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# Cultivation of oleaginous microalgae for removal of nutrients and heavy metals from biogas digestates

Suo Yang [a], Jin Xu [a, b, c], Zhong-Ming Wang [b], Lian-Jun Bao [a], Eddy Y. Zeng [a, *]

[a] School of Environment, Guangzhou Key Laboratory of Environmental Exposure and Health, and Guangdong Key Laboratory of Environmental Pollution and Health, Jinan University, Guangzhou 510632, China
[b] Key Laboratory of Renewable Energy, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences, Guangzhou 510640, China
[c] Department of Ecology, Jinan University, Guangzhou 510632, China

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ABSTRACT

Cultivation of microalgae in anaerobically digested effluent is beneficial for removal of nutrients, such as nitrogen and phosphorus, and heavy metals at reduced cost and generation of biomass to produce biofuels. In the present study, four strains of oleaginous microalgae, separated from fresh water in South China, were evaluated for their ability to remove nitrogen and phosphorus. The results showed that *Scenedesmus* sp. (GN 171) was capable of removing nitrogen and phosphorous in BG-11 medium at the rates of 97% and 99%. Two types of anaerobically digested effluents, i.e., grass anaerobically digested effluent and molasses wastewater anaerobically digested effluent, were chosen as the nutrient sources. These anaerobically digested effluents diluted in BG-11 medium or with tap water were used as the substitute medium to cultivate GN 171; other pretreatment methods, such as sterilization and dilution ratio, were also taken into consideration. A ratio of 1/3 for unsterilized grass anaerobically digested effluent/tap water mixture (designated as G4) was the optimal proportion for growth of GN 171 and removal of nitrogen and phosphorus at a large scale. The dry weight of GN 171 reached 3.2 g $L^{-1}$, and the total lipid, carbohydrate and protein contents were 34%, 30% and 16% in G4. The efficacies for removal of total nitrogen, ammonia nitrogen, phosphorus and selected heavy metals were generally satisfactory. It seems possible to use ADE, rather than any artificial medium, as the sole nutrient source for microalgae production.



## 1. Introduction

Biogas production is one of the environmentally friendly technologies, which is widely used as an important source of renewable energy. Anaerobically digested effluent (ADE) is a main by-product of biogas production, commonly used as a valuable fertilizer as it contains abundant nutrients (Haraldsen et al., 2011). Nitrogen in ADE causes substantial environmental pollution in the forms of ammonia and nitrogen oxides. Eutrophication of water body is worsen by excessive nitrogen and phosphorus (Azevedo et al., 2015). Anaerobically digested effluent may also contain heavy metals and organic pollutants which can be accumulated in crops, posing potential risk to human health (Stenchly et al., 2017). The treatment techniques often involve nitrification and denitrification for removing nitrogenous compound by releasing $NH_3$ to the atmosphere (Tiwary et al., 2015). Chemical precipitation was also used to remove phosphorus through the use of ferrous chloride (Kiran et al., 2014). These methods are not environmentally friendly and cost-effective, and at the same time produce sludge as by-product.

Microalgae have been considered as the next generation energy feedstock (Chen et al., 2015). However, biofuel production has not been exploited industrially because of the high cost related to microalgae cultivation, which requires large amounts of water, nutrients and trace elements. The first attempt to cultivate microalgae in wastewater was made in the 1950s (Golueke and Oswald, 1959). Microalgae can be cultivated in urban (Caporgno et al., 2015) or animal wastewater (Lu et al., 2015) using freshwater or marine strains, so as to depurate wastewater and produce biomass. A combination of wastewater treatment and microalgae biomass production is potentially an environmentally friendly approach for achieving waste reduction and energy reutilization.

* Corresponding author. Tel:. +86-20-85226853.
*E-mail address:* eddyzeng@jnu.edu.cn (E.Y. Zeng).

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Anaerobically digested effluent often contains abundant dissolved nutrients and trace elements for microalgae growth, which has stimulated research efforts in recent years. Uggetti et al. (2014) found that microalgae growth was satisfactory using anaerobic digestate as a substrate but was reduced with increasing amount of anaerobic digestate. Most studies have used ADEs from dairy (Wang et al., 2010) and swine manure (Cheng et al., 2015), poultry litter (Singh et al., 2011) and municipal wastewater (Batista et al., 2015); microalgae can efficiently uptake nutrients from ADE while producing valuable biomass that can be used for the production of bioenergy. Energy grass (Li et al., 2015) and molasses wastewater (Li et al., 2014a) become the feedstock for anaerobic digestion in recent years. Use of these ADEs to cultivate microalgae has not been explored. Because energy grass and molasses wastewater are the main raw materials to generate biogas, development of low-cost technologies such as microalgae cultivation to treat ADEs is environmentally significant. However, the use of ADEs as the medium for growing microalgae may reduce growth rates, alter biochemical constituents and promote the growth of undesired organisms (Vasseur et al., 2012). The occurrence of ammonia in ADEs often inhibits microalgae growth (Källqvist and Svenson, 2003). The high turbidity of ADEs leads to low photosynthetically active radiation, thereby reduce the growth of microalgae (Wang et al., 2010). These problems can be overcome by using ADEs at suitable concentrations. Deionized water (Ledda et al., 2016) and seawater (Sepúlveda et al., 2015) have been used as diluent, but microalgae growth rates were lower than those reported with pure synthetic media. The difference between tap water and normal medium used as diluent has not been studied, but searching for affordable diluents is important for developing cost-effective methods for scale-up microalgae cultivation. Another key point in using ADEs to cultivate microalgae is the negative effect of bacteria on microalgae growth and biomass biochemical constituents. Whether sterilization would affect microalgae growth is worth examining. It is worthwhile to investigate the use of ADEs from different fermentation materials as nutrient sources in microalgae growth and biomass production.

The objectives of the present study were therefore to (1) select suitable oleaginous strains for removal of nitrogen and phosphorous; (2) determine the optimal conditions for culturing the selected microalgae in ADEs for removal of nitrogen, phosphorus and heavy metals in large scale and (3) assess the ability of the selected microalgae cultured in ADEs to produce biomass and energy substances. To accomplish these objectives, four strains of oleaginous microalgae were evaluated. One strain with the greatest ability to uptake nitrogen and phosphorous was chosen for cultivation in ADEs. The present results are of interest for the integration of ADE treatment and microalgae cultivation, turning waste into treasure and also achieving the goal of resource recycling.

## 2. Material and methods

### 2.1. Sources of anaerobically digested effluents and microalgae strains

Anaerobically digested effluents used in the present study were obtained from a biogas laboratory at the Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences. There are two types of ADEs originating from different fermentation materials. One is energy grass, harvested from the north campus of South China University of Technology (Guangzhou, China). Molasses-alcoholic wastewater was collected from Wuming Heli Biological Chemical (Guangxi, China). Anaerobically digested effluents produced from energy grass and molasses-alcoholic wastewater was designated as grass anaerobically digested effluent (GADE) and molasses wastewater anaerobically digested effluent (MADE), respectively. All ADEs were filtered by six layers of gauze to remove large suspended solids and centrifuged at 3500 rpm for 5 min with an Eppendorf centrifuge; the supernatants were collected for cultivating microalgae. The four oleaginous strains used in the present study were *Scenedesmus* sp.-1 (GN 28), *Chloroccucom* sp. (GN 38), *Chlorella* sp. (GN 44) and *Scenedesmus* sp.-2 (GN 171), which have been preserved at the Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences upon separation and purification from rivers and lakes in South China.

