Effects of influent C/N ratios and treatment technologies on integral biogas upgrading and pollutants removal from synthetic domestic sewage

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Three different treatment technologies, namely mono-algae culture, algal-bacterial culture, and algal-fungal culture, were applied to remove pollutants from synthetic domestic sewage and to remove CO₂ from biogas in a photobioreactor. The effects of different initial influent C/N ratios on microalgal growth rates and pollutants removal efficiencies by the three microalgal cultures were investigated. The best biogas upgrading and synthetic domestic sewage pollutants removal effect was achieved in the algal-fungal system at the influent C/N ratio of 5:1. At the influent C/N ratio of 5:1, the algal-fungal system achieved the highest mean chemical oxygen demand (COD) removal efficiency of 81.92% and total phosphorus (TP) removal efficiency of 81.52%, respectively, while the algal-bacterial system demonstrated the highest mean total nitrogen (TN) removal efficiency of 82.28%. The average CH₄ concentration in upgraded biogas and the removal efficiencies of COD, TN, and TP were 93.25 ± 3.84% (v/v), 80.23 ± 3.92%, 75.85 ± 6.61%, and 78.41 ± 3.98%, respectively. These results will provide a reference for wastewater purification ad biogas upgrading with microalgae based technology.

In rural areas of China, one of the main water pollution sources is domestic sewage, which contains abundant carbon, nitrogen, and phosphorus compounds. Recently, more than 90% of the domestic sewage in rural areas was directly discharged into natural waters without appropriate treatment, eventually causing water eutrophication¹. Many studies indicated that conventional water treatment processes, including oxidation ditch processes², anaerobic-anoxic-oxic (A²/O)³, University of Cape Town (UCT)⁴, Bardenpho, and sequencing batch reactor (SBR)⁵, achieved moderate success in removing pollutants. However, these processes generally entail enormous land requirements, operational costs, complex operations, and large volumes of waste sludge production and are therefore not practicable in rural areas in China.

Conventional activated sludge systems will consume around half of the whole energy to convert chemical oxygen demand (COD) into the greenhouse gas CO₂. The pollutants (i.e. carbon, nitrogen and phosphorus sources) in wastewater can be assimilated by microalgal species as nutrients for heterotrophic or mixotrophic growth⁶. Hence, as an alternative for the purification of domestic sewage, biological wastewater treatment system using microalgae is currently attracting increased interest because of its low construction and maintenance costs, minimal energy consumption, freedom from spatial restriction during operation, as well as high removal efficiency⁷. Study has shown that 85–88% of COD, 78–83% of total nitrogen (TN), and 73–80% of total phosphorus (TP) could be removed from wastewater by cultivating the algae Chlorella sp.⁸. Kumar et al. (2010) reported that treating digested piggery effluent with Chlorella vulgaris (C. vulgaris.) achieved removal efficiencies of 100% for TP and 78% for NH₄⁺-N⁹. In addition, culturing certain species of microalgae with sanitary sewage might enable the harvest of potentially high added value microalgae biomass and the metabolic products, such as proteins and fatty acids¹⁰,¹¹. Therefore, microalgae-based technology is suitable for wastewater treatment because of its high effectiveness and low-cost¹²,¹³.

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In previous studies, significant improvement in the removal of xenobiotics from wastewaters has been observed with algae-bacteria/fungus–algae co-cultivation14, 15. The success of this technology is dependent on a mutually beneficial relationship, that is, by fixing CO₂ which is produced by fungi/bacteria, algae can synthesize carbon source in the form of sugars and nutrients for fungi/bacteria through photosynthesis16. Therefore, pretreating wastewater by algal-fungal culture or algal-bacterial culture has an advantage on higher pollutants removal efficiencies comparing with mono-cultivation of algae cells, thereby offering a promising and efficient way to treat wastewater.

Biogas represents a renewable energy source based on its high CH₄ content. Raw biogas usually consists of methane (47–65%), carbon dioxide (34–41%), and other trace compositions including hydrogen sulfide, water vapor, etc.17. However, the CH₄ content of more than 90% (v/v) in biogas is required for vehicle fuel according to the natural gas standard18. The relatively high content of CO₂ in raw biogas will lower its heat content as well as treatment by using microalgae only23. Besides, the high cost generated from microalgae biomass harvesting is one and biogas upgrading is indispensable because of its highly economically convenient comparing with wastewater simultaneously treating wastewater and upgrading biogas in recently years. Combination of wastewater treatment and biogas upgrading by CO₂ removal, the biological sequestration of CO₂ using photosynthetic microalgae has been receiving considerable attention because of the relatively high CO₂ fixation capability of microalgae19, 20. There are a limited number of studies on the integration of digestate treatment procedures and biogas upgrading via microalgal culture systems8. Accordingly, using microalgal growth to remove CO₂ from crude biogas is an economic and efficient method for biogas upgrading, which makes the biogas upgrading an economically convenient technique when used in conjunction with wastewater treatment14, 19.

