Science of the Total Environment 768 (2021) 144462

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Swine digestate treatment by prior nitrogen-starved *Chlorella vulgaris*: The effect of over-compensation strategy on microalgal biomass production and nutrient removal

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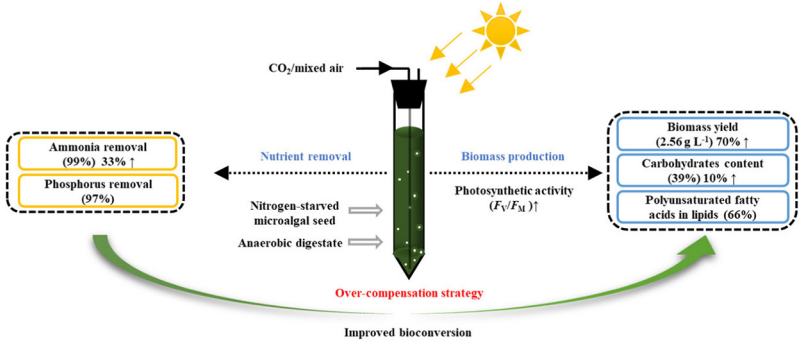
HIGHLIGHTS

- Microalgae are effective in recycling digestate up to 350 mg L^{-1} ammonia nitrogen.
- Microalgal growth was improved by 70% using nitrogen-starved seed.
- Over-compensation boosted ammonia nitrogen removal from the digestate to 99%.
- Microalgae with over-compensation had nearly triple photosynthetic activity.
- Biomass has 39% carbohydrates and lipids contain 66% polyunsaturated fatty acids.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 September 2020
Received in revised form 30 November 2020
Accepted 7 December 2020
Available online 6 January 2021

Editor: Jose Julio Ortega-Calvo

Keywords:
Microalgae
Digestate
Nitrogen-starved seeds
Nutrient removal
Microalgal products

ABSTRACT

Anaerobic digester effluent containing high levels of ammonia poses a threat to the environment. To hinder this issue, a modern and promising treatment method incorporates both microalgae and their bioconversion potential. When culturing *Chlorella vulgaris* at a 1:7 digestate supernatant dilution ratio, biomass concentration was 1.33 g L^{-1} and 66% of ammonia nitrogen was removed. Furthermore, a prior nitrogen-starved seed method, namely over-compensation strategy, was applied to improve both biomass production and nutrient removal. By using nitrogen-starved seeds after a 48 h nitrogen-free stimulation, biomass yield increased by 1.7-times to 2.56 g L^{-1}. Simultaneously, ammonia nitrogen and total phosphorus removal efficiencies reached 99% and 97% respectively. The enhanced production corresponds to higher chlorophyll fluorescence in the middle and late stages of the culture. In addition, the bioproduct contained 39% carbohydrates, and the proportion of polyunsaturated fatty acids in lipids was 66%. These findings demonstrated that the over-compensation strategy contributed to greater nitrogen removal and high-value bioproduct production in the microalgae-digestate treatment system.

1. Introduction

With the rapid development of the agriculture industry, livestock manure generated is approximately 4 billion tons per year in China,

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https://doi.org/10.1016/j.scitotenv.2020.144462
0048-9697/

consisting greatly of pig manure (Ouyang et al., 2015). Accordingly, more than 113,000 biogas projects through anaerobic digestion have been built in China as of 2016. These anaerobic digesters are expected to treat waste as well as produce clean energy, about 14.5 billion m^3 of biogas annually reported by the ministry of agriculture of China. However, a large amount of digestate sewage is improperly disposed of into nature, putting tremendous pressure on the surroundings through harmful effects such as air pollution by ammonia volatilization and eutrophication (Godos et al., 2010). These environmental threats call for digestate treatment technologies to be developed urgently to further improve the sustainability of the digestion process and to improve the agricultural applications of the digestate (Huang et al., 2014). Traditional physicochemical methods (e.g. ammonia stripping and struvite precipitation) are costly, and pollutant removal efforts are limited due to the downsides of single pollutant removal at a time (Lei et al., 2007). In addition, potential biotreatment faces the restriction of high ammonia nitrogen concentrations (Kaparaju and Rintala, 2008). Therefore, the environmental concerns shown for sustainable biogas projects and digestate should be the main consideration, with economic feasibility of the digestate recovery methods following.

Anaerobic digestate provides a potential cultivation system for microalgae as it contains substantial nitrogen, phosphorus, micronutrients, and water resources. Microalgal culture paired with digestate effluent provides numerous benefits such as yielding little to no cost and providing digestate treatment, proven to be feasible by various research (Garcia et al., 2017; Kobayashi et al., 2013; Singh et al., 2011). However, the industrial application of microalgal cultures is hindered by low removal efficiency of ammonia nitrogen. To help alleviate this bottleneck problem, the over-compensation strategy arose. An unusually high rate of nitrogen assimilation was observed if nitrogen became available to nitrogen-starved cells (Cho and Komor, 1984; Xie et al., 2017). When re-exposed to nitrogen-rich surroundings, microalgal cells were triggered to uptake more nitrogen than that necessary for survival. This phenomenon may be related to the level of keto-acids which form the carbon skeleton in the assimilation process of ammonium to glutamate. Millbank (1957) proved that the level of keto-acids was much higher under the condition of nitrogen starvation as opposed to a regular autotrophic state of *Chlorella*. Consequently, digestate treatment using nitrogen-starved microalgae is proposed to achieve efficient nitrogen removal.

In addition to treatment potential, microalgal biomass is a kind of value-added animal feed and biorefinery feedstock. Microalgae are able to synthesize considerable amounts of polyunsaturated fatty acids (PUFAs) in the biomass (Bellou et al., 2014). More specifically, linolenic (C18:3) and linoleic acids (C18:2) belonging to omega-3 and omega-6 families are good nutritional supplements for various applications, such as in marine fish food for fisheries (Bellou et al., 2014; Radwan, 1991). In addition, carbohydrates accumulated by microalgae are a potential feedstock for bulk chemicals, e.g., bioethanol, acetone and butanol (Castro et al., 2015).

The objective of this work is to recycle digestate through the use of microalgae by applying the over-compensation strategy. Microalgae were cultivated in a swine digestate system bubbling with CO_2-mixed air. Microalgal growth, photosynthetic activity, nitrogen and phosphorus removals were studied to optimize the prior nitrogen starvation process. Moreover, biomass product was evaluated based on its biochemical composition.

2. Materials and methods

2.1. Microorganisms and wastewater

The experimental microalga, *Chlorella vulgaris* FACHB-8, was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). Microalgae were pre-cultured in modified SE medium (Xie et al., 2012). Microalgal cells were collected by centrifugation at 3000 ×*g* for 2 min and the pellet was used as the inoculation seed.