### 2.2. Anaerobically digested effluent cultivation

Suitable candidates were selected from four strains of oleaginous microalgae, with the potential for growth and effectively removing nitrogen and phosphorus. The selected strain was used to inoculate glass bubble–column photobioreactor containing ADEs diluted with BG-11 medium or tap water. The optimal volume ratio in the present study was determined in trial experiments. The GADE/tap water ratios of 1/3, 1/1, and 3/1 in volume were tested, and a ration of 1/3 was the best for microalgae growth. BG-11 medium and tap water were used as diluent to evaluate the effects of the fertilizers. Sterilization and unsterilization were used to observe the effects of the co-occurrence of algae bacteria. Ten experiments were carried out (Table 1), in duplicates.

### 2.3. Culture conditions

The microalgae cells were expanded to the logarithmic phase to inoculate with an initial optical density of $OD_{750} = 0.5$. Cultures were incubated in a glass bubble–column photobioreactor (6.0 cm diameter and 60 cm height) at 25 ± 1 °C, and agitated by aeration enhanced with 1%–2% of $CO_2$ under continuous white fluorescent illumination at a light intensity of 300 μmol photon $m^{-2}$ $s^{-1}$.

### 2.4. Sample collection

During the cultivation period, medium samples were collected immediately upon resuspension (0 day) and every two days afterwards to determine dry weight, cell density, total pigment (chlorophyll *a*, chlorophyll *b* and carotenoid), total nitrogen (TN), nitrate nitrogen ($NO_3$-N), nitrite nitrogen ($NO_2$-N), ammonia ($NH_3$-N), total phosphorus (TP), chemical oxygen demand (COD), pH and metal elements (As, Cr, Mn, Fe, Co, Ni, Cu, Zn, Mo, Cd, Ba and Pb). A certain volume of microalgae was harvested by centrifugation every four days and used to determine total lipid, carbohydrate and protein contents.

### 2.5. Determination of growth parameters

Cell density was measured through direct counting by optical microscopy. Water samples were filtrated through pre-dried and pre-weighed 0.45 μm acetate cellulose filters, and the filters were dried at 80 °C in an oven until constant weight was reached, cooled down in a desiccator and weighed again to obtain dry sample weights. Pigment contents in microalgae were measured with an ultraviolet spectrophotometer after microalgae were extracted with methanol until they were colorless. Pigment contents were calculated by

$$\text{Chl. } a\left(\text{mg L}^{-1}\right) = 16.72 \times OD_{665} - 9.16 \times OD_{652} \quad (1)$$

**Table 1**
Experimental designs.

| Trials | Parallel group | Dilution | | ADE | | Whether sterilization | |
|---|---|---|---|---|---|---|---|
| | | BG-11 medium | Tap water | GADE | MADE | Sterilization | Unsterilization |
| G1 | 2 | 75% | | 25% | | Sterilization | |
| G2 | 2 | 75% | | 25% | | | Unsterilization |
| G3 | 2 | | 75% | 25% | | Sterilization | |
| G4 | 2 | | 75% | 25% | | | Unsterilization |
| G5 | 2 | | | 100% | | Sterilization | |
| G6 | 2 | | | 100% | | | Unsterilization |
| M7 | 2 | 75% | | | 25% | Sterilization | |
| M8 | 2 | 75% | | | 25% | | Unsterilization |
| M9 | 2 | | 75% | | 25% | Sterilization | |
| M10 | 2 | | 75% | | 25% | | Unsterilization |

$$\text{Chl. } b\left(\text{mg L}^{-1}\right) = 34.09 \times \text{OD}_{652} - 15.28 \times \text{OD}_{665} \quad (2)$$

$$\text{Carotenoid}\left(\text{mg L}^{-1}\right) = (1000 \times \text{OD}_{470} - 1.63 \times \text{Chl. } a - 104.96 \times \text{Chl. } b)/221 \quad (3)$$

where Chl. *a*, Chl. *b* and Carotenoid represent the contents of chlorophyll *a*, chlorophyll *b* and carotenoid and $OD_{750}$, $OD_{665}$, $OD_{652}$ and $OD_{470}$ are optical densities at 750, 665, 652 and 470 nm, respectively.

### 2.6. Determination of physical and chemical parameters

The collected samples were centrifuged at 3500 rpm for 5 min and supernatants were filtered with 0.45 μm pore-size membranes. TN, TP, $NO_3$-N, $NO_2$-N, $NH_3$-N and COD in the supernatants were measured with a Hach DR2700 spectrophotometer (Loveland, CO, USA) coupled with a Hach DRB200 rector (Loveland, CO, USA). Values of pH were measured with a Mettler-Toledo SG2-T pH meter (Zurich, Switzerland). Concentrations of As, Cr, Mn, Fe, Co, Ni, Cu, Zn, Mo, Cd, Ba and Pb were determined with a Pekin Elmer NexIon 300× inductively coupled plasma mass spectrometer (Waltham, MA, USA) after digestion with nitric acid (Krejcova et al., 2006).

### 2.7. Determination of biochemical constituents

Algae samples were centrifuged at 3500 rpm for 5 min and the supernatants were removed. The filtered algae samples were dried in a vacuum freeze dryer at −80 °C for 72 h, and the freeze dried samples were used for determination of total lipids, carbohydrates and protein. Total lipids were extracted from algae using a previously described method (Khozin-Goldberg et al., 2005) with slight modifications. Lipids were extracted with dimethylsulfoxide-methanol, diethyl ether and hexane. The combined extract was mixed with water to form a ratio of 1/1/1/1 in volume. The upper layer containing lipids was collected and evaporated to dryness under a gentle $N_2$ stream. Intracellular carbohydrates were extracted by acid hydrolysis with 0.5 N $H_2SO_4$ for 2 h and were measured by a phenol-sulphuric method (Dubois et al., 1956). Contents of proteins extracted with 0.5 N NaOH for 2 h were estimated using the Lowry method (Lowry et al., 1951) and Protein Quantitation Kit offered by Nanjing Jiancheng Biological Engineering Institute.

## 3. Result and discussion

### 3.1. Evaluation of microalgae strains in BG-11 medium

The cultured strains of microalgae were evaluated in terms of their growth in biomass and cell density, ability to generate biochemical constituents and capacity for removal of nitrogen and phosphorus. Evolution of the dry weights and cell densities of GN 28, GN 38, GN 44 and GN 171 in BG-11 medium (Fig. 1a and b) indicated that the four strains of microalgae were able to adapt to the new conditions quickly and grew at a rapid pace after 2 days. The growth became steady after 10 days, and the maximum dry weights reached 4.1, 3.2, 2.8 and 4.5 g $L^{-1}$ (Fig. 1a and Supplementary Data (SD) Table S1). The change in cell density (Fig. 1b) followed a typical pattern for batch cultivation. The cell densities of GN 28 and GN 171 were much lower than those of GN 38 and GN 44, but their dry weights were greater than those of GN 38 and GN 44 (Fig. 1b). This was because the cell sizes of GN 28 and GN 171 are generally larger than those of GN 38 and GN 44. The pigment contents varied with microalgae species, but the trends were similar, i.e., they increased rapidly in the early stage and became steady or declined slowly in the late period (SD Fig. S1).