Microalgal biomass production exhibits potentials for both pollutants reduction and CO₂ removal. However, microalgal growth is also affected by organic matter and nutrient concentrations in wastewater as well as the presence of other heterotrophic microorganisms20. Fungi and bacteria can also have strong degradation abilities for certain wastewater contaminants and can be associated with microalgae to form immobilization systems of algal-fungal culture or algal-bacterial culture with multiple functions. The advantages of these associations in wastewater treatment are: (i) improved collection of biomass from wastewaters, (ii) easily recycled and manipulated consortia, (iii) improved features of microalgae such as thermal stability and productivity, and (iv) harvestable bioresource from proliferated microalgae biomass21, 22. The use of biogas and sewage as raw materials can not only output high-grade biogas through the microalgae photosynthesis to assimilate carbon dioxide, but also can purify sewage by accumulating C, N, and P in sewage with microalgae. When compared with the traditional biogas upgrading and sewage purification technology, environmental friendly and cost-effective are the biggest advantages. Some researches on biogas upgrading or sewage purification resulted in methane loss and energy consumption, or lead to the increase of effluent13. Microalgae-based technology was therefore developed for simultaneously treating wastewater and upgrading biogas in recently years. Combination of wastewater treatment and biogas upgrading is indispensable because of its highly economically convenient comparing with wastewater treatment by using microalgae only20. Besides, the high cost generated from microalgae biomass harvesting is one of the major bottlenecks for the industrialization of algae-based technology because of the small microalgal size, negative surface charge, and low biomass concentration of microalgae20. Co-culture of microalgae with fungi/bacteria can well solve this problem. The bio-flocculation based microalgae assisted with fungi/bacteria is regarded as one of the best ways to realize the large-scale separation and microalgae recovery25. The energy consumption and the cost of the subsequent enrichment process have been significantly reduced14. At present, the few studies on couple biogas upgrading with wastewater treatment in photobioreactors provide little information on the influence of operational conditions on strain composition. Influent C/N ratio is the main factor affecting biological wastewater treatment processes26. However, thus far, only a few studies have evaluated the effects of different influent C/N ratios on the efficiency of domestic sewage treatment by microalgae27-29. Overload or overhigh C/N ratios in the influent may result in low growth rate of microalgae and low removal efficiencies of nitrogen and phosphate in wastewater. In addition, the C/N ratio is also vital to the growth of microalgae when co-cultivation with fungi or bacteria.

This study proposes an integrated approach for synthetic domestic sewage treatment and biogas upgrading through three algal culture methods (mono-algal culture, algal-fungal culture, and algal-bacterial culture). The purposes of this study were to (1) evaluate the effects of three different microalgae culture methods on sewage purification and biogas upgrading; and (2) identify the optimal influent C/N ratio to achieve the highest efficiencies of pollutants removal combined with biogas upgrading in synthetic domestic sewage.

**Results and Discussion**

**Physicochemical variations.** Changes in values of the sewage pH and DO are shown in Table 1. These changes were similar under all tested influent C/N ratios. The pH ranged from 7.11 to 7.54 in the influent, which were optimum values for the microalgae cultivation and the precipitation of ammonia toxicity and phosphate precipitation30. No significant difference (p > 0.05) was observed among the three algae source cultures for pH values in the influent or effluent. However, for all treatments, pH of the influent was significantly higher (p < 0.05) than that in the effluent, which was typically below pH 8.0 (Table 1). The pH values observed in this study were consistent with those found by Papazi et al. (2008), who reported that pH values slightly varied between 6.0 and 7.5 under CO₂ 30% (v/v) with Chlorella minutissima31. This phenomenon meant that the biogas CO₂ content in this research met the requirement for microalgae growth. Therefore, the CO₂ consumption of the suspension culture only slightly affected the pH, which was rather related to the removal of organic pollutants. For instance, the
Pollutants removal efficiencies. Pollutants uptake by the three selected cultures significantly contributed to COD, TN and TP removal from the wastewater. Figures 1–3 indicated the effects of various influent C/N ratios and culture methods on COD, TN, and TP removal efficiencies using microalgal-based technologies. All the cultures studied efficiently removed pollutants (COD, TN, and TP) from the synthetic domestic sewage. Pollutant removal efficiencies fluctuated for the three algae source culture during the operational period, and the variation tendencies of pollutant removal were similar under different influent C/N ratios. The COD removal efficiencies varied between 50.06% and 89.47% for the microalgal monoculture (Fig. 1a), between 52.36% and 93.87% for the microalgal-fungal co-culture (Fig. 1b), and between 50.35% and 93.78% for the algal-bacterial co-culture (Fig. 1c) under different COD or TN level treatments. The TN removal efficiency variations for the microalgal monoculture ranged from 50.06% to 89.47% for the microalgal monoculture (Fig. 1a), between 52.36% and 93.87% for the microalgal-fungal co-culture (Fig. 1b), and between 50.35% and 93.78% for the algal-bacterial co-culture (Fig. 1c). The TP removal efficiencies for the microalgal monoculture varied between 42.17% and 89.57% (Fig. 3a), for the microalgal-fungal co-culture from 54.25% to 94.28% (Fig. 2b), and for the algal-bacterial co-culture from 49.23% to 93.97% (Fig. 2c). The TP removal efficiencies fluctuated for the three algae source culture during the operational period, and the variation tendencies of pollutant removal were similar under different influent C/N ratios. The COD removal efficiencies varied between 49.36% and 93.77% (Fig. 3b), and for the algal-bacterial co-culture between 47.14% and 92.54% (Fig. 3c).

Table 1. Means ± standard deviations of the physico-chemical parameters of influent and effluent for domestic sewage of three different methods of treatments at different influent C/N ratio.