The anaerobic digestate was collected from the pilot test base, digesting piggery wastewater (Chengdu, China). The slurry was stored at 4 °C temporarily. The digestate effluent was centrifuged at 4000 ×*g* for 5 min to remove most of the residual solids and then filtered using a 0.22 μm pore membrane (Jinteng Co., Ltd., Tianjin, China) to remove the remaining microorganisms. The main characteristics of the pretreated piggery digestate are given in Table 1.

2.2. Microalgal culture

The tubular photoreactors are made of quartz glass with a diameter of 45 mm, a height of 450 mm, and a working capacity of 500 mL. The photoreactors were placed in an illuminating frame under continuous illumination of 200 μmol photons m^{-2} s^{-1} at a constant temperature of 25 ± 1 °C. In addition, these tubes were equipped with silicone plugs and capillary aeration tubes with an inner diameter of 0.9 mm. The gas of 2% CO_2-mixed air was bubbled into the tubular reactors at a flow rate of 0.2 vvm.

To evaluate microalgal adaptability in swine digestate, four initial digestate concentrations were studied. The prepared concentrations were 1:1, 1:3, 1:5 and 1:7 digestate supernatant to deionized water ratios. The samples were analyzed every day to evaluate nutrient removal and microalgal growth. Additionally, dry cell weight and value-added biocomponents such as lipids, proteins, and carbohydrates were assessed at the conclusion of the cultures. All the experiments were conducted in triplicate.

To perform the over-compensation strategy, the microalgae seeds were stimulated by different durations of nitrogen starvation (12, 24, 36, and 48 h, respectively) in a nitrogen-free SE medium. Cultures using the seeds without nitrogen-starvation were carried out as the control. Parameter measurements were the same as above. Additionally, the control experiments without microalgae were conducted in the 1:1, 1:3, 1:5, and 1:7 diluted digestates for quantifying the volatile ammonia. Except for no inoculation, the other conditions remained the same with the corresponding experimental group, and system pH was adjusted daily.

2.3. Analytical methods

2.3.1. Microalgal growth

Optical density (OD) of the culture medium at a wavelength of 680 nm was measured daily as an algal density indicator using a spectrophotometer (AOE, UV/Vis A-360, Shanghai, China). At the end of culture, the harvested biomass yield was estimated from the dry cell weight. Triplicate samples were filtered through pre-weighted glass microfiber filters (GF/C, Whatman, UK) and dried overnight at 105 °C.

Table 1
Characteristics of the experimental piggery digestate.

Ingredient	Undiluted digestate	Ingredient	Undiluted digestate
pH	8.3–8.5	K (mg L^{-1})	875.5
TN (mg L^{-1})	1610–1659	Na (mg L^{-1})	498.0
NH_3-N (mg L^{-1})	1402–1492	Ca (mg L^{-1})	42.4
TP (mg L^{-1})	37.1–42.0	Zn (mg L^{-1})	0.3
COD[a] (mg L^{-1})	1300–1400	Fe (mg L^{-1})	2.0
TS[b] (g L^{-1})	4.8 ± 0.0	Cu (mg L^{-1})	0.1
VS[c] (g L^{-1})	1.7 ± 0.1	Mn (mg L^{-1})	0.6
TSS[d] (g L^{-1})	3.8 ± 0.2	Ni (mg L^{-1})	0.1
VSS[e] (g L^{-1})	1.1 ± 0.2	Cr (mg L^{-1})	0.3

[a] COD, chemical oxygen demand.
[b] TS, total solids.
[c] VS, volatile solids.
[d] TSS, total suspended solids.
[e] VSS, volatile suspended solids.

The derivation of the average specific growth rate (*SGR*, d^{-1}) in exponential phase is described as follows:

$$SGR = \frac{\ln OD_2 - \ln OD_1}{t_2 - t_1} \quad (1)$$

where OD_1 and OD_2 are the optical densities at times t_1 and t_2 (d), respectively.

To quantify the photosynthetic activity of microalgae, chlorophyll fluorescence was determined using a portable chlorophyll fluorometer Os30p+ (Opti-sciences, USA). The parameter F_V/F_M represents the maximum potential quantum efficiency of photosystem II of microalgae (PS II), which can reflect microalgal photosynthetic activity (Markou and Muylaert, 2016), calculated as:

$$F_V/F_M = (F_M - F_0)/F_M \quad (2)$$

where F_0 and F_M are respectively, the minimum and maximum fluorescence of PS II.

For the determination of pigment concentration, 2 mL of samples were centrifuged at 9000 ×*g* for 2 min. The supernatant was removed, and the cells were washed twice with deionized water. The microalgae pellets were then resuspended in absolute methanol. Following that step, an ultrasound pretreatment (high intensity ultrasonic processor, 250 W model) at an amplitude of 30% for 2 min and On/Off pulses of 5/5 was applied to avoid overheating of the samples. Subsequently, the methanol liquid was sealed for 24 h in the dark at 4 °C. Chlorophyll a (Chl-a) concentration was measured at 665.2 nm and 652.4 nm using the spectrophotometer. Chl-a concentration ($C_{Chl\text{-}a}$, mg L^{-1}) was calculated from the equation (Lichtenthaler, 1987):

$$C_{Chl-a} = 16.72Abs_{665.2nm} - 9.16Abs_{652.4nm} \quad (3)$$

2.3.2. Wastewater parameters

The analysis of pH was performed through use of a pH meter (ARK, pHS—4C+, China). The chemical oxygen demand (COD), ammonia nitrogen, and total phosphorus were examined with standard APHA methods (APHA, 1998). Total nitrogen was quantified by an alkaline potassium persulfate digestion-UV spectrophotometric method (Nydahl, 1978). The concentrations of metal irons were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent 720, USA).

The removal efficiency (*RE*, %) and average removal rate (*ARR*, mg L^{-1} d^{-1}) of nitrogen and phosphorus were defined as:

$$RE(\%) = \left(1 - \frac{C_t}{C_0}\right) \times 100 \quad (4)$$

$$ARR\left(\text{mg L}^{-1}\text{ d}^{-1}\right) = \frac{C_0 - C_t}{t - t_0} \quad (5)$$

where C_0 (mg L^{-1}) is the initial concentration of nitrogen and phosphorus, while C_t (mg L^{-1}) is the concentration of nitrogen and phosphorus at time *t* (d).

2.3.3. Biochemical component

Carbohydrates analysis was done using an anthrone-sulfuric acid colorimetric method (Laurentin and Edwards, 2003). Proteins were analyzed using a BCA Protein Assay Kit (Beyotime Biotech Inc., China). Lipids were extracted with a chloroform-methanol solution (1:1, v/v) and quantified gravimetrically (Bligh and Dyer, 1959). After methyl esterification, the fatty acid methyl ester (FAME) composition was analyzed by gas chromatography and mass spectrometry (GC/MS, QP2010, Shimadzu, Japan), following the conditions described by Liu et al. (2012).