The concentrations of TN in culture medium dropped rapidly within the first two days and declined slowly afterwards (Fig. 1c), attaining removal efficiencies of 36%, 82%, 67% and 97% for GN 28, GN 38, GN 44 and GN 171 (SD Table S1). The initial concentration of TP was 16.7 mg $L^{-1}$, and the four strains of microalgae were able to quickly remove phosphorus in the early stage of culture (Fig. 1d), with a greater than 99% removal efficiency (SD Table S1). GN 171 had the greatest capacity of uptaking nitrogen and phosphorus among the four strains of microalgae. Effective removal of TN and TP in the early stage of culture indicated that microalgae cultivation can remove nitrogen and phosphorus without acclimation process. Microalgae acclimation was not necessary in removing nitrogen and phosphorus, as opposed to what was suggested previously (Osundeko et al., 2014). Nitrogen and phosphorus were absorbed by microalgae for synthesis of proteins, phospholipids, enzymes and nucleic acids, which largely dictate microalgae metabolism.

The occurrence of biochemical constituents in microalgae is a crucial indicator of the utility of microalgae in production of biodiesel and animal feed. Lipid content was reduced within the first four days, and increased by day 8; it constituted more than 30% of the microalgae dry weights in GN 38 and GN 44, which were higher than those in GN 28 and GN 171 in the late growth stage (Fig. 2a and SD Table S2). The decline of lipid content during the first four days may be attributed to the cell division under favorable growth conditions. The carbohydrate contents reached maxima at different time points for different microalgae strains, e.g., GN 28 and GN 44 in day 8, GN 38 in day 12 and GN 171 in day 16 (Fig. 2b). GN 28

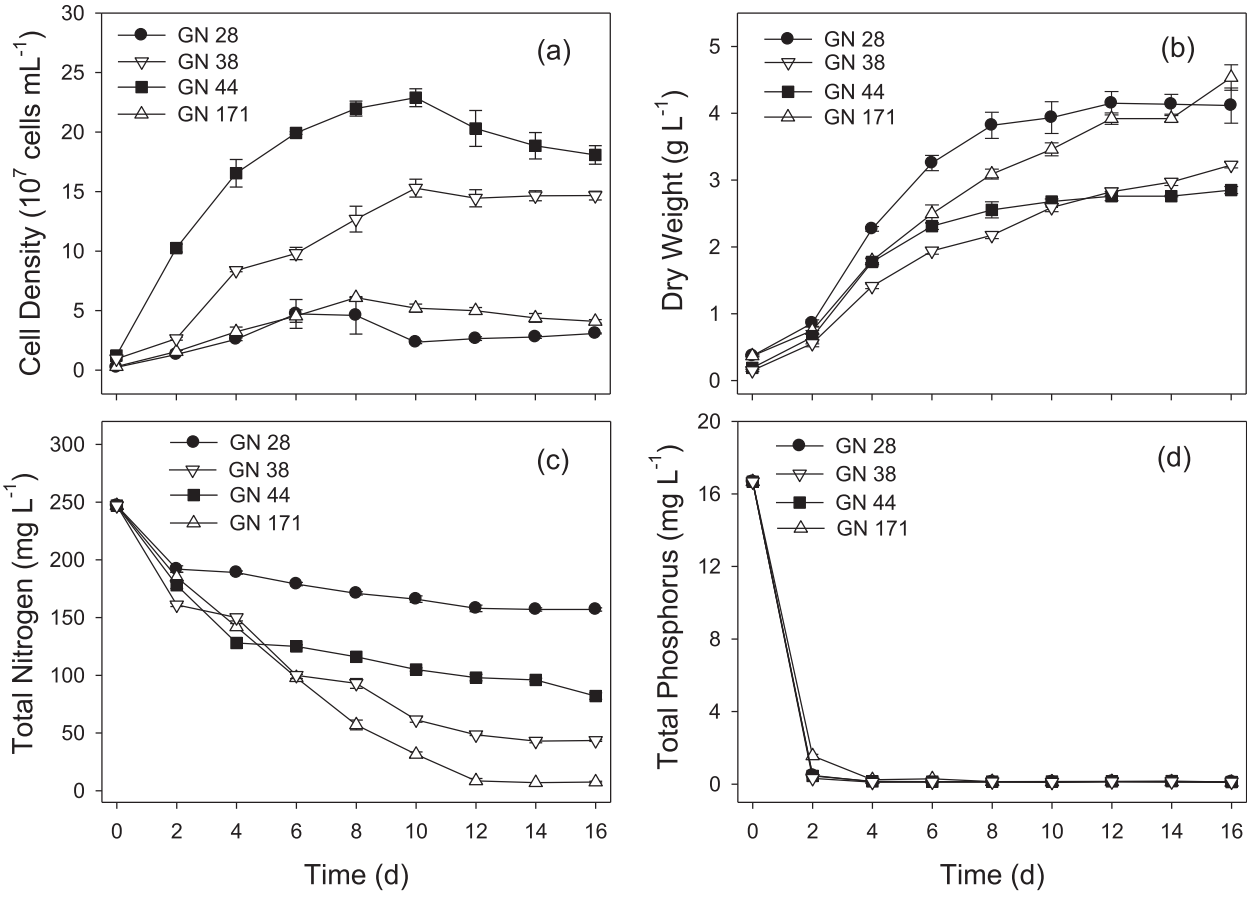


**Fig. 1.** Microalgae growth and nutrients concentration in BG-11 medium for *Scenedesmus* sp.-1 (GN 28), *Chloroccucom* sp. (GN 38), *Chlorella* sp. (GN 44) and *Scenedesmus* sp.-2 (GN 171) over cultivation time. (a) dry weight, (b) cell density, (c) total nitrogen and (d) total phosphorus.

contained the highest carbohydrate content, reaching approximately 50% of microalgae dry weight, followed by GN 171, GN 44 and GN 38 (Fig. 2b and SD Table S2). The carbohydrate content of GN 171 reached 38% in day 16 (SD Table S2). Protein contents in four strains of microalgae showed similar declining trends during cultivation (Fig. 2c), which can be explained by breakdown of proteins to other substances while energy was released for synthesis of lipids and carbohydrates. Nitrogen and phosphorus were removed from the culture medium, and the metabolic flux of microalgae was changed, leading to altered biomass biochemical constituents. The present results inferred that GN 28 and GN 171 prefer to produce carbohydrate and GN 38 and GN 44 are likely to generate lipid.

Based on the above results, GN 171 was selected as the microalgae strain to further cultivate in the ADEs for assessment of biomass production and removal of nitrogen and phosphorus.

### 3.2. Removal of pollutants by cultivated GN 171

Initial values of pH in the ADEs were 8–9 and increased to 10–11 when the ADEs were sterilized (Fig. 3), e.g., the initial pH in G5 was 11, higher than that in G6 (8.2) (SD Table S3). The difference in pH values between sterilization and unsterilization can be attributed to decomposition of some substances in the ADEs at high temperatures to produce $OH^-$ and therefore higher pH. The pH in the culture media decreased in the first two days and varied in a narrow range of 7–8, but it was higher in G5 and G6 (8–9; Fig. 3a). The drop in pH within first two days was probably due to injection of $CO_2$ and release of $NH_3$. Ammonium may be generated in alkaline culture medium by $NH^{4+} + OH^- \leftrightarrows NH_3 + H_2O$. In this case, stirring promoted the release of $NH_3$ to air, resulting in decreased pH (MartıNez et al., 2000). The pH of the trials G5–G6 was high due to high concentrations of ammonia nitrogen.