| C/N ratio | Influent | Effluent | Influent | Effluent |
|-----------|----------|----------|----------|----------|
|           | pH       | Do (mg L⁻¹) | pH       | Do (mg L⁻¹) |
| Mono-algae culture |                |          |          |          |
| C2.5N1-COD100 | Low COD level | 7.42 ± 0.23 | 6.45 ± 0.31 | 6.37 ± 1.92 |
| C5N1-COD200 | Medium COD level | 7.31 ± 0.19 | 6.11 ± 0.16 | 6.52 ± 1.41 |
| C10N1-COD400 | High COD level | 7.19 ± 0.11 | 6.32 ± 0.14 | 1.95 ± 1.13 |
| C10N1-TN20 | Low TN level | 7.28 ± 0.25 | 7.03 ± 0.11 | 6.14 ± 1.02 |
| C5N1-TN40 | Medium TN level | 7.51 ± 0.28 | 7.12 ± 0.19 | 6.43 ± 1.27 |
| C2.5N1-TN80 | High TN level | 7.26 ± 0.13 | 6.39 ± 0.24 | 5.78 ± 0.82 |
| Algal-fungal culture |                |          |          |          |
| C2.5N1-COD100 | Low COD level | 7.44 ± 0.17 | 8.12 ± 2.11 | 6.87 ± 0.31 | 6.09 ± 1.17 |
| C5N1-COD200 | Medium COD level | 7.11 ± 0.26 | 7.63 ± 1.55 | 6.93 ± 0.15 | 6.28 ± 1.56 |
| C10N1-COD400 | High COD level | 7.52 ± 0.15 | 2.54 ± 1.18 | 7.04 ± 0.19 | 2.17 ± 1.08 |
| C10N1-TN20 | Low TN level | 7.47 ± 0.19 | 6.91 ± 1.94 | 6.39 ± 0.21 | 6.32 ± 1.26 |
| C5N1-TN40 | Medium TN level | 7.33 ± 0.21 | 7.72 ± 1.83 | 6.57 ± 0.16 | 6.71 ± 1.34 |
| C2.5N1-TN80 | High TN level | 7.35 ± 0.32 | 6.53 ± 0.97 | 6.41 ± 0.18 | 6.03 ± 1.27 |
| Algal-bacterial culture |                |          |          |          |
| C2.5N1-COD100 | Low COD level | 7.54 ± 0.27 | 8.51 ± 1.84 | 6.58 ± 0.16 | 6.92 ± 1.57 |
| C5N1-COD200 | Medium COD level | 7.41 ± 0.18 | 7.93 ± 0.92 | 6.77 ± 0.18 | 6.31 ± 1.25 |
| C10N1-COD400 | High COD level | 7.48 ± 0.29 | 2.39 ± 1.46 | 6.32 ± 0.14 | 1.84 ± 0.93 |
| C10N1-TN20 | Low TN level | 7.34 ± 0.15 | 6.77 ± 1.01 | 6.21 ± 0.17 | 6.04 ± 1.16 |
| C5N1-TN40 | Medium TN level | 7.53 ± 0.24 | 7.02 ± 0.87 | 6.49 ± 0.16 | 6.23 ± 1.28 |
| C2.5N1-TN80 | High TN level | 7.41 ± 0.12 | 6.15 ± 0.59 | 6.63 ± 0.26 | 5.93 ± 1.29 |

Growth of the three selected cultures at various influent C/N ratios. Biomass productivity is a key parameter to analyze the potential of the three selected cultures to remove CO₂. It generally varies with operational factors, such as light intensity, pH, working volume of the photobioreactor, and initial CO₂ concentration in the simulated biogas. Studies have found that biomass growth is coupled not only with higher N/C and P/C ratios, but also with lower N/P ratios in many heterotrophic organisms, including bacteria. Table 2 shows the microalgal mean daily productivity, sewage pollutant removal efficiencies and CH₄ content in biogas under three cultures and various C/N ratios. The behavior of the three selected cultures varied even under identical environmental conditions and media. In both the mono-algae and the algal-fungal culture, biomass grew faster in the C5N1-COD200 treatment than in the other treatments (p < 0.05). In the present study, biomass productivity of the algal-fungal culture was higher than the other two cultures. The highest biomass productivity (0.44 g L⁻¹ d⁻¹, Table 2) for algal-fungal culture was found in C5N1-COD200, although there was no differences among other C/N ratios (p < 0.05).
than the other treatments. (78.42%) and C5N1-TN40 treatments (81.66%) (p < 0.05), but no significant difference (p > 0.05) was observed in removal efficiencies for COD and TN among C/N variation treatments by the mono-algae culture. For the algal-fungal culture, the C5N1-COD200 treatment had a higher TN removal efficiency (79.35%) than C2.5N1-COD100 (75.08%) and C10N1-COD400 (75.54%) (p < 0.05), but the influent C/N ratios showed no statistically significant effects (p > 0.05) on COD removal. For the algal-bacterial culture, the highest TN (79.48%) and TP (69.34%) removal efficiencies were achieved by C5N1-COD200 (74.79%) and C5N1-COD400 (74.79%) treatments (p < 0.05).

Under N addition treatments, the C5N1-TN40 treatment had a higher COD removal efficiency (77.64%) than the C10N1-TN20 (78.17%) and C2.5N1-TN80 treatments (77.42%) (p < 0.05). The C5N1-TN40 treatment had significantly lower (p < 0.05) the TN removal efficiency (79.13%) than the C2.5N1-TN80 (79.35%) and C10N1-TN20 treatments (80.59%) (p < 0.05). However, in the algal-bacterial culture, the C2.5N1-TN80 treatment had significantly lower (p < 0.05) COD (75.54%) and TN (75.11%) removal efficiencies than the C2.5N1-COD100 (74.79%) and C5N1-COD200 treatments (77.41%) (p < 0.05).

Under N addition treatments, the C5N1-TN40 treatment had a higher COD removal efficiency (77.64%) than the C10N1-TN20 (78.17%) and C2.5N1-TN80 treatments (77.42%) (p < 0.05). The C5N1-TN40 treatment had significantly lower (p < 0.05) the TN removal efficiency (79.13%) than the C2.5N1-TN80 (79.35%) and C10N1-TN20 treatments (80.59%) (p < 0.05). However, in the algal-bacterial culture, the C2.5N1-TN80 treatment had significantly lower (p < 0.05) COD (75.54%) and TN (75.11%) removal efficiencies than the other treatments.

As shown in Table 3, the interaction of treatment methods and influent C/N ratios as well as the treatment methods significantly influenced pollutants removal efficiencies (p < 0.05). Only TN and TP removal efficiencies were significantly affected (p < 0.05) by influent C/N ratios. This shows that appropriate selection of treatment methods and influent C/N ratios is a simple and effective strategy to increase pollutants removal efficiencies.