2.4. Statistical analyses

All the values were reported as mean ± standard deviation from three independent experiments. Analysis of differences was performed in the form of ANOVA using trial SPSS software V20 (SPSS, USA). The significance threshold was set at a probability level of $p = 0.05$.

3. Results and discussion

3.1. Microalgal cultivation in digestate

3.1.1. Microalgal growth

Microalgal growth curves in a series of digestate dilutions using CO_2/air bubbling photobioreactors are shown in Fig. 1a. With the 1:3, 1:5, and 1:7 dilution ratios, all underwent a lag phase of 1 to 2 days and then entered a rapid growth period. The specific growth rate of *C. vulgaris* was the highest (0.89 ± 0.01 d^{-1}) under the 1:7 dilution ratio, followed by 0.84 ± 0.01 d^{-1} with the 1:5 dilution ratio and 0.35 ± 0.01 d^{-1} with the 1:3 dilution ratio (Table 2). The maximum optical density (OD_{680} 3.4 ± 0.2) occurred for the 1:7 dilution ratio, which was an increase of 1.89-fold compared to the 1:3 dilution ratio (OD_{680} 1.8 ± 0.1). After 3 days, microalgal cultures with the 1:7 and 1:5 dilution ratios reached the plateau phase, while the 1:3 dilution ratio needed twice the amount of time. The tardive cell replication and long lag phase observed at the 1:3 dilution ratio was due to high ammonia nitrogen (349.1 ± 2.7 mg L^{-1}) (Kumar et al., 2010). Nevertheless, minimizing the lag phase and maximizing exponential growth better satisfied the objective for bioprocess design in the 1:7 and 1:5 dilution ratios (Najafpour, 2015). Similarly, the harvested cell dry weight proved to be greatest for the 1:7 dilution ratio, being 1.33 ± 0.15 g L^{-1} (Table 2). The following was 1.15 ± 0.03 g L^{-1} and 0.65 ± 0.04 g L^{-1} for the 1:5 and 1:3 dilution ratios, respectively. As shown in Table 1, the digestate supernatant provided essential macro elements of nitrogen and phosphorus as well as several suitable metal elements (e.g., K, Na, Ca, Zn and Fe) for microalgae. Moreover, carbon dioxide as an additional inorganic carbon source was mixed in the bubbling air, in consideration of a low carbon to nitrogen molar ratio (0.9; the ideal value of 6.6) (Redfield, 1958) and low BOD_5 proportion (BOD_5:COD = 0.25) in piggery digestate. However, this high strength digestate was not suitable for microalgae, even if it was diluted twice. No microalgal growth was observed under the 1:1 dilution ratio, and the biomass yield could be negligible (Fig. 1a). There were two possible reasons for no growth or growth hindrance in the low dilution ratios (1:1 and 1:3). First off was the dark brown color that reduced the illumination transmittance, impeding the growth of the microalgae. The other reason for little to no growth was the toxicity of the highly-concentrated free ammonia present in the alkaline digestate liquid (Collos and Harrison, 2014; Hargreaves and Tucker, 2004; Peccia et al., 2013). Ammonia leads to the death of algae via affecting the oxygen evolution complex (OEC) in photosystem I (PS I) (Azov and Goldman, 1982) along with uncoupling of electron transport in photosystem II (PS II) (Belkin and Boussiba, 1991; Oyala et al., 2015). After multiple dilutions, microalgae were able to adapt to the digestate environment. Considering that more dilution brought about exorbitant operational costs and that the microalgae needed adequate nutrient supplies, the 1:7 dilution ratio was adopted for the microalgae-digestate system.

The pH changes along with culture time are shown in Fig. 1a. System pH was kept in the range of 8–9, which was suitable for *Chlorella* (Khalil et al., 2010). Generally, microalgal photosynthesis utilizing carbon dioxide contributes to an increased pH (Chi et al., 2011; Peccia et al., 2013). On the other hand, the absorption of ammonium results in a decreased medium pH due to the release of H^+ ions (Collos and Harrison, 2014). When *C. vulgaris* was cultivated in digestate supernatant without the addition of carbon dioxide (Fig. S1), the pH value dropped sharply, leading to microalgae growth inhibition and rapid decay, similar to Ho et al. (2015). It was suggested that insufficient photosynthesis cannot balance

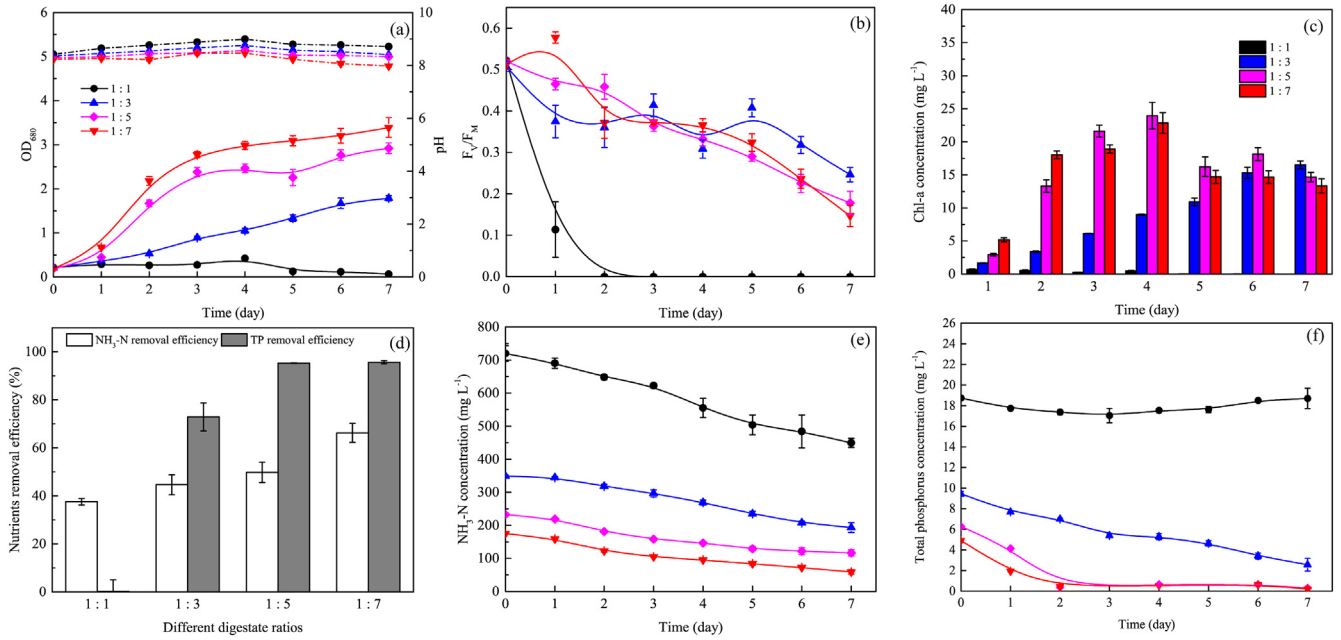

Fig. 1. Microalgae cultivation in different dilutions of anaerobic digestate: (a) Optical density (OD_{680}, solid line) and pH (dash dot line); (b) Fluorescence parameter of F_V/F_M; (c) Chlorophyll a concentration; (d) Nutrient removal efficiency; (e) NH_3-N concentration; (f) Total phosphorus concentration. All the values were reported as the average of the triplicates and the standard deviation.