The profiles of TN, $NH_3$-N, $NO_3$-N and TP concentrations with culture time in all trials are depicted in Fig. 4. The concentrations of TN and $NH_3$-N decreased when culture medium was sterilized (Fig. 4a–d), e.g., the initial concentrations of TN and $NH_3$-N in G6 were 685 and 594 mg $L^{-1}$ higher than those in G5 (430 mg $L^{-1}$ for TN and 400 mg $L^{-1}$ for $NH_3$-N) which was sterilized (SD Table S3). The decline of TN and $NH_3$-N concentrations was probably resulted from decomposition of some unstable nitrogenous substances upon autoclaving. Although the TN concentrations were different in all the trials, nitrogen was mostly in the form of $NH_3$-N, e.g., the initial concentration of TN in G4 was 178 mg $L^{-1}$, with that of $NH_3$-N at 159 mg $L^{-1}$ (SD Table S3). Similar result has been reported recently (Xia and Murphy, 2016). The removal efficiency was 20%–92% of TN, 33%–100% of $NH_3$-N, 10%–74% of $NO_3$-N and 7%–98% of TP (SD Table S1). The concentrations of TN, $NH_3$-N, $NO_3$-N and TP in all trials decreased due to consumption and absorption of nutrients by GN 171. The removal efficiency of TN was higher in G1–G4 than in G5–G6, suggesting that GADE diluted with BG-11 medium or tap water may be more suitable for GN 171 cultivation; the removal efficiency of TN was higher in G1–G4 than in M7-M10, suggesting that GADE may be more suitable for GN 171 cultivation. The residual TN concentrations in all trials declined with increasing culture time, except for G5 where the TN concentration slightly

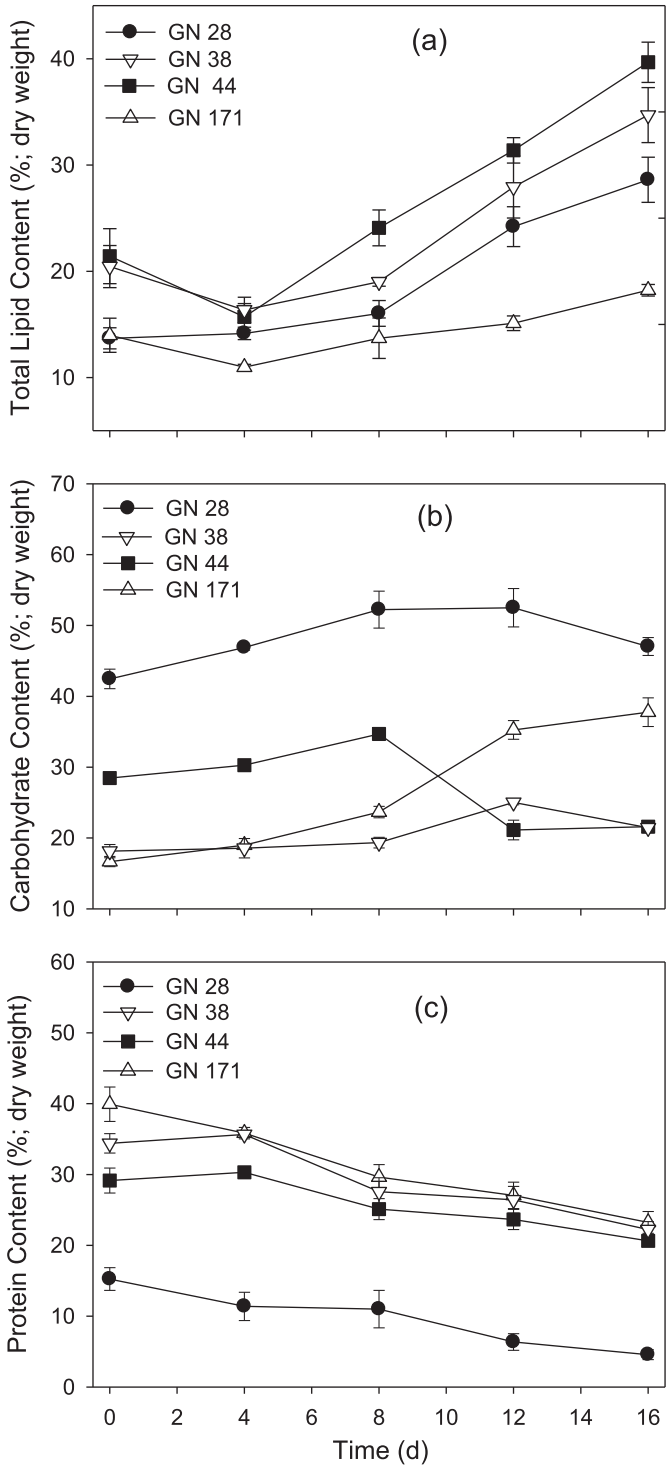


**Fig. 2.** Variations of total lipid, carbohydrate and protein of *Scenedesmus* sp.-1 (GN 28), *Chloroccucom* sp. (GN 38), *Chlorella* sp. (GN 44) and *Scenedesmus* sp.-2 (GN 171) cultured in BG-11 medium during the cultivation.

declined in the first six days, increased from day 6 to day 10 and decreased again at the late stage (Fig. 4a). The decline of TN concentration in the first six days and the second decline were both attributed to volatilization of $NH_3$, while the increase between the declines might be caused by dead microalgae cells, which released nitrogenous substances to the medium.

The concentration of $NH_3$-N decreased with cultivation time (Fig. 4c and d) but the $NO_3$-N concentration remained largely unchanged (Fig. 4e and f). This indicated that nitrogen was consumed mainly in the form of ammonia nitrogen. In G1, there was no ammonia nitrogen after day 4 (Fig. 4c); the concentration of $NO_3$-N started to decrease after 2 days (Fig. 4e). Apparently GN 171 preferred to utilize $NH_3$-N and did not use $NO_3$-N until $NH_3$-N was considerably depleted. Similar results were also observed in other studies, e.g., $NH_3$-N was first utilized when microalga *Scenedesmus* sp. LX1 was cultivated with nitrogen of different sources (Xin et al., 2010). In all trials, the concentration of $NO_2$-N remained low, in the ranges of 0.035–0.1 mg $L^{-1}$ initially and 0.04–0.4 mg $L^{-1}$ in the final stage of the cultivation (SD Table S3). The $NO_2$-N concentration in G1–G2 and M7–M8 sharply increased in cultivation (SD Fig. S2), possibly because $NO_3$-N was more abundant in these trials than in other trials and was converted to $NH_3$-N which can be utilized by GN 171 to produce $NO_2$-N.

The concentrations of residual phosphorus dropped considerably during the first two days of culturing in trials G1–G4, G6 and M8–M10 (Fig. 4g and h), which is attributable to consumption of GN 171 and formation of insoluble precipitates. GN 171 could effectively metabolize both nitrogen and phosphorus without suppressing assimilation of any nutrients. The concentration of TP fluctuated in G5 (Fig. 4g), but in M7 it rose slightly during the first two days of culturing, decreased substantially from day 4 to day 10 and stayed steady after 10 days (Fig. 4h). The increase in the TP concentration may have resulted from metabolism of microalgae, generating phosphorous substances in the medium.