Carbon is a basal element for microalgae growth, contributing to up to about 50% of microalgal biomass35. A previous study accessed the carbon mass balance and found that assimilation into biomass was the main carbon removal pathway36. Dissolved nitrogen and phosphorus in wastewater could be efficiently removed through

Table 2. Mean values ± SD of the removal efficiency of biogas CO₂ and pollutants removal of different C/N ratio at three different methods of treatments. Values with different superscript letters in the same column for the same method and influent C/N ratios are significantly different at p ≤ 0.05 according to Duncan’s multiple range tests.

| C/N ratio          | Mono-algae culture | Algal-fungal culture | Algal-bacterial culture |
|--------------------|--------------------|----------------------|-------------------------|
|                    | Effluent Upgraded biogas | Biomass productivity | Effluent Upgraded biogas | Biomass productivity | Effluent Upgraded biogas | Biomass productivity |
| C2.5N1-COD100      | Low COD level       | 78.65 ± 4.02         | 69.08 ± 4.02             | 74.79 ± 3.56         | 69.08 ± 4.02             | 74.79 ± 3.56         |
|                    | Medium COD level    | 81.92 ± 4.57         | 75.08 ± 3.27             | 75.08 ± 3.27         | 75.08 ± 3.27             | 75.08 ± 3.27         |
|                    | High COD level      | 79.11 ± 2.43         | 79.35 ± 4.26             | 79.35 ± 4.26         | 79.35 ± 4.26             | 79.35 ± 4.26         |
| C10N1-COD400       | Low TN level        | 79.13 ± 3.77         | 78.42 ± 3.17             | 78.42 ± 3.17         | 78.42 ± 3.17             | 78.42 ± 3.17         |
|                    | Medium TN level     | 80.64 ± 3.92         | 81.60 ± 6.61             | 81.60 ± 6.61         | 81.60 ± 6.61             | 81.60 ± 6.61         |
|                    | High TN level       | 79.35 ± 5.08         | 75.15 ± 5.05             | 75.15 ± 5.05         | 75.15 ± 5.05             | 75.15 ± 5.05         |

Table 2. Mean values ± SD of the removal efficiency of biogas CO₂ and pollutants removal of different C/N ratio at three different methods of treatments. Values with different superscript letters in the same column for the same method and influent C/N ratios are significantly different at p ≤ 0.05 according to Duncan’s multiple range tests.
continuous microalgal growth. Total N was mainly reduced via microalgal assimilation, as algae cells require nitrogen for protein, nucleic acid, and phospholipid synthesis. Previous work has documented that in wastewater, simple organic nitrogen, including urea and amino acids, can be assimilated by microalgae. Thus, microalgae growth is essential for nitrogen removal via uptake, decay, and sedimentation. On the other hand, partial loss of TN could be attributed to the physical absorption by strain complexes because of their unique structure.

In addition, phosphorus is an important nutrient in algal production as a constituent of phospholipids (for cell membranes) and adenosine triphosphate (to supply energy for cell functions), although it constitutes less than 1% of the biomass. In a similar study, Yan et al. (2014) obtained a phosphorus removal efficiency of 73.89% for Chlorella sp. in biogas slurry.

Carbon dioxide dissolving in sewage will provide a substrate for the microalgal photosynthesis. The dissolution of carbon dioxide promoted the growth of microalgae and therefore played a key role in nitrogen and phosphorus removal. Accordingly, coculture of microalgal and fungi or bacteria promotes the purification of sewage and biogas upgrading. The rich carbon dioxide in the biogas slurry promotes the growth of algal bacteria and increases the biomass of algae bacteria, thus increases the absorption of N and P by algae and improves the sewage purification capacity of algae bacteria.

Several researchers have suggested that denitrification rates in a reactor depend largely upon the amounts of nitrate N and organic carbon as well as on environmental conditions such as pH, temperature, and DO concentration. Here, influent pH levels (7.11–7.54) were within the optimum range for denitrification. In practice, nitrogen removal is highly dependent on pollution load levels. In this study, different influent C/N ratios resulted in different denitrification rates, and high TN removal efficiency occurred only if the C/N ratio was 5:1. Consequently, influent C/N ratio plays a crucial role in wastewater treatment. Some scholars have suggested that influent C/N ratio is a domain value, rather than a specific value, because when the experimental influent C/N ratio approaches this value, optimal pollutant removal efficiencies can be achieved. In this research, the balance of carbon and nitrogen nutrient sources in the influent affected the COD removal efficiency over time under various influent C/N ratios for three algae source cultures: (a) microalgal monoculture, (b) microalgal-fungal co-culture, and (c) algal-bacterial co-culture.
growth of the culture, thereby affecting the nutrient removal efficiencies of the microalgae. On the other hand, the synthetic wastewater used in this research contained carbamide; a high C/N ratio might thus inhibit the removal of ammonia nitrogen due to the competition for DO demand. Therefore, when carbon sources were lacking (C:N = 2.5:1) or nitrogen sources were insufficient (C:N = 10:1), pollutants removal decreased. Considering the combined removal efficiencies for all pollutants, the optimal C/N ratio in this research was 5:1. These results are in agreement with the findings of Yan et al., who reported that influent C/N ratio significantly affected nutrient removal efficiency and that the highest removal effect was found with a medium influent C/N ratio.

Influent C/N ratio also affected effluent phosphorus concentrations, and at C/N 5:1, all treatment systems reached their highest TP removal efficiencies. In the nitrogen addition treatments, the highest average removal efficiencies for the three algae source culture occurred at the C/N ratio of 5:1 (Table 2). These results could be explained by the combined carbon and nitrogen effects for removal of TP. Overall, the results showed that appropriate control of carbon and nitrogen input were necessary to achieve the efficient phosphorus removal.