pH with deficient carbon dioxide. On the contrary, photosynthesis and ammonium utilization achieved a good balance in the experimental bubbling system with CO_2/air gas, and thus the pH remained stable. Under these pH conditions, the initial free ammonia concentrations were calculated theoretically to be 86, 38, 23, and 14 mg L^{-1} for the 1:1, 1:3, 1:5, and 1:7 dilution ratios respectively, based on Hargreaves and Tucker (2004) and ideal value (Fig. S2). However, about 20 mg L^{-1} of free ammonia would reduce the rate of microalgal photosynthesis by half (Azov and Goldman, 1982). In this context, the free ammonia concentrations for the 1:1 and 1:3 digestate ratios greatly exceeded 20 mg L^{-1}, and thus microalgal metabolism was inhibited as shown by their growth.

The maximum potential quantum efficiencies were detected for the microalgae in PS II in terms of F_V/F_M (Fig. 1b). The F_V/F_M value descended dramatically to zero after the inoculum was mixed with high strength digestate (1:1 of dilution ratio). The blend of inoculum and high strength digestate resulted in chloroplast damage of microalgal PS II from the high concentration of free ammonia (Dai et al., 2012), being consistent with no growth. Furthermore, the decreasing trend in F_V/F_M with time was eased by dilution during the culture period, slightly offsetting this trend. In examining the F_V/F_M value for the 1:7 dilution ratio, it proved to rise slightly on the first day, indicating that the photosystem was not impaired by the new surrounding wastewater. However, the F_V/F_M value mainly decreased. The decrease in F_V/F_M was not only linked to free ammonia concentration, but also related to its own growth and depletion of nutrients (Qi et al., 2019).

Pigments also play an important role in microalgal photosynthesis, both absorbing and transmitting light energy, as well as converting it into electrical energy (Li et al., 2019). Fig. 1c exhibits the accumulation of Chl-a in biomass. A significant difference was observed between the 1:3, 1:5, and 1:7 dilution ratios ($p < 0.05$). In the first 4 days, the Chl-a concentrations in the 1:5 and 1:7 ratios increased rapidly, and reached their highest levels on the 4th day, being 24.0 ± 2.0 mg L^{-1} and 22.9 ± 1.6 mg L^{-1} respectively (Table 2). After that, they were reduced on the 5th day and kept stabilized until the end of cultivation. In contrast, the Chl-a concentration of the 1:3 ratio slowly increased until the end of the culture period (16.5 ± 0.6 mg L^{-1}). The difference in pigment accumulation over time may be due to the limitation of phosphorus in the high dilution of digestate. With exogenous phosphorus restriction,

Table 2
Summary of various parameters obtained from different dilution ratios of digestate supernatant and from different nitrogen starvation periods.

Dilution ratio/N-starved period	Parameters								
	μ_m[a] (d^{-1})	Chl-a (mg L^{-1})	Biomass (g L^{-1})	Average removal rate (mg L^{-1} d^{-1})		Removal efficiency (%)		Ammonia stripping (%)	Microalgal uptake (%)
				NH_3-N	TP	NH_3-N	TP		
1:1	NA[b]	NA	NA	43.2 ± 1.3	NA	37.5 ± 1.3	NA	24.1 ± 2.9	NA
1:3	0.35 ± 0.01	16.5 ± 0.6	0.65 ± 0.04	23.5 ± 0.8	1.8 ± 0.2	44.6 ± 4.2	72.9 ± 5.8	5.9 ± 0.6	38.7 ± 3.7
1:5	0.84 ± 0.01	24.0 ± 2.0	1.15 ± 0.03	25.8 ± 2.4	2.9 ± 0.1	49.8 ± 4.2	95.3 ± 0.1	4.9 ± 0.5	44.9 ± 3.9
1:7	0.89 ± 0.01	22.9 ± 1.6	1.33 ± 0.15	26.2 ± 2.2	3.0 ± 0.2	66.2 ± 4.0	95.6 ± 0.4	2.5 ± 0.9	63.7 ± 3.6
1:7 + N-starved 0 h	0.79 ± 0.02	20.7 ± 0.6	1.51 ± 0.01	34.2 ± 0.2	2.7 ± 0.1	77.5 ± 1.0	95.5 ± 0.5	2.3 ± 0.3	75.1 ± 0.8
N-starved 12 h	1.38 ± 0.03	27.3 ± 0.5	2.20 ± 0.08	39.5 ± 6.9	3.7 ± 0.4	86.2 ± 0.3	96.6 ± 1.1		83.9 ± 0.4
N-starved 24 h	1.35 ± 0.05	26.3 ± 0.7	1.97 ± 0.05	34.5 ± 1.7	2.4 ± 0.1	88.3 ± 1.5	95.6 ± 0.6		86.0 ± 1.8
N-starved 36 h	1.47 ± 0.01	30.9 ± 1.2	2.46 ± 0.03	37.6 ± 0.7	2.4 ± 0.0	97.3 ± 1.4	95.9 ± 0.6		95.0 ± 1.2
N-starved 48 h	1.45 ± 0.03	32.9 ± 0.8	2.56 ± 0.08	39.9 ± 1.4	2.8 ± 0.1	99.1 ± 0.2	96.5 ± 0.6		96.8 ± 0.4

All the data were reported as mean ± standard deviation ($n = 3$).
[a] μ_m, the maximum specific growth rate in the exponential phase.
[b] NA, not available.

there may be an impact on Chl-a pigment concentration (Collier and Grossman, 1992; Geider et al., 1993). In addition, the Chl-a concentration of the 1:1 dilution ratio was always close to zero throughout the cultivation process ($p > 0.05$), which coincided with no growth.

