_{\text{NH}_4}$ and $Y_P$ in the dark indicated less biomass synthesis on nutrients. Furthermore, the inverse of $Y_{\text{NH}_4-N}$ and $Y_P$ reflected the nutrient content in the algae cell. Based on that, N content was 4.2–4.8% in the light and 8.3–10% in the dark, and P content was 1.5–1.7% in the light and 2.2–2.5% in the dark. The results demonstrated that N and P content of algae cells in the dark was higher than that in the light, which was consistent with research of Wang et al. (2016) who found that C and N content of C. sorokiniana in the dark was higher than that in the light.

**Performance comparison between C. sorokiniana and activated sludge at HRT of 8 h**

Under static and mixing conditions, DO was exhausted over time and P was released by phosphorus accumulating organisms (PAOs) to effluent, therefore static and mixing conditions were not included in comparison. In Figure 4(a), COD removal of C. sorokiniana was comparable to that of activated sludge except in the condition of light-M/O, in which COD removal of C. sorokiniana (73%) was inferior to activated sludge (82%). In the case of dark-aeration (Figure 4(b)), despite the different COD removal dynamics of C. sorokiniana (zero-order reaction) and activated sludge (first-order reaction), at the end of 8 h the same amount of COD was removed. From this study and previous research (Mujtaba et al. 2018), Chlorella sp. was capable of removing the same amount of COD as activated sludge. It's worth noting that the synthetic influent in this study only consisted of glucose and acetate, which were preferred carbon sources for algae, and the resilient and toxic organic compounds in raw wastewater would influence the biodegradation process and removal efficiency of algae (Goncalves et al. 2017). Microalgae are considered a sustainable remediation for removing heavy metals and some toxic organic compounds (Maeng et al. 2018).

In Figure 4(c), NH$_4$-N removal of algae (50%–61%) exceeded that of activated sludge (44%–51%). In particular, NH$_4$-N removal of algae in the dark was apparently higher than that of activated sludge. The assimilation capacity of NH$_4$-N of algae and activated sludge is compared in Figure 4(d). After 6 h aeration, N assimilation of activated sludge was saturated at 8 mg/L, while N assimilation of C. sorokiniana varied from fast to slow, and it was still not saturated at the end of 16 h, when N assimilation capacity was 1.4 times greater than that of activated sludge. The results demonstrated that N assimilation capacity of algae was higher than activated sludge, due to the higher N content in algae (5.51–8.25%) than in activated sludge (4.04–4.93%) (Wang et al. 2016; Zhu et al. 2019).

In Figure 4(e), P removal of algae is 2.2–2.5 times more than that of activated sludge under aeration condition. However, under M/O condition, the difference between algae (71–85%) and activated sludge (65–72%) was narrowed, since M/O provided an anaerobic/aerobic phase for PAOs, which exhibited P release in the anaerobic phase and luxury P uptake in the aerobic phase. Compared with C and N removal, algae presented more apparent advantage for P removal over activated sludge. Under aeration condition, fast assimilation of P occurred during the initial 6 hours, at the end of which P assimilation capacity of algae and activated sludge was 3.6, and 1.4 mg L$^{-1}$, respectively (Figure 4(f)). The P assimilation capacity was associated with the P content of biomass. In general, the P content of bacteria and algae was 2–3%, 0.21–3.85% respectively (Powell et al. 2009). Specially, luxury P uptake was involved not only in PAOs but also in algae (Crimp et al. 2017). When PAOs functioned under M/O condition, the P assimilation capacity of activated sludge quickly rose to 3 mg L$^{-1}$ which was still lower than that of algae (Figure 4(f)). Thus algae outcompeted activated sludge in terms of the P removal efficiency and P assimilation capacity.

Comparison between algae and activated sludge in view of carbon and nutrient removal is listed in Table 3. COD removal of activated sludge was 0%–10% higher than that of algae. N removal efficiency through biomass assimilation of algae and activated sludge was 50%–100%, 20%–30% respectively, while N removal of 50%–55% by activated sludge could be obtained if nitrification was involved (aeration condition). Algae half-saturation constant for N (0.1–0.2 mg L$^{-1}$) was lower than that of activated sludge (0.2–1 mg L$^{-1}$) (Solimeno & Garcia 2017); as a result, the affinity of algae to N was better and more effective than activated sludge. Besides, N and P removal by activated sludge fluctuated largely, since bacterial species and DO have an important impact on nitrification, denitrification and PAOs. It should be mentioned that concentrations of activated sludge in this study and other literatures (Table 3) was much lower than 2,500 mg L$^{-1}$ that is usually adopted in WWTPs. 2,500 mg L$^{-1}$ was a relatively high concentration for algae and led to self-shading, therefore algae were used as an assistant for activated sludge rather than an alternative. Wastewater treatment could benefit from the complementary functions of algae and activated sludge.
CONCLUSIONS

HRT of algae for wastewater treatment was shortened from 6 d to 8 h. Algal biomass with HRT of 16 h in the light was 1.3–1.7 times more than that in the dark. Biomass decline in the dark only decreased P removal while it had no adverse impact on COD and NH$_4$–N removal. Mixing was good for COD removal while aeration was suitable for nutrients.
removal enhancement. COD assimilation capacity of algae was similar to that of activated sludge; however, COD removal of algae was a zero-order dynamic reaction while that of activated sludge was first-order reaction. N and P removal of *C. sorokiniana* was 5%-10% and 10%-55%, respectively, higher than that of activated sludge, due to the obviously higher capacity for nutrient assimilation.

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