In the present study, among the three selected cultures, the algal-fungal culture showed the highest pollutants removal efficiencies (COD, TN and TP). The sphere structure of the fungus–algae complex was stable and did not easily break into small pieces (data not shown), which might partially explains the high pollutants removal efficiency. According to the references, when fungi associated with microalgae grew in wastewater, the fungus was pelletized with microalgae cells through bioflocculation and non-bioflocculation. The coagulative mechanism is spore coagulation resulting in accumulation of pellets. The non-coagulative mechanism includes that the spores germinate into hyphae and then intertwines into pellets. In the symbiotic system of algae-fungi/bacteria, on one hand, the extracellular metabolites produced and secreted by the microalgae can be effectively taken up by the surrounding fungi/bacteria for the growth and reproduction. On the other hand, the bacteria in the metabolic process not only can produce the necessary nutrients and growth factors for the growth of microalgae, but also can directly or indirectly regulate the growth environment of microalgae. This forms a mutually beneficial symbiotic relationship. The intergrowth of microalgae and fungi enhances the specific surface area of

Figure 2. TN removal efficiency over time under various influent C/N ratios for three algae source cultures: (a) microalgal monoculture, (b) microalgal-fungal co-culture, and (c) algal-bacterial co-culture.
algae-fungi symbionts and thus the nutrient intake capacity\(^43\). According to Table 2, coculture of microalgae and fungi resulted in higher nutrient removal as well as CO\(_2\) removal except for TN removal. The algal-bacterial culture showed higher TN removal efficiency than algal-fungi culture maybe attribute to the nitrification of bacteria in activated sludge\(^44\).

**Biogas upgrading.** At the end of the experiment, the CH\(_4\) contents (v/v) of the biogas and the CO\(_2\) removal efficiency (%) were investigated to evaluate differences in biogas upgrade with varying influent C/N ratios for the three selected cultures (Fig. 4a–c). The results showed that the tendencies of CH\(_4\) contents and CO\(_2\) removal efficiencies were similar to biomass productivities of the three selected culture, as shown in Table 2.

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**Table 3.** P-values of factors and combined effects of factors for each parameter based on analysis of variance. Influent C/N ratios: C variation treatments (C2.5N1-COD100, C5N1-COD200, and C10N1-COD400) and N variation treatments (C10N1-TN20, C5N1-TN40, and C2.5N1-TN80); treatment methods: microalgal monoculture, microalgal-fungal coculture and microalgal-activated sludge coculture (*\(p < 0.05\)).

| Factor                          | COD RE (%) | TN RE (%) | TP RE (%) | CO\(_2\) RE (%) |
|---------------------------------|------------|-----------|-----------|-----------------|
| Influent C/N ratios            | 0.059      | 0.027*    | 0.039*    | 0.067           |
| Treatment methods               | 0.034*     | 0.018*    | 0.044*    | 0.025*          |
| Influent C/N ratios × Treatment methods | 0.028*     | 0.032*    | 0.017*    | 0.011*          |

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**Figure 3.** TP removal efficiency over time under various influent C/N ratios for three algae sources cultures: (a) microalgal monoculture, (b) microalgal-fungal co-culture, and (c) algal-bacterial co-culture.
During biogas upgrading, CO₂ could be effectively removed. In all treatments using algal-bacterial culture, the CO₂ removal percentage (%) ranged from 74.71% to 90.26%, which was slightly higher than that observed for mono-algae culture (62.89–91.56%) and algal-fungal culture (65.42–84.98%). The overall CO₂ removal percentage followed similar trends and reached the highest values of 91.56 ± 2.36%, 84.98 ± 2.36%, and 90.26 ± 1.76% in the C5N1-TN40 treatment by mono-algae culture, algal-fungal culture, and algal-bacterial culture, respectively. Under the different C and N variation treatments, no significant differences (p > 0.05) in biogas CO₂ removal efficiencies were found between C5N1-COD200 and C5N1-TN40 among the three microalgal cultures, but their corresponding removal efficiencies were significantly higher (p < 0.05) than that in C2.5N1-COD100, C10N1-COD400, C10N1-TN20, and C2.5N1-TN80.

During the experiment, biogas upgrading was affected by the initial pollutants concentrations, and the CH₄ content (v/v) increased with CO₂ contents (v/v) decreasing. The differences in the various treatments for the CH₄ contents (v/v) of the biogas mainly resulted from the variations in the CO₂ removal, because CH₄ and CO₂ contents (v/v) in biogas were significantly negatively correlated. The CH₄ contents (v/v) enriched by algal-bacterial culture in upgraded biogas in the treatments C2.5N1-COD100, C5N1-COD200, C10N1-COD400, C10N1-TN20, C5N1-TN40, and C2.5N1-TN80 were 90.58 ± 1.25%, 93.04 ± 2.01%, 89.37 ± 1.88%, 91.04 ± 2.16%, 93.25 ± 1.74%, and 88.83 ± 1.69%, respectively.

The CO₂ in biogas was utilized for algal photosynthesis. As a result, the CH₄ content in biogas was increased. The CH₄ content in biogas upgraded by algal-bacterial culture was 88.8%–93.3%, which was higher than those by mono-algae culture and algal-fungal culture. The CH₄ contents (v/v) enriched through removing CO₂ by algal-bacterial culture reached the standard of the fuel (CH₄ > 90%, v/v) in the treatments C5N1-COD200, C10N1-TN20, and C5N1-TN40. Only in C10N1-COD400 and the C2.5N1-TN80, algal-fungal culture, the CH₄ content

**Figure 4.** CO₂ removal efficiencies and final CH₄ contents (v/v) in biogas under six nutrient concentration levels for three algae source cultures: (a) microalgal monoculture, (b) microalgal-fungal co-culture, and (c) algal-bacterial co-culture.
mainly because approximately half of the microalgal DW was CO2-derived carbon45. When the microalgae were
a CO2-concentrating mechanism to adapt to the changes in the CO2 concentration46, 47.  