3.1.2. Nitrogen and phosphorus removal

As for ammonia nitrogen and total phosphorus removal efficiency (Fig. 1d), the highest ammonia nitrogen removal efficiency was 66.2 ± 4.0% by the 1:7 dilution ratio, while less than 50% in others. Apart from that, the total phosphorus removal efficiency of both 1:5 and 1:7 was more than 95% on the 2nd day, yet merely 72.9 ± 5.9% in the 1:3 dilution ratio by the end of cultivation. The nitrogen removal was related to biological absorption (directly utilized by microalgae) and physical escape (ammonia stripping from the bubbling system) (Table 2). The content of un-ionized aqueous ammonia was dependent upon pH and temperature (Collos and Harrison, 2014; Li et al., 2019). Hargreaves and Tucker (2004) observed that approximately 30–40% free aqueous ammonia occurred when pH was close to 9.0 at 25 °C. Similarly, about 24.1–37.5% of ammonia nitrogen was lost in the 1:1 digestate without microalgae and with inactive microalgae, because their pH fluctuated around 8.7–9.0. It indicated that the loss of ammonia nitrogen for 1:1 digestate wastewater was mainly due to ammonia volatilization (Lei et al., 2007). For the other ratios, the free ammonia contents were calculated within the range of 8–12% according to the initial pH value. The proportions of free ammonia in digestate were far less than ammonia nitrogen removal efficiencies (44–66%). Accordingly, the assimilation efficiencies of microalgae were 38.7% ± 3.7%, 44.9% ± 3.9% and 63.7% ± 3.6% for the 1:3, 1:5 and 1:7 digestates respectively, after subtracting ammonia nitrogen volatilization (Table 2). Based on these data, microalgal uptake was the main factor for the ammonia nitrogen removal from the digestate in effective microalgae-digestate systems.

In fact, nitrogen supply is an inevitable requirement for sustainable microalgae production. Having ammonia nitrogen as the main nitrogen source of the anaerobic digestate is preferred since inorganic nitrogen must be reduced to ammonium inside algal cells before being assimilated into amino acids or proteins (Fernandez and Galvan, 2008). The concentration of ammonia nitrogen decreased gradually over cultivation time (Fig. 1e). The ammonia nitrogen average removal rate was 26.2 ± 2.2 mg L^{-1} d^{-1} in the 1:7 dilution ratio, followed by 25.8 ± 2.4 mg L^{-1} d^{-1} and 23.5 ± 0.8 mg L^{-1} d^{-1} in the 1:5 and 1:3 dilution ratios (Table 2). Furthermore, the highest ammonia nitrogen removal rates of the 1:5 and 1:7 ratios were obtained on the 2nd day, while on the 6th day for the 1:3 ratio, suggesting that the adaptation of microorganisms to the new environment was inevitable when there was no acclimation period (Najafpour, 2015).

Phosphorous, as the other major macronutrient, is required for organisms and can form many important substances, such as nucleic acids, coenzymes, proteins, and ATP molecules (Peccia et al., 2013; Solovchenko et al., 2019). The total phosphorus degradation curves in different digestate ratios are exhibited in Fig. 1f. Obviously, there was almost no consumption of total phosphorus in the 1:1 dilution ratio. The highest total phosphorus removal rate was obtained in the 1:7 digestate, being 3.0 ± 0.2 mg L^{-1} d^{-1}, followed by 2.9 ± 0.1 mg L^{-1} d^{-1} and 1.8 ± 0.2 mg L^{-1} d^{-1} for the 1:5 and 1:3 dilution ratios, respectively (Table 2). After the 2nd day, little phosphorus in the digestate of the 1:5 and 1:7 ratios (< 0.3 mg L^{-1}) could not afford the growth of algae, resulting in a state of phosphorus-starvation. Geider et al. (1993) found that the Chl-a quota in the diatom *Phaeodactylum tricornutum* decreased from 0.35 pg $cell^{-1}$ to 0.05–0.1 pg $cell^{-1}$ in the phosphorus-starved culture. Also Collier and Grossman (1992) attested chlorophyll accumulation continued for several hours after phosphorus deprivation, and then cellular chlorophyll content decreased because of cell division. These findings supported the phenomenon that the pigment (Chl-a) concentration of 1:5 and 1:7 maintained a decrease from the 4th day (phosphorus limitation) but no loss in Chl-a concentration was observed in the 1:3 ratio, along with the slow consumption of phosphorus throughout the culture period.

Based on these results, the feasibility of anaerobic digestate treatment through *C. vulgaris* cultivation showed potential, even with some residual ammonia nitrogen. However, it was necessary to develop new methods to boost the assimilation of ammonia nitrogen by microalgae to further improve the feasibility.

3.2. Over-compensation strategy

3.2.1. Microalgal growth using nitrogen-starved seed

The cell growth (OD_{680}) and pH of *C. vulgaris* after experiencing different durations of nitrogen starvation were studied in the digestate supernatant of the 1:7 dilution ratio (Fig. 2a). It was observed that with longer prior starvation times, improved microalgal growth in the wastewater occurred ($p < 0.05$). The maximum OD of the no-starvation control group was approximately half of the nitrogen-starved 48 h group. There was a significant difference between the control and experimental groups for their growth rates. In Table 2, the highest specific growth rate for the exponential phase in the no-starvation control group was 0.79 ± 0.02 d^{-1}, while the specific growth rates of the nitrogen-starved 36 and 48 h groups (1.47 ± 0.01 d^{-1} and 1.45 ± 0.03 d^{-1}) were 1.86- and 1.84-times greater than the control. The results indicated that prior nitrogen-starvation made a contribution to accelerate microalgal growth. The final dry cell weight was consistent with the growth curve. The highest value (2.56 ± 0.08 g L^{-1}) was obtained under the nitrogen-starved 48 h group, and it increased by 70% as opposed to the control (1.51 ± 0.01 g L^{-1}). From these results, it can be seen that a prior nitrogen-starved approach stimulated cell propagation, resulting in a large boost of microalgal biomass when re-exposed to a nitrogen-rich environment.

One of the most critical factors in microalgae-based wastewater treatment is pH when the main nitrogen source is ammonia nitrogen. Many studies have confirmed that medium pH decreased dramatically after ammonia nitrogen was consumed by microalgae (Collos and Harrison, 2014; Ho et al., 2015; Kim et al., 2013). The overall trend of pH was stable or slightly decreasing, and the pH of the nitrogen-starved seeds was lower than the control. Indication of greater ammonia nitrogen assimilation by the starved microalgae was inferred. Nonetheless, the value of pH was still suitable for microalgae and it did not threaten the growth of *C. vulgaris*. Overall, the appropriate pH was conducive to algal growth, resulting in abundant biomass harvest.

