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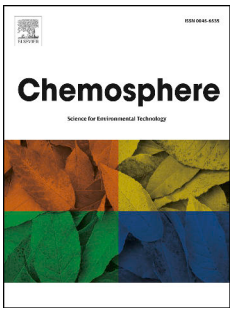

Towards high-quality biodiesel production from microalgae using original and anaerobically-digested livestock wastewater

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Gang Li [a], Jiang Zhang [a], Huan Li [b, e, f], Ruichen Hu [a], Xiaolong Yao [c, d], Ying Liu [b, e, f], Yuguang Zhou [b, e, f *], Tao Lyu [g *]

[a] School of Materials and Mechanical Engineering, Beijing Technology and Business University, No.11 Fuchenglu, Haidian District, Beijing 100048, China

[b] Bioenergy and Environment Science & Technology Laboratory, College of Engineering, China Agricultural University, Beijing 100083, China

[c] Key Laboratory of Cleaner Production and Integrated Resource Utilization of China National Light Industry，Beijing Technology and Business University，Beijing 100048，China

[d] Department of Environmental Science and Engineering, Beijing Technology and Business University, No.11 Fuchenglu, Haidian District, Beijing 100048, China

[e] Key Laboratory of Clean Production and Utilization of Renewable Energy, Ministry of Agriculture and Rural Affairs, Beijing 100083, China

[f] National Center for International Research of BioEnergy Science and Technology, Ministry of Science and Technology, Beijing 100083, China

[g] Cranfield Water Science Institute, Cranfield University, College Road, Cranfield, Bedfordshire, MK43 0AL, UK

[*] Correspondance: zhouyg@cau.edu.cn (Y. Zhou), t.lyu@cranfield.ac.uk (T. Lyu)

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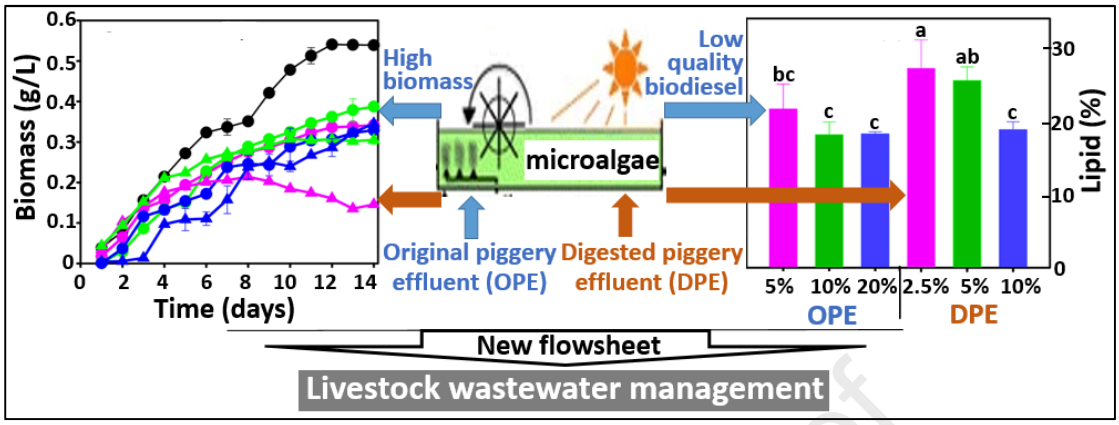
Graphical Abstract



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[a] School of Materials and Mechanical Engineering, Beijing Technology and Business University, No.11 Fuchenglu, Haidian District, Beijing 100048, China

[b] Bioenergy and Environment Science & Technology Laboratory, College of Engineering, China Agricultural University, Beijing 100083, China

[c] Key Laboratory of Cleaner Production and Integrated Resource Utilization of China National Light Industry，Beijing Technology and Business University，Beijing 100048，China

[d] Department of Environmental Science and Engineering, Beijing Technology and Business University, No.11 Fuchenglu, Haidian District, Beijing 100048, China

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[f] National Center for International Research of BioEnergy Science and Technology, Ministry of Science and Technology, Beijing 100083, China