In this research, the effect of biogas upgrading followed similar trends as biomass productivity (Table 2),
mainly because approximately half of the microalgal DW was CO2-derived carbon45. When the microalgae were
cultivated in synthetic domestic sewage, CO2 was used for microalgal photosynthesis. The microalgae developed
a CO2-concentrating mechanism to adapt to the changes in the CO2 concentration46, 47.  

**Economic efficiency of the energy consumption.** The economic efficiency of energy consumption on
synthetic domestic sewage purification and biogas upgrading showed a similar variation (Table 4). The highest
economic efficiency of pollutants and CO2 removal with different culture methods can be found at the C/N ratio
of 5:1 with the medium TN level. This finding is corresponded to the variation of the microalgal biomass
productivity. To be specific, algal-fungal culture achieved the highest economic efficiency of COD and TP removal;
algal-bacterial culture achieved the highest economic efficiency of TN and CO2 removal. The ANOVA showed
that there was no significant difference in COD, TN and TP removal rates between algal-fungi and algal-bacteria
cultures (p > 0.05), and there was no significant difference on energy efficiency CO2 removal by the three cultures
(p > 0.05). For these two cocultivations, the energy efficiencies of pollutant removal were higher, but energy effi-
ciencies of CO2 removal were lower of than that of mono-algae culture. Microalgae will grow well in the influent
C/N ratio of 5:1 and improve the sewage purification, as a result, the economic efficiency of energy consumption
are increased13. For algal-bacteria culture, due to nitrification and denitrification of activated sludge, the eco-
nomic efficiency of nitrogen removal achieve higher than algal-fungal culture39, 44.

**Conclusion**  
Both of the microalgal culture methods and C/N ratios had significant effects on of synthetic domestic sewage
purification and biogas upgrading. The medium level of C/N ratio showed higher pollutants and CO2 removal
efficiencies than low and high C/N ratios. Co-culture of C. vulgaris with G. lucidum or activated sludge in photo-
bioreactors was more effective than mono-cultivation on removing sewage pollutants and CO2 in biogas simulta-
neously according to the data in this study. Coculture of microalgae with fungi was the suitable treatment
technology for wastewater purification and biogas upgrading under the C/N ratio of 5:1.

**Methods**  
**Algae sources and culture conditions.** The C. vulgaris (FACHB-31) was used for sewage treatment and
biogas upgrading based on its high biogas tolerance and rapid growth in high-strength wastewater58. The strain
was purchased from the FACHB-Collection, Institute of Hydrobiology, Chinese Academy of Sciences. The BG-11
culture media was prepared for growing the microalgal culture49, with initial pH adjusted to 6.9. The culture

| C/N ratio  | Economic efficiency (USD−1) |
|-----------|----------------------------|
|           | COD | TN | TP | CO2 |
| Mono-algae culture |     |     |    |     |
| C2.5N1-COD100 Low COD level | 37.34± ± 2.28 | 37.42± ± 2.37 | 27.28± ± 1.74 | 29.58± ± 1.94 |
| C5N1-COD200 Medium COD level | 39.81± ± 2.74 | 39.09± ± 2.58 | 36.44± ± 2.06 | 37.63± ± 3.18 |
| C10N1-COD400 High COD level | 36.16± ± 1.97 | 36.77± ± 2.61 | 28.36± ± 1.93 | 35.44± ± 2.86 |
| C10N1-TN20 Low TN level | 39.96± ± 2.45 | 42.33± ± 2.53 | 37.24± ± 2.32 | 35.89± ± 3.05 |
| C5N1-TN40 Medium TN level | 43.27± ± 3.18 | 43.07± ± 3.18 | 40.31± ± 2.17 | 38.76± ± 2.89 |
| C5N1-TN80 High TN level | 35.83± ± 1.84 | 42.58± ± 2.72 | 35.57± ± 2.09 | 28.07± ± 2.77 |
| Algal-fungal culture |     |     |    |     |
| C2.5N1-COD100 Low COD level | 44.28± ± 3.22 | 42.26± ± 2.34 | 43.19± ± 2.75 | 31.42± ± 2.35 |
| C5N1-COD200 Medium COD level | 46.16± ± 2.83 | 45.78± ± 2.57 | 45.98± ± 2.37 | 38.17± ± 2.49 |
| C10N1-COD400 High COD level | 45.07± ± 2.71 | 42.63± ± 2.28 | 43.52± ± 2.63 | 30.61± ± 2.78 |
| C6N1-TN20 Low TN level | 45.19± ± 3.05 | 43.92± ± 3.08 | 44.13± ± 2.79 | 37.94± ± 3.37 |
| C5N1-TN40 Medium TN level | 45.75± ± 3.29 | 45.99± ± 3.11 | 46.37± ± 3.07 | 38.23± ± 2.34 |
| C5N1-TN80 High TN level | 45.41± ± 2.98 | 42.18± ± 2.62 | 42.23± ± 2.66 | 34.73± ± 3.39 |
| Algal-bacterial culture |     |     |    |     |
| C2.5N1-COD100 Low COD level | 41.62± ± 2.65 | 42.34± ± 2.37 | 36.05± ± 2.17 | 35.59± ± 2.68 |
| C5N1-COD200 Medium COD level | 42.87± ± 3.01 | 45.58± ± 2.68 | 42.51± ± 3.79 | 38.21± ± 3.27 |
| C10N1-COD400 High COD level | 39.25± ± 2.42 | 42.41± ± 2.26 | 36.87± ± 2.96 | 35.79± ± 2.96 |
| C6N1-TN20 Low TN level | 43.83± ± 2.79 | 45.72± ± 2.89 | 40.94± ± 2.34 | 37.32± ± 3.11 |
| C5N1-TN40 Medium TN level | 45.32± ± 2.61 | 46.65± ± 3.42 | 42.98± ± 3.83 | 38.63± ± 3.09 |
| C5N1-TN80 High TN level | 42.12± ± 2.83 | 42.52± ± 2.63 | 41.22± ± 2.62 | 28.85± ± 2.83 |