The chlorophyll fluorescence of *C. vulgaris* cells are shown in Fig. 2b. The starved curves were similar to the previous curves for the 1:7 dilution ratio (Fig. 1b). All of the curves reached their maximum value on the first day, and then declined continuously. The initial values of F_V/F_M were 0.43 ± 0.03, 0.45 ± 0.03, 0.37 ± 0.01, 0.31 ± 0.01, and 0.24 ± 0.03 in the starved 0, 12, 24, 36, and 48 h groups, respectively. This indicated that the photosynthetic activities of microalgae seeds were severely affected by the long-term nitrogen deprivation (longer than 12 h) (Juergens et al., 2015). Although, the photosynthetic activity was slightly increased by appropriate starvation stimulation (12 h). Combining Fig. 2a and b, the values of F_V/F_M from all the groups were greater than 0.5 before the third day when microalgal growth was in the logarithmic phase. The high photosynthetic efficiency of each group resulted in a large accumulation of microalgal biomass. Gradually, algal growth entered the stable period on account of the reduced values of F_V/F_M. However, the values of F_V/F_M in the experimental groups fell more slowly than that of the control after the third day, indicating that the nitrogen-stress-suffered cells possessed more resistance in the digestate system. These results proved that the over-compensation strategy helped stimulate biological activity of microalgal cells and therefore maximizing the role of microalgae in the digestate treatment process.

The accumulation of Chl-a is shown in Fig. 2c under a variation of prior nitrogen-starved times. The rapid accumulation of pigment mainly

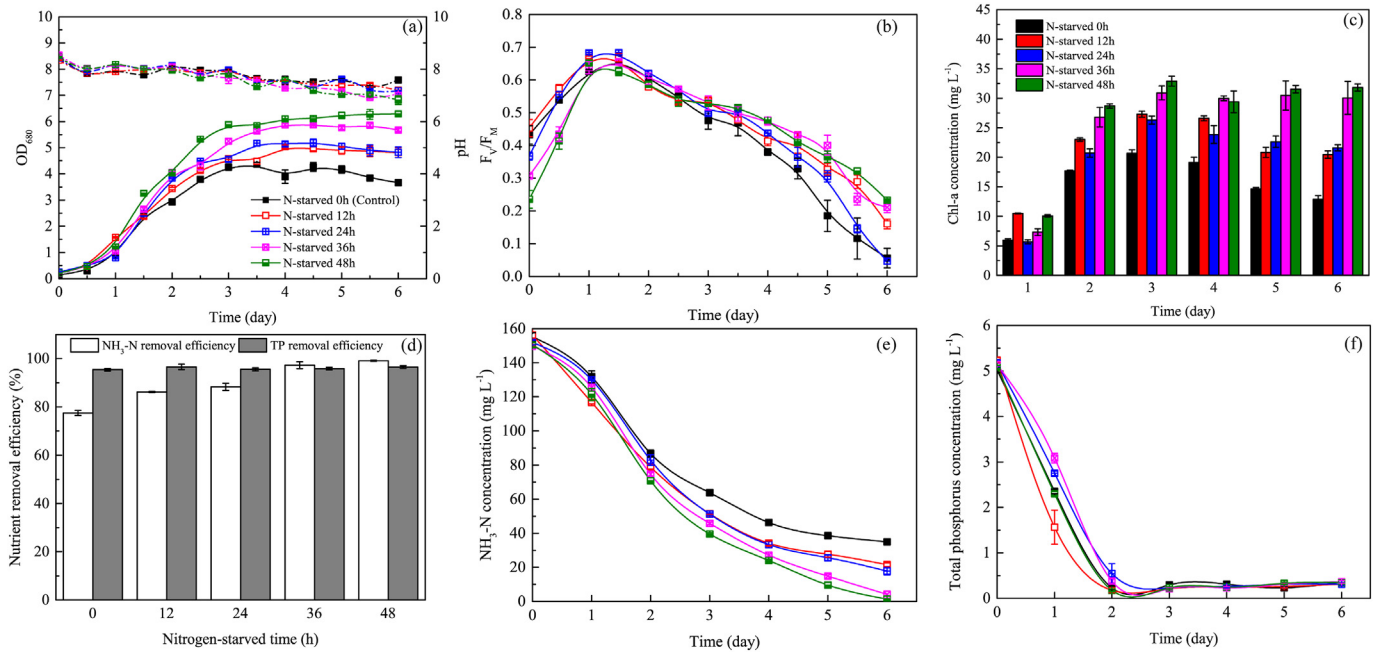

Fig. 2. Microalgae cultivation in the 1:7 ratio of anaerobic digestate via over-compensation strategy: (a) Optical density (OD_{680}, solid line) and pH (dash dot line); (b) Fluorescence parameter of F_V/F_M; (c) Chlorophyll a concentration; (d) Nutrient removal efficiency; (e) NH_3-N concentration; (f) Total phosphorus concentration. All the values were reported as the average of the triplicates and the standard deviation.

occurred in the initial 3 days, resulting from rapid cell proliferation (Fig. 2a). In the nitrogen-starved 36 h and 48 h conditions, the productivities of Chl-a were improved to 19.4 ± 1.1 and 18.7 ± 0.2 mg L^{-1} d^{-1} respectively, having been 1.7- and 1.6-times larger than the control (11.8 mg L^{-1} d^{-1}). On the third day, all the groups achieved the highest concentration of pigment. The highest Chl-a concentration was recorded in the nitrogen-starved 48 h group (32.9 ± 0.8 mg L^{-1}), while only 20.7 ± 0.6 mg L^{-1} in the control group. The results demonstrated that the prior nitrogen starvation stimulation caused microalgal cells to accumulate lavish biomass and resulted in augmentation largely for pigment. After the third day, microalgal pigment concentration of the control gradually decreased. The nitrogen-starved 12 h and 24 h groups declined slightly and then became stable in the last two days for Chl-a concentration, whereas it always maintained a higher level under nitrogen-starved 36 h and 48 h groups (Fig. 2c). The potential reason for less pigment synthesis during the stationary phase was because of continued reduction of the photosynthetic efficiency (F_V/F_M) after the first day, thereby resulting in a minimal, purely life-sustaining energy uptake (Fig. 2b). Accordingly, the pigment concentration of nitrogen-stress-suffered cells had little to no effect, owing to higher biological activity compared to the control.

3.2.2. Nutrient removal through over-compensation

Fig. 2d and e exhibit the change of ammonia nitrogen concentration in digestate. From the degradation curves of ammonia nitrogen (Fig. 2e), the residual ammonia nitrogen concentrations of the starved 36 h and 48 h groups were 4.1 ± 2.1 mg L^{-1} and 1.3 ± 0.3 mg L^{-1} on the 6th day (less than 15 mg L^{-1}), meeting the integrated wastewater discharge standard (GB 8978-1996, China). The maximum ammonia nitrogen removal rate of the control group was 34.2 ± 0.2 mg L^{-1} d^{-1}, while that of the nitrogen-starved 48 h group was 39.9 ± 1.4 mg L^{-1} d^{-1} (Table 2). Additionally, the highest removal efficiency of ammonia nitrogen reached 99.1% ± 0.2% under the nitrogen-starved 48 h group (Fig. 2d). It is worth noticing that only 2.3% ± 0.3% ammonia was escaped, and biological absorption accounted for 96.8 ± 0.4% of ammonia nitrogen removal from the digestate (Table 2). On the other hand, the removal efficiency of ammonia nitrogen from the no-starvation control was only 77.5 ± 1.0%, and plentiful amounts of inorganic nitrogen (35 mg L^{-1}) remained in the treated digestate. Compared to literatures focused on the treatment of anaerobic digestate using *Chlorella*, the ammonia nitrogen removal efficiency of the control group using normal seeds was consistent with a range of 30–80% (Table 3). However, controlling the nitrogen-starved duration of *C. vulgaris* could almost

Table 3
Comparison of *Chlorella* treatment of anaerobic digestates.