Efficiency of COD removal in all trials except M9 was 6%–49%; the COD concentration in M9 slightly increased from 634 mg $L^{-1}$ to 752 mg $L^{-1}$ (SD Table S3). The increase of COD was perhaps derived from generation of organic matter during metabolism of microalgae. Concentration of COD slightly declined or fluctuated within a small range in all trials except M9 (SD Fig. S3). Previous studies proposed that COD concentration may be lowered with microalgae cultivation. For example, Travieso et al. (2006) and Cheng et al. (2015) reported COD removal rates of 88% and 79%, which were much higher than the removal rates in the present study and a previous study (28%–38%) (Wang et al., 2010). The low removal rates can be explained by the presence of recalcitrant organic compounds in the remaining COD which are hardly degradable by microalgae cultivation (Tan et al., 2014). Some organic compounds can be either naturally excreted via microalgae growth or suddenly released via cell lysis (Hadj-Romdhane et al., 2013), thus affecting COD removal.

Anaerobically digested effluents contained various metal elements at different concentrations, such as Al, Mn, Fe, Zn, Mo, Ba and Cu at >100 μg $L^{-1}$ and Cr at < 5 μg $L^{-1}$ (SD Table S4). Although heavy metals can be prohibitive to microalgae growth, the concentrations of Cr, Cd and Pb in ADEs were too low to cause negative effects. Inhibition of *Stichococcus bacillaris* growth was negligible with lead concentration at lower than 1.036 mg $L^{-1}$ (Pawlikskowrońska, 2002). During the cultivation, the removal efficiencies for 12 metals varied widely from 17% to 97% (Table 2). Cheng et al. (2015) also obtained removal rates of 35%–90% for heavy metals in ADEs. Table 2 indicates that removal efficiency was different in different trials even for the same metal, possibly because the ADEs were obtained from different reactors with various fermentation materials. However, removal efficiencies for some metals were high in all trials, e.g., Mn (51%–98%), Fe (48%–87%) and Cu (32%–90%) (Table 2). This was likely due to absorption of the metals by GN 171 to maintain its growth. Different removal rates for different metals in the same trial were attributed to the dependence of the removal capacity by GN 171 on the type of metal elements. GN 171 has demonstrated the potential for removal of heavy metals while growing in the ADEs (Table 2). Chan et al. (2014) also achieved the

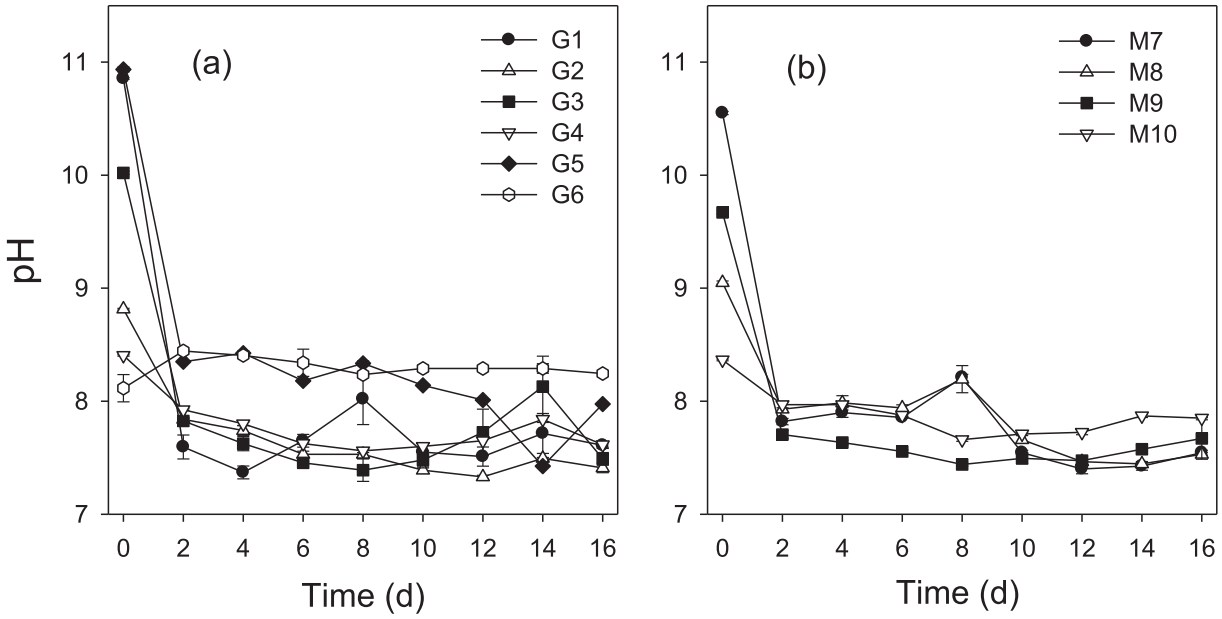


**Fig. 3.** Variation of pH values in all trials during the cultivation time. G1 (grass anaerobically digested effluent (GADE)/BG-11 = 1/3 in volume; sterilized), G2 (GADE/BG-11 = 1/3 in volume; unsterilized), G3 (GADE/tap water = 1/3 in volume; sterilized), G4 (GADE/tap water = 1/3 in volume; unsterilized), G5 (pure GADE; sterilized), G6 (pure GADE; unsterilized), M7 (molasses wastewater anaerobically digested effluent (MADE)/BG-11 = 1/3 in volume; sterilized), M8 (MADE/BG-11 = 1/3 in volume; unsterilized), M9 (MADE/tap water = 1/3 in volume; sterilized) and M10 (MADE/tap water = 1/3 in volume; unsterilized).

same results using two microalgae strains to remove heavy metals from secondary effluent. Heavy metals can be eliminated through a combined process of biosorption and bioaccumulation (Jahan et al., 2004), but the removal capacity of microalgae may be affected by metal concentration, metal species, culture conditions and pH among others (Chipasa, 2003).

### 3.3. Growth of GN 171 in anaerobically digested effluents

Compared to pure GADE for microalgae culture, diluted GADE enhanced biomass growth, e.g., the dry weights of trials G1–G4 were 4.0, 4.3, 3.2 and 3.2 g $L^{-1}$, higher than G6 (1.7 mg $L^{-1}$) and G5 without any growth (SD Table S4). GN 171 could not even survive in the pure GADE medium with sterilization (G5), mainly because of high pH (~11) (SD Table S3). High pH favors generation of free ammonia from ammonia nitrogen, which is harmful to microalgae (Abeliovich and Azov, 1976). GN 171 experienced a long lag at the beginning of culturing in G6 and started to grow after 6 days (Fig. 5a), probably because GN 171 underwent physiological adjustments to the complex composition of ADEs. The temporal trends of the dry weights in M7–M10 were similar to those in G1–G4, increasing with cultivation time (Fig. 5a and b); the dry weights in M7–M10 were 1.9, 2.7, 1.4 and 1.9 g $L^{-1}$, lower than those in G1–G4 (SD Table S4). The temporal trends were different for the cell densities of G1–G4 and M7–M10, i.e., the cell densities of the strain cultured in M7–M10 substantially trailed those cultured in G1–G4 during the first eight days (Fig. 5c and d). GADE is more suitable for GN 171 growth than MADE, as MADE contained excessive organics inhibiting the growth of microalgae (SD Table S3), which was also corroborated by a previously reported finding that higher than 1000 mg $L^{-1}$ concentration of COD inhibited the growth of microalgae (Travieso et al., 2006).