Table 4. The economic efficiency of energy consumption on synthetic domestic sewage purification and biogas
upgrading at different C/N ratio with three different culture methods. Values with different superscript letters
in the same column for the same method of treatments indicate significant differences at p = 0.05 according to
Duncan’s multiple range tests.
conditions were as follows: the wavelength spectrum and photosynthetic photon flux density (PPFD) of cool-white LED light were 360–720 nm and 200 μmol m$^{-2}$ s$^{-1}$, respectively. The temperature and light period were 25 ± 0.5°C and 12 h light-12 h dark, respectively. The cultures were intermittently shaken three times a day (8:00 a.m., 2:00 p.m., and 8:00 p.m.).

Algal-fungal culture conditions. Based on the preliminary experiment results, we found that *Ganoderma lucidum* (*G. lucidum*, 5.765) achieved high growth rate and high performance of pelletization with *C. vulgaris*. Therefore, the *Ganoderma lucidum* stain obtained from China General Microbiological Culture Collection Center was selected for this study. An inoculum was prepared by inoculating 100 mL of a synthetic medium with 25 mycelial discs. The composition of synthetic medium was as follows: glucose, 10 g L$^{-1}$; NH$_4$NO$_3$, 2.0 g L$^{-1}$; K$_2$HPO$_4$, 1.0 g L$^{-1}$; NaH$_2$PO$_4$·H$_2$O, 0.4 g L$^{-1}$; MgSO$_4$·7H$_2$O, 0.5 g L$^{-1}$; yeast extract, 2.0 g L$^{-1}$; pH 6.5. The prepared inoculum was then incubated at 25 ± 1°C on a rotary shaker at 160 rpm for 7 d. The obtained biomass was washed with sterile distilled water and homogenized with 100 mL of sterile distilled water in a laboratory blender. Subsequently, the obtained cultures were used for the co-cultivation of microalgal cells.

Each 100 mL Microalgal suspension (158.37 ± 14.26 mg L$^{-1}$) was mixed with 5 mL *G. lucidum* suspension (82 ± 8 mg L$^{-1}$) for pelletization. The fungal-algal mixtures were shaken at 160 rpm for 7 d under constant PPFD (200 μmol m$^{-2}$ s$^{-1}$) at 25 °C. All experiments were performed in triplicate.

Algal-bacterial Culture conditions. The synthetic domestic sewage was inoculated with 1 L cultured microalgae strain and 200 mL activated sludge. The total suspended solid (TSS) of microalgae and activated sludge were 0.75 g TSS L$^{-1}$ and 4.14 g TSS, respectively. The activated sludge came from a wastewater treatment plant in Jiaxing, Zhejiang, China. The light intensity and temperature were the same as the algal-fungi culture.

Photobioreactor. Two interconnected 16.8 L (individual) glass cylinder blocks with a height of 0.6 m and a diameter of 0.2 m were used as a photobioreactor (Fig. 5). The reactors were hermetically sealed with rubber stoppers. The sampling outlet consisted of a plug and rubber gasket. The synthetic domestic sewage was pumped from the right to the left cylinder block of the photobioreactor in one time. The raw biogas was blown into the photoreactors from the raw biogas inlet until the air in the headspace was expelled.

Synthetic domestic sewage and biogas. To facilitate comparison with similar experiments, this study used the synthetic domestic sewage, a modification of the OECD standard sewage$^{44}$. The concentrations of TN and COD were adjusted, whereas the TP was not. The experiment was divided into two groups. In group 1, low (100 mg L$^{-1}$), medium (200 mg L$^{-1}$), and high (400 mg L$^{-1}$) levels of COD (fixed TN/TP levels at medium strength) were designated as C2.5N1-COD100, C5N1-COD200, and C10N1-COD400. In group 2, low (20 mg L$^{-1}$), medium (40 mg L$^{-1}$), and high levels (80 mg L$^{-1}$) of N (fixed COD/TP levels at medium strength) were designated as C2.5N1-TN80, C5N1-TN40, C10N1-TN20. Medium for group 1 was prepared by mixing the following components: 100, 200, and 400 g m$^{-3}$ glucose, respectively; 80 g m$^{-3}$ carbamide; 15 g m$^{-3}$ NaH$_2$PO$_4$·1.5 g m$^{-3}$ KH$_2$PO$_4$; 4 g m$^{-3}$ CaCl$_2$; and 2 g m$^{-3}$ MgSO$_4$. Medium for group 2 was: 200 g m$^{-3}$ glucose; 40, 80, and 160 g m$^{-3}$ carbamide; 15 g m$^{-3}$ NaH$_2$PO$_4$·1.5 g m$^{-3}$ KH$_2$PO$_4$; 4 g m$^{-3}$ CaCl$_2$; and 2 g m$^{-3}$ MgSO$_4$. Table 5 shows the characteristics of the synthetic domestic sewage.

Biogas was obtained from a farm biogas plant in JiaYuan Green Meadow. Prior to the experiments, the biogas was pretreated via chemical absorption to reduce the H$_2$S content less than 0.005% (v/v). The raw biogas mainly
Under different influent C/N ratios, growth rate and pollutants removal efficiencies of the three algal source cultures and their roles in biogas upgrading were determined. Gas samples (100 mL) were drawn daily at the sampling port of the photobioreactor, to monitor the COD, TN, and TP concentrations.