Strain	Wastewater resource	Dilution fold	Light intensity	NH_3-N removal efficiency (%)	Biomass (g L^{-1})	Carbohydrates yield (mg L^{-1})	PUFAs[a] (%)		Reference
							C18:2	C18:3	
Chlorella vulgaris	Anaerobic piggery digestate	8.0	200 μmol m^{-2} s^{-1}	99.13	2.56	991.01	23.24	25.77	This study
Chlorella sp.	Anaerobic dairy digestate	20	200 μmol m^{-2} s^{-1}	100.00	1.88[b]	NA[c]	32.40	NA	Wang et al. (2010)
Chlorella vulgaris	Biogas slurry	8.0	600 μmol m^{-2} s^{-1}	29.43	1.36	836.40	NA	NA	F. Tan et al. (2016)
Chlorella sorokiniana	Anaerobic digestate	16.7	75–80 μmol m^{-2} s^{-1}	41[d]	0.506	113.34	NA	NA	Singh et al. (2011)
Chlorella vulgaris	Municipal wastewater	NA	8000–10,000 lx	NA	1.86	837.00	29.90	5.64	He et al. (2013)
Chlorella sorokiniana	Cattle manure digestate	10.0	160 μmol m^{-2} s^{-1}	74.70	0.280	57.92	15.00	10.00	Kobayashi et al. (2013)
Chlorella sp	Anaerobic digestion	Undiluted	80–100 μmol m^{-2} s^{-1}	63.47	0.41	NA	NA	NA	Ouyang et al. (2015)
Chlorella pyrenoidosa	Anaerobically digested sludge	1.5	127 μmol m^{-2} s^{-1}	75.90	2.26	NA	20.46	7.17	X.-B. Tan et al. (2016)

[a] PUFAs, polyunsaturated fatty acids.
[b] The value was estimated by optical density.
[c] NA, not available.
[d] Total nitrogen removal efficiency.

completely recover ammonia nitrogen in the digestate supernatant, with acceleration of the nitrogen recovery rate. It was demonstrated that the approach of prior nitrogen-starvation increased the saturation value of microalgae for nitrogen assimilation. Specifically, this phenomenon is a nitrogen-stress response where the microalgae are initially starved of nitrogen, then immersed in a nitrogen-rich environment, resulting in greater nitrogen uptake (Kudela and Cochlan, 2000). Additionally, Millbank (1957) and Cho and Komor (1984) found that the level of keto-acids (2-oxaloglutarate and oxaloacetate) and the rate of nitrogen assimilation were considerably high in *Chlorella* under nitrogen-starved conditions. The high rate of nitrogen assimilation was connected to carbon skeletons in the form of keto-acids in the assimilation process of ammonium, resulting in L-glutamine synthesis (Fernandez and Galvan, 2008). The previous statements could be attributed to the fact that the nitrogen-free environment boosted enzyme activity related to the nitrogen assimilation pathway, thereby enhancing ammonium uptake through nitrogen deficiency (Dortch et al., 1982). The application of prior nitrogen-starvation enhanced microalgal cell metabolism through efficiently absorbing nitrogen that was present in the digestate. Therefore, the applied over-compensation strategy proves to be beneficial in enhancing nitrogen recovery from wastewater.

The use of nitrogen starvation in hopes of improved total phosphorus removal from the anaerobic digestate was not significant (Fig. 2f). The highest removal rate was 3.7 ± 0.4 mg L^{-1} d^{-1} under the nitrogen-starved 12 h group, and the rest were 2.7 ± 0.1, 2.4 ± 0.1, 2.4 ± 0.0, and 2.8 ± 0.1 mg L^{-1} d^{-1} under the nitrogen-starved 0, 24, 36, and 48 h groups, respectively. The change in the total phosphorus average removal rate was also related to photosynthetic activity because the slight increase of initial F_V/F_M only occurred in the nitrogen-starved 12 h group (Fig. 2b). Furthermore, the removal efficiencies of total phosphorus (Fig. 2d) were just slightly higher than or similar to the control group (95%), which were 96–97% under the nitrogen-starved 12, 24, 36 and 48 h groups ($p > 0.05$). In addition to the high removal efficiency of the control group itself, the phosphorus absorption was not greatly affected by the system-stressed pressure brought upon by nitrogen. Wu et al. (2012) found that the phosphorus-starvation mode could increase the rate of phosphorus assimilation, however nitrogen uptake was affected slightly in phosphorous-starvation and luxury-nutrient mode. This result confirmed that microalgae have specific feedback mechanisms when encountering single nutrient stress.

3.3. Bioproduct harvest

The content and yield of bioproducts including lipids, proteins, and carbohydrates are shown in Fig. 3a and b. It is worth noting that carbohydrate content and yield were enhanced prominently. The carbohydrate content and yield of the nitrogen-starved 48 h group increased from 29.2 ± 0.9% (the control) to 38.7 ± 0.4% and from 440.7 ± 16.2 mg L^{-1} (the control) to 991.0 ± 30.7 mg L^{-1}. In particular, carbohydrate accumulation in the nitrogen-starved 48 h group may be triggered by the nitrogen depletion (Chen et al., 2013). The carbohydrate-rich cells were used usually for applications such as biofuel and biorefining. Nevertheless, the contents of a system's products do not always parallel its yield, specifically when microalgae grow in wastewater. For instance, F. Tan et al. (2016) found that carbohydrate concentration of *C. vulgaris* present in a biogas slurry increased from 41% (artificial medium) to 55%, but the yield was reduced from 1130.86 mg L^{-1} to 487.90 mg L^{-1} because of biomass decline from 2.79 g L^{-1} to 0.89 g L^{-1}. In addition, the contents of lipids and proteins were in the scopes of 22–23% and 16–19% respectively (Fig. 3a). Despite no evident changes occurring in the proportions of lipids and proteins to biomass between the control and experimental groups, an advance in the accumulation of lipids and proteins through the prior nitrogen-starved stimulation resulted. Under the nitrogen-starved 48 h group, the lipid and protein yields peaked (574.2 ± 21.0 mg L^{-1} and 418.1 ± 40.3 mg L^{-1}), which