BG-11 medium diluted GADE (G1 and G2) was more suitable than GADE diluted by tap water (G3 and G4) for GN 171 growth (Fig. 5c), mainly because tap water lacked certain necessary elements for microalgae growth. Sterilization had no effect on cultivation of GN 171 with GADE (1/3 in volume) as the medium because the dry weights were not significantly different in G1 and G2 (*t*-test; $p = 0.10$). However, the dry weight in the diluted MADE without sterilization (M8) was heavier than that with sterilization (M7) (Fig. 5b), as a result of the co-occurrence of algae and bacteria. The bacteria can be the "helpers" for microalgae growth (de-Bashan et al., 2004). Sterilization impacted GN 171 growth differently in two ADEs, as different bacteria in the ADEs played different roles in microalgae growth. Bacteria decompose organic matter in ADEs and supply carbon to the culture, enhancing microalgae production (Uggetti et al., 2014). However, C. *pyrenoidosa* stimulated better growth in wastewater with sterilization than without sterilization, i.e., bacterial activities in wastewater may impose negative influences on microalgae growth (Tan et al., 2014). Some bacteria, such as *Myxobacter*, Cytophaga *Vibrio*, *Pseudomonas*, *Flavobacterium* and *Alteromonas*, can excrete some extracellular substances which inhibit or kill microalgae cells (Kim et al., 2007). It is important to identify which bacteria are beneficial to microalgae growth and can be utilized in large-scale culture. It is also important to understand the mechanism on how bacteria improve microalgae production, which currently remains largely unclear.

### 3.4. Biochemical constituents of GN 171

The lipid contents in G1–G6 and M7–M10 increased gradually from day 4 to day 16 (Fig. 6a and b). Lipid accumulation was a response to the deficiency of nitrogen. The tap water diluted ADE was more suitable for microalgae to accumulate lipid, as it contains relatively low TN concentration. For example, the lipid content was higher in G3 than in G1 (Fig. 6a) and higher in M9 than in M7 at the end of cultivation (Fig. 6b). Among all groups, the highest lipid content was achieved in G3, accounting for 34% of the dry weight (SD Table S5). The TN concentration in G3 was the lowest at day 6 (Fig. 4a), which limited microalgae growth and thrusted lipid accumulation. Some previous studies also reported the effects of nitrogen limitation on lipids accumulation. For example, *C. vulgaris* accumulated lipid to 50% of dry weight at low nitrogen concentrations (Markou, 2015). High lipid content (53%) was accumulated

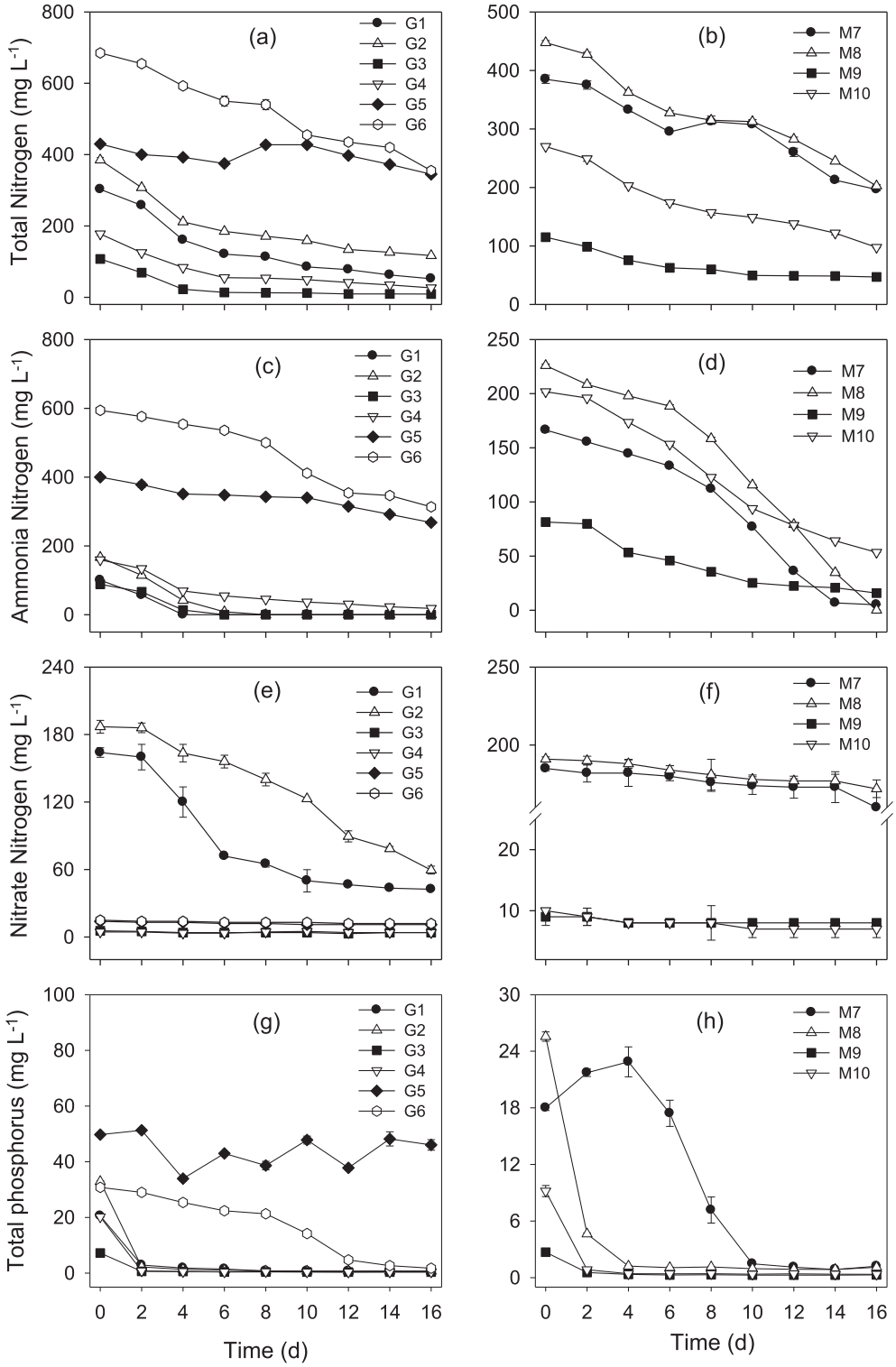


**Fig. 4.** Variations of total nitrogen (TN), ammonia nitrogen ($NH_3$-N), nitrate nitrogen ($NO_3$-N) and total phosphorus (TP) concentration in groups during culture periods. G1 (grass anaerobically digested effluent (GADE)/BG-11 = 1/3 in volume; sterilized), G2 (GADE/BG-11 = 1/3 in volume; unsterilized), G3 (GADE/tap water = 1/3 in volume; sterilized), G4 (GADE/tap water = 1/3 in volume; unsterilized), G5 (pure GADE; sterilized), G6 (pure GADE; unsterilized), M7 (molasses wastewater anaerobically digested effluent (MADE)/BG-11 = 1/3 in volume; sterilized), M8 (MADE/BG-11 = 1/3 in volume; unsterilized), M9 (MADE/tap water = 1/3 in volume; sterilized) and M10 (MADE/tap water = 1/3 in volume; unsterilized).

in *C. protothecoides* with depleted nitrogen, which was substantially higher than that (22%) with co-depletion of N and P (Li et al., 2014b).