[g] Cranfield Water Science Institute, Cranfield University, College Road, Cranfield, Bedfordshire, MK43 0AL, UK

[*] Correspondance: zhouyg@cau.edu.cn (Y. Zhou), t.lyu@cranfield.ac.uk (T. Lyu)

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Abstract

In this study, we conducted proof-of-concept research towards the simultaneous treatment of livestock wastewater and the generation of high-quality biodiesel, through microalgae technology. Both original (OPE) and anaerobically-digested (DPE) piggery effluents were investigated for the culture of the microalgae, *Desmodesmus* sp. EJ8-10. After 14 days' cultivation, the dry biomass from microalgae cultivated in OPE increased from an initial value of 0.01 g/L to 0.33-0.39 g/L, while those growing in DPE only achieved a final dried mass of 0.15-0.35 g/L, under similar initial ammonium nitrogen (NH_4^+-N) concentrations. The significantly higher microalgal biomass production achieved in the OPE medium may have been supported by the abundance of both macronutrient, such as phosphorus (P), and of micronutrients, such as trace elements, present in the OPE, which may not been present in similar quantities in the DPE. However, a higher lipid content was observed (19.4-28%) in microalgal cells from DPE cultures than those (18.7-22.3%) from OPE cultures. Moreover, the fatty acid compositions in the microalgae cultured in DPE contained high levels of monounsaturated fatty acids (MUFAs) and total C16-C18 acids, which would afford a superior potential for high-quality biodiesel production. The N/P ratio (15.4:1) in OPE was much closer to that indicated by previous studies to be the most suitable (16:1) for microalgae growth, when compared with that determined from the DPE culture medium. This may facilitate protein synthesis in the algal cells and induce a lower accumulation

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of lipids. Based on these findings, we proposed a new flowsheet for sustainable livestock waste management.

Keywords*:* *Desmodesmus* sp.; microalgae technology; wastewater treatment; lipid accumulation; fatty acid composition

1. Introduction

The rapid increase in of worldwide consumption of fossil fuels has led to serious environmental consequences, such as emission of greenhouse gases and water pollution (Stern et al., 2016). In order to contain such environmental risks and overcome a possible future energy shortage, alternative clean and renewable energy sources have attracted considerable attention (Li et al., 2019a). Although studies on renewable energies have focussed on solar, wind, and hydropower, the use of biodiesel has gained considerable attention among the scientific community due to its energy density, thermal efficiency (Manigandan et al., 2020a), and relatively simple integration into present transport technology. Previous studies have demonstrated that the addition of biodiesel into combustion engines could significantly increase their performance and reduce emissions (Manigandan et al., 2020b). The current cost of biodiesel production does not have significant advantage than petroleum fuels (Oláh et al., 2017), however, extensive efforts have been expended on the development of relevant technology/resources, as it has been deemed as a promising eco-friendly renewable energy. Non-edible phytomass, such as *Jatropha curcas L.* (Maroušek et al., 2013a),

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and rapeseed *Brassica napus L.* (Maroušek et al., 2013b), have been proven to be cost-effective biomass for biodiesel production (Maroušek et al., 2015). However, the cultivation of such species may still occupy arable land which might be otherwise given over to food production, and their growth is heavily dependent on seasonal factors and use of fertilizers, often synthetic, which could limit their larger scale production.

Microalgae, with the advantages of high photosynthetic efficiency, rapid growth, high lipid content, and lack of need for arable land, have been considered to be one of the most promising sources for biofuel production (Li et al., 2019b). As a photosynthetic microorganism, most autotrophic microalgae can be initiated by natural light to capture CO_2 and take up nutrients, e.g. nitrogen (N) and phosphorous (P), from wastewater. After short-term cultivation (usually within weeks), microalgae may synthesise lipids at levels up to 56% of the dry biomass, for potential biofuel production (López-Rosales et al., 2019). Moreover, the residual algal biomass could also be reused as valuable by-products, such as biochar (Maroušek et al., 2019) and fertiliser additives (Pan et al., 2018). However, notwithstanding the significant lipid accumulation, further investigation and manipulation of microalgae technology, towards the large-scale production of high-quality biofuel, are urgently needed.