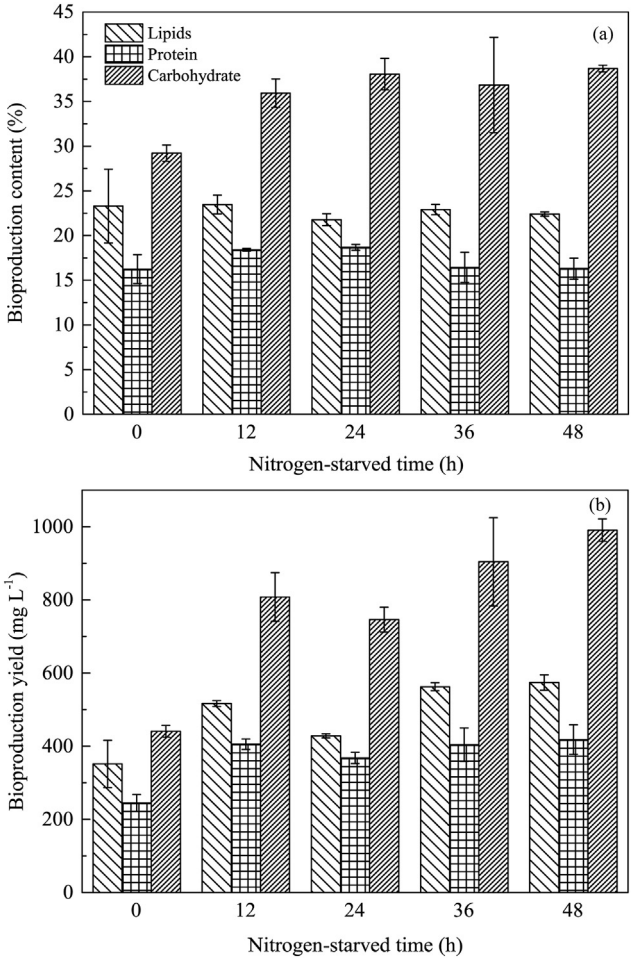

Fig. 3. Bioproduct content (a) and yield (b) of the harvested microalgal biomass. All the values were reported as the average of the triplicates and the standard deviation.

were 1.6- and 1.7-times greater than that of the control (351.7 ± 64.4 mg $L^{-1}$and 244.9 ± 23.3 mg L^{-1}), respectively (Fig. 3b).

Microalgal fatty acid compositions under the prior nitrogen-starved 0 h and 48 h groups were analyzed (Fig. 4). The primary fatty acid components were saturated fatty acids (SFAs) of palmitic acid (C16:0) and stearic acid (C18:0), monounsaturated fatty acids (MUFAs) of linoleic acid (C18:1), and polyunsaturated fatty acids (PUFAs) of linoleic acid (C18:2) and linolenic acid (C18:3). Under both the control and experimental groups, aliphatic chains with 16–18 atoms that possess higher energy, exceeded 95% of total fatty acids (96.2 ± 0.9% and 97.7 ± 0.4% respectively). After the 48 h group was subjected to nitrogen starvation, the sum of SFAs and MUFAs decreased from 69.7 ± 4.9% to 34.2 ± 1.8%, mainly caused by a decline of C18:0, C16:1 and C18:1. On the contrary, an increase in the concentration of PUFAs from 29.1 ± 6.2% in the control to 65.8 ± 1.8% resulted from complete MUFA (C18:1) to PUFA conversion. Thus, PUFAs were mainly presented in the form of α-linolenic acid (C18:3 n-3, 26%) and linoleic acid (C18:2 n-6, 23%) except for C16:2, C16:3 and C16:4. Similar results for high linolenic acid contents were described by Zhukova and Aizdaicher (1995) for *Chlorella* sp. (22%). X.-B. Tan et al. (2016) also obtained a high percentage of PUFAs (65%) when culturing *Chlorella pyrenoidosa* at a high pH level (8.3–8.8) in the effluent of anaerobically digested activated sludge. Additionally, linolenic acid is important in the biosynthesis of several bioactive signal molecules (Radwan, 1991) with the absence of linolenic acid in the control. The data and analysis previously shown suggested that the prior nitrogen-stress strategy was beneficial for the accumulation of high-value PUFAs in *C. vulgaris* lipids.

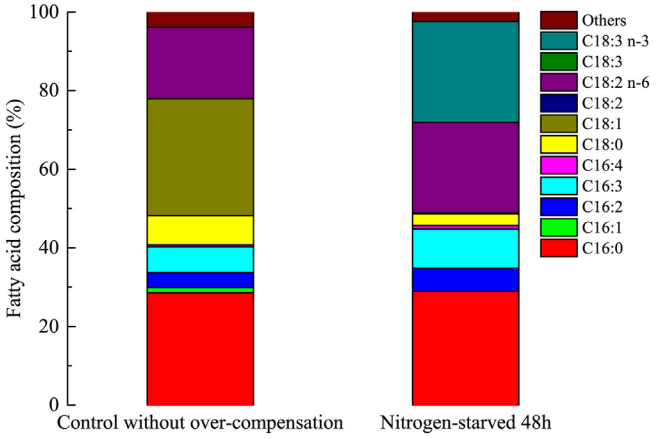

Fig. 4. Fatty acid composition of microalgal lipids (% of total fatty acids) with and without over-compensation. All the values were reported as the average of the triplicates and error bars were not shown.

4. Conclusions

The prior nitrogen-starvation seed was used to enhance ammonia nitrogen removal and microalgae production in piggery digestate by the over-compensation strategy. It was demonstrated that microalgae have the potential to treat digestate through nitrogen and phosphorus removal, and the ability of nitrogen assimilation has been greatly improved. When using the nitrogen-starved 48 h seed, ammonia nitrogen removal efficiency and rate reached 99% and 40 mg L^{-1} d^{-1} respectively. Meanwhile, microalgal biomass yield increased by 70%. Furthermore, its carbohydrate content and yield were increased to 39% and 991 mg L^{-1}, and the PUFA proportion accounted for 66% of total fatty acids. Overall, the innovative application of the over-compensation strategy is effective in the microalgae-digestate system. It could be applied for other nutrient-rich wastewater treatments to promote nutrient recovery and bioproduct production.

CRediT authorship contribution statement

Chaogang Ran: Formal analysis, Investigation, Data curation, Writing – original draft. **Xinyu Zhou:** Investigation. **Changhong Yao:** Supervision. **Yongkui Zhang:** Conceptualization. **Wu Kang:** Funding acquisition. **Xiaolong Liu:** Funding acquisition. **Colton Herbert:** Writing – review & editing. **Tonghui Xie:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (Grant No. 21506132), Science and Technology on Reactor Fuel and Materials Laboratory, Sichuan University "Chemical Star" Excellent Young Talents Training Program, and International Visiting Program for Excellent Young Scholars of SCU.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.144462.

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