The carbohydrate contents in all trials were 5%–30% of dry weight at the end of cultivation, lower than those in the control group cultivated with BG-11 medium (Fig. 6c and d). The carbohydrate content in G5 was only 5% of dry weight, but was over 11% in other groups (Fig. 6c and d). The possible reason is that high pH

**Table 2**
Removal efficiency (%) of heavy metals in G1-G6 and M7-M10.[a]

| Heavy metals | G1 | G2 | G3 | G4 | G5 | G6 | M7 | M8 | M9 | M10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Fe | 87 | 86 | 68 | 84 | 54 | 61 | 67 | 48 | 76 | 64 |
| Co | 61 | 61 | 62 | 66 | 29 | 32 | 49 | 18 | 69 | 56 |
| Ni | 28 | 30 | 41 | 57 | 31 | 40 | 51 | 23 | 82 | 61 |
| Cu | 88 | 90 | 80 | 83 | 32 | 50 | 76 | 60 | 82 | 73 |
| Zn | 51 | 60 | 58 | 67 | 41 | 62 | 70 | 36 | 77 | 66 |
| Mn | 95 | 97 | 77 | 87 | 73 | 90 | 75 | 71 | 51 | 59 |
| Ba | 36 | 57 | 29 | 28 | 57 | 25 | 53 | 16 | 49 | 50 |
| Mo | 93 | 95 | 61 | 88 | 22 | 82 | 33 | 29 | 27 | 30 |
| As | 39 | 41 | 34 | 34 | 38 | 44 | 41 | 42 | 39 | 42 |
| Cd | 73 | 75 | 73 | 72 | 67 | 75 | 67 | 66 | 70 | 70 |
| Pb | 50 | 62 | 47 | 28 | 42 | 61 | 53 | 49 | 63 | 56 |
| Cr | 63 | 55 | 72 | 50 | 45 | 35 | 49 | 32 | 63 | 42 |

[a] G1 = grass anaerobically digested effluent (GADE)/BG-11 (1/3 in volume; sterilized); G2 = GADE/BG-11 (1/3 in volume; unsterilized); G3 = GADE/tap water (1/3 in volume; sterilized); G4 = GADE/tap water (1/3 in volume; unsterilized); G5 = pure GADE (sterilized); G6 = pure GADE (unsterilized); M7 = molasses wastewater anaerobically digested effluent (MADE)/BG-11 (1/3 in volume; sterilized); M8 = MADE/BG-11 (1/3 in volume; unsterilized); M9 = MADE/tap water (1/3 in volume; sterilized) and M10 = MADE/tap water (1/3 in volume; unsterilized).

or great nutrient concentrations in pure ADE inhibited the biosynthesis of carbohydrates and affected the growth of microalgae cells. It took microalgae a long time to adapt to such high nutrient concentrations, which consumed large amounts of energy to establish self-protection against potentially harmful effects of environmental toxins, e.g., forming antioxidant pigments and thickening of cell walls (Osundeko et al., 2014). The underlying mechanism, however, remains to be determined. GN 171 cultivated in G1–G4 contained higher carbohydrate contents (25%–30%) than those (11%–21%) cultivated in M7–M10 (SD Table S5). This can be attributed to the dark color and composition of MADE, which may have inhibited the synthesis of carbohydrates. The color of ADE may alter light absorption over the entire visible spectrum and substantially affect the photosynthesis of microalgae, as reported in the growth of *Scenedesmus* sp (Marcilhac et al., 2014).

The protein contents of GN 171 in culture medium decreased with cultivation time, except for G5 and G6 (Fig. 6e and f). The protein content of GN 171 cultured in G5 declined from 40% to 16% within four days and started to increase from day 4 to day 16. In addition, it declined from 40% to 22% within four days and reached 32% by day 8 in G6 (Fig. 6e). A plausible explanation is that many impurities in pure ADE (G5 and G6) contained low contents of proteins, resulting in relatively low protein contents in GN 171 on the 4th day; proteins might degrade to other intracellular substances to resist environmental stress. After a few days, GN 171 gradually adapted to the pure ADE and began to grow rapidly. Anaerobically digested effluent could generate a large number of low molecular weight compounds after autoclaved sterilization treatment. These compounds could be easily assimilated by GN 171,

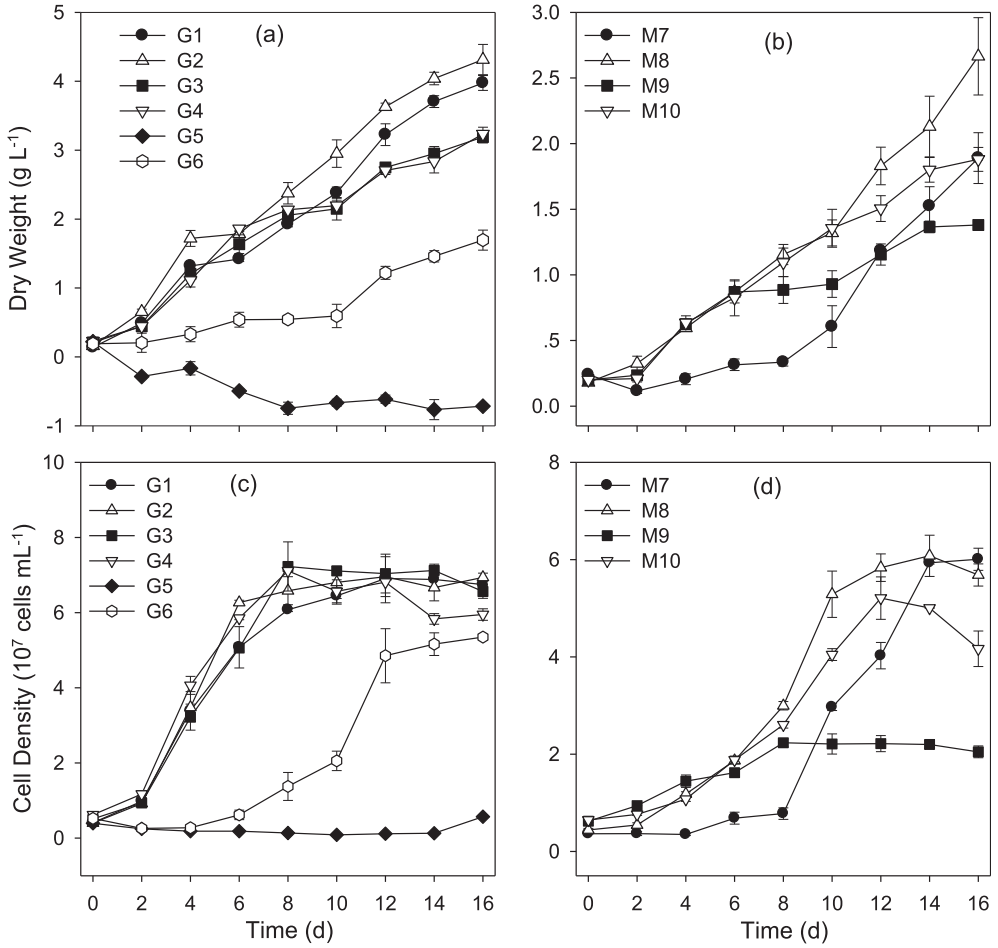


**Fig. 5.** Growth of *Scenedesmus* sp-2 (GN 171) during the batch culture at different trials. G1 (grass anaerobically digested effluent (GADE)/BG-11 = 1/3 in volume; sterilized), G2 (GADE/BG-11 = 1/3 in volume; unsterilized), G3 (GADE/tap water = 1/3 in volume; sterilized), G4 (GADE/tap water = 1/3 in volume; unsterilized), G5 (pure GADE; sterilized), G6 (pure GADE; unsterilized), M7 (molasses wastewater anaerobically digested effluent (MADE)/BG-11 = 1/3 in volume; sterilized), M8 (MADE/BG-11 = 1/3 in volume; unsterilized), M9 (MADE/tap water = 1/3 in volume; sterilized) and M10 (MADE/tap water = 1/3 in volume; unsterilized).