### Experimental procedure

The photobioreactor was filled with 14 L of raw biogas and 2.8 L of synthetic domestic sewage and illuminated on 200 μmol m$^{-2}$ s$^{-1}$ by six fluorescent lamps arranged in a circular configuration (20 W, 110 V) around the left cylinder block (Fig. 1). The initial dry weight (DW) of the three selected cultures was measured according to the reference. Daily biomass productivity ($P$, g L$^{-1}$ d$^{-1}$) was calculated using Equation (1):

$$P = \frac{(D_t - D_0)}{(t_t - t_0)}$$  \hspace{1cm} (1)

where $D_t$ is the biomass concentration (g L$^{-1}$) at time $t_t$ (d) and $D_0$ is the initial biomass concentration (g L$^{-1}$) at $t_0$ (d).

The culture filtrates were analyzed for COD, TN, and TP concentrations by using standard methods. The pH value and dissolved oxygen (DO) was measured using a pH (Orion 250 Aplus ORP Field Kit, USA) and oxygen probes (Model 862 Aplus, USA). Pollutants (COD, TN, and TP) removal efficiency was calculated as follows:

$$R = (1 - C_t/C_0)$$  \hspace{1cm} (2)

where $R$ is the pollutant removal efficiency (%), $C_0$ and $C_t$ are the pollutant concentrations in the initial synthetic domestic sewage and in the filtrates of the cultures (mg L$^{-1}$), respectively.

### Sampling and analyses

The DW of the three algal source cultures was measured according to the reference. The economic efficiency of the energy consumption for nutrient removal in sewage and CO$_2$ removal in biogas were calculated by Eq. (3)

$$E = \frac{R}{KTP}$$  \hspace{1cm} (3)

### Table 5. Parameters of influent synthetic sewage and crude biogas in the photobioreactor.

| C/N ratio                | Influent concentration (mg L$^{-1}$) |
|-------------------------|--------------------------------------|
|                         | COD       | TN          | TP          |
| Mono-algae culture      |           |             |             |
| C2.5N1-COD100 Low COD level | 102.16 ± 3.21 | 43.12 ± 3.54 | 5.21 ± 0.42 |
| C5N1-COD200 Medium COD level | 203.77 ± 6.19 | 41.08 ± 2.75 | 5.39 ± 0.57 |
| C10N1-COD400 High COD level | 408.35 ± 8.67 | 44.63 ± 3.18 | 5.11 ± 0.74 |
| C10N1-TN20 Low TN level | 202.49 ± 5.11 | 42.42 ± 2.06 | 5.33 ± 0.62 |
| C5N1-TN40 Medium TN level | 205.18 ± 4.09 | 43.71 ± 2.83 | 5.46 ± 0.83 |
| C2.5N1-TN80 High TN level | 206.03 ± 7.16 | 82.75 ± 4.35 | 5.08 ± 0.49 |
| Algal-fungal culture    |           |             |             |
| C2.5N1-COD100 Low COD level | 103.83 ± 4.28 | 42.07 ± 2.98 | 5.41 ± 0.72 |
| C5N1-COD200 Medium COD level | 204.39 ± 5.93 | 44.24 ± 3.04 | 5.22 ± 0.63 |
| C10N1-COD400 High COD level | 403.58 ± 8.47 | 42.35 ± 3.76 | 5.28 ± 0.57 |
| C10N1-TN20 Low TN level | 206.34 ± 7.53 | 22.31 ± 2.53 | 5.05 ± 0.66 |
| C5N1-TN40 Medium TN level | 208.37 ± 8.95 | 41.64 ± 2.32 | 5.14 ± 0.58 |
| C2.5N1-TN80 High TN level | 203.67 ± 7.02 | 84.09 ± 3.84 | 5.23 ± 0.67 |
| Algal-bacterial culture |           |             |             |
| C2.5N1-COD100 Low COD level | 106.25 ± 3.89 | 40.86 ± 3.21 | 5.39 ± 0.84 |
| C5N1-COD200 Medium COD level | 208.02 ± 5.92 | 42.24 ± 2.95 | 5.04 ± 0.62 |
| C10N1-COD400 High COD level | 407.11 ± 7.81 | 43.31 ± 3.76 | 5.28 ± 0.25 |
| C10N1-TN20 Low TN level | 205.27 ± 6.47 | 22.26 ± 2.99 | 5.19 ± 0.57 |
| C5N1-TN40 Medium TN level | 208.81 ± 8.43 | 41.06 ± 4.33 | 5.23 ± 0.65 |
| C2.5N1-TN80 High TN level | 201.34 ± 7.29 | 84.32 ± 3.27 | 5.37 ± 0.53 |

The photobioreactor contained microalgal for wastewater treatment. Under different influent C/N ratios, growth rate and pollutants removal efficiencies of the three algal source cultures and their roles in biogas upgrading were determined.
where E is the energy consumption for nutrient removal in sewage and CO₂ removal in biogas, USD⁻¹; R is the removal efficiency in Eq. (2); k stands for the electric power charge per unit of energy consumption, USD kw⁻¹h⁻¹; T is the illumination time according to photoperiod; h is the LED electrical power consumption, W. The electric power charge per unit of energy consumption k is around 0.08826 USD kw⁻¹h⁻¹ in local after conversion.¹

**Statistical analyses.** All statistical analyses were performed using the package SPSS (SPSS 2013). A one-way analysis of variance (ANOVA) was used to test the statistical differences of the 6 C/N ratios for the same microalgae cultures. Duncan's multiple range tests was employed to further test for significant differences among the treatments with different C/N ratios. A two-way ANOVA was used to test for differences among the effects of different light wavelengths, algae sources, and the interaction between any two of these factors on treatment performance. The threshold for statistical significance was set at p = 0.05. Error bars in the figures showed the standard deviation with n = 3.

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Author Contributions
Jie Xu, Xue Wang and Shiqing Sun conducted the experiments. Yongjun Zhao and Changwei Hu prepared the figures and wrote the main manuscript text. Shiqing Sun and Yongjun Zhao revised the manuscript. All authors reviewed the manuscript.

Additional Information
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