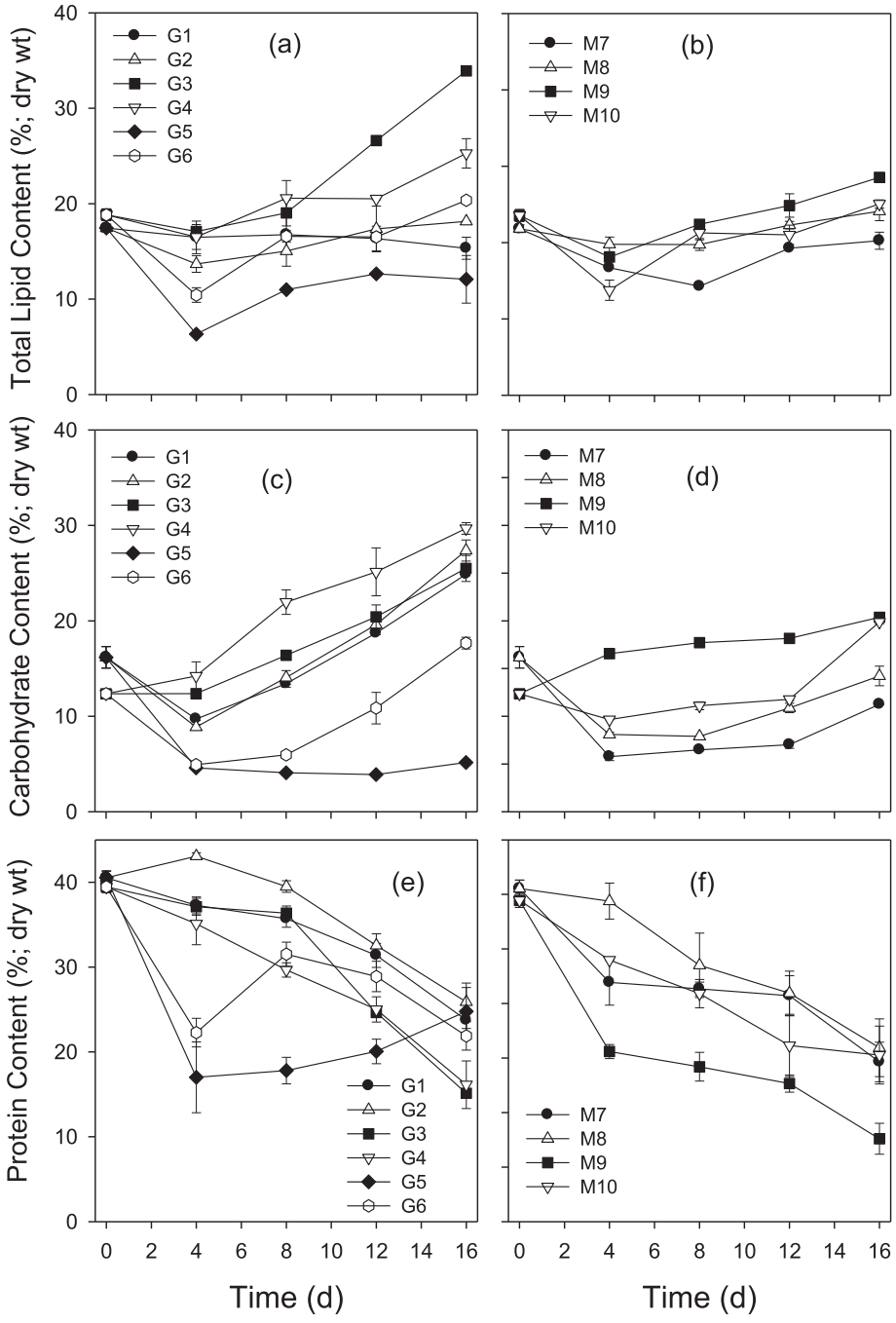


**Fig. 6.** Variations of total lipid contents, carbohydrate contents and protein contents of *Scenedesmus* sp-2 (GN 171) cultured in different trials during cultivation. G1 (grass anaerobically digested effluent (GADE)/BG-11 = 1/3 in volume; sterilized), G2 (GADE/BG-11 = 1/3 in volume; unsterilized), G3 (GADE/tap water = 1/3 in volume; sterilized), G4 (GADE/tap water = 1/3 in volume; unsterilized), G5 (pure GADE; sterilized), G6 (pure GADE; unsterilized), M7 (molasses wastewater anaerobically digested effluent (MADE)/BG-11 = 1/3 in volume; sterilized), M8 (MADE/BG-11 = 1/3 in volume; unsterilized), M9 (MADE/tap water = 1/3 in volume; sterilized) and M10 (MADE/tap water = 1/3 in volume; unsterilized).

which accounted for the increase in the protein contents of GN 171 from day 4 to day 16. Under stress conditions such as nitrogen and phosphorus starvation, microalgae alter their metabolism and often accumulate carbohydrates and/or lipids as energy storage. Markou et al. (2012) found that *A. platensis* accumulated carbohydrates up to 65% and lipids up to 7.5%, but showed reduced protein contents under phosphorus limitation. Depraetere et al. (2015) observed an increase in the amount of carbohydrates and a reduction in the amount of proteins under nitrogen deprivation. If the protein content in microalgae cells is low, the cultivation conditions may be adjusted to alter the metabolic processes of microalgae cells and drive up protein contents. However, adjusting cultivation conditions to increase protein contents is costly and the related technology is immature. It is better to explore the alternative uses of microalgae biomass, e.g., as raw materials to produce biomass energy, such as bioethanol and biodiesel.

## 4. Conclusions

The integration of both microalgae biomass production and ADE treatment is positive and technically feasible. The optimal conditions for microalgae biomass production and ADE treatment determined in the present study are valuable for scale-up cultivation, which have not been adequately addressed in previous studies. Among the four strains of microalgae examined, GN 171 could be cultivated in ADEs and achieved considerable biomass concentrations and high efficiency for removal of nitrogen and phosphorus, as well as certain heavy metals, such as Mn, Fe, Cu, Mo, Co, Ni, Zn and Cd. The GADE is more suitable for GN 171 cultivation than MADE, but should be diluted to achieve high biomass concentration. The culture medium can be diluted with tap water to reduce the cost of ADE treatment and microalgae cultivation, at the same time ensuring highest lipid contents in microalgae biomass. As sterilization adds additional expenditures and constraints to scale–up microalgae cultivation with the use of ADEs, unsterilization should be the better choice. The composition of the culture medium also affected the biochemical constituents of microalgae, with lipid, carbohydrate and protein content being close relation of nutrients in ADE. The biomass of GN 171, containing high contents of lipids and carbohydrates, may be used as the feedstock of biodiesel.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jclepro.2017.06.221.

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