The considerable increasing demand for livestock products has been brought about by human population pressure, and, as a consequence, vast amounts of generated manure and wastewater need to be treated in order to address any potential

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environmental issues (Luo et al., 2017). Anaerobic digestion (AD) technology has been successfully implemented to treat such concentrated waste streams, particularly for the removal of organics (Maroušek et al., 2020a). However, after conversion of the majority of the organics to renewable biogas, the remaining AD effluent still contains significant amounts of nutrients, e.g. N and P (Li et al., 2020). Currently, this AD slurry is often used in agriculture as a bio-fertiliser to increase crop yield (Ma et al., 2017). However, this approach may involve high transportation costs when farmland is distant to the AD plant and, additionally, demand for the product could be very limited during the non-growing season. Thus, appropriate measures to treat the AD effluent are required in order to meet the appropriate discharge regulations and to recover/reutilise the nutrients.

Previous studies have proven that microalgae technology could effectively purify nutrient-rich AD wastewater before discharge (Stiles et al., 2018). However, from the perspective of potential biofuel generation, AD treatment might alter the N/P ratio in the original livestock effluent, as well as remove trace elements (e.g. Zn and Fe) and micronutrients (e.g. amino acids and vitamins), which could in turn hinder microalgal growth (Uggetti et al., 2014). It may be hypothesised that, without AD treatment, the composition of nutrients in original livestock wastewater could facilitate rapid microalgal growth, but that these conditions may not lead to higher lipid concentrations (the main composition of the biofuel) in the microalgae cell (Luo et al., 2017). We, therefore hypothesise that microalgae cultivation in digested livestock wastewater

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would benefit the generation of biofuel compared with culturing in the original un-digested livestock wastewater. Moreover, the high levels of nitrogen (Procházka et al., 2012) and phosphorus (Mancipe-Jiménez et al., 2017) have been demonstrated to inhibit the AD process. Thus, we further hypothesise that microalgae cultivation in original livestock wastewater could consume such nutrients and then the treated waste (remaining high in organics) could be further used in AD facilities.

The aim of this study was to evaluate production and quality of biofuel obtained from microalgae during the treatment of different livestock wastewaters. Wild-type *Desmodesmus* sp. EJ 8-10 was selected as the model microalgal species. The original piggery effluent (OPE) and digested piggery effluent (DPE) were both collected as the culture media for comparison purposes. During the experiment, the wastewater nutrient dynamics were monitored in order to assess nutrient uptake and wastewater treatment performance. In addition, algal biomass growth, lipid accumulation and fatty acid compositions were determined in order to evaluate the potential quantity and quality of biodiesel generation. With the results, this study could support a new strategy for high-quality biodiesel production from microalgae and we proposed a new flowsheet for sustainable livestock effluent management.

2. Materials and Methods

2.1 Algae strain and growth medium preparation

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Desmodesmus sp., has been proven as a promising microalgae species for biodiesel generation along with excellent performance in the removal of nutrients from wastewater (Ji et al., 2014). The wild-type algal strain *Desmodesmus* sp. EJ 8-10 (hereafter noted as EJ 8-10) was obtained from a freshwater source in Fangshan District, Beijing, China. EJ 8-10 was purified by serial dilutions and plate streaking in 1.5% BG11 medium (Rippka et al., 1979). Constituents of the growth medium are noted in Table S1. The pH of the medium was adjusted to 7.5 with 1 M NaOH or HCl. The seed cultures were inoculated at 10% (*v/v*) in 250 mL Erlenmeyer flasks containing BG-11 medium (100 mL). The cultivation conditions were as follow: illumination intensity: 120±2 μmol/m^2/s; temperature: 27±1 °C; illumination duration: 14 h:10 h (light:dark). The pre-cultured samples were streaked on BG11 enriched agar plates and cultured for a further 1-3 weeks. Single colonies on agar were removed and inoculated into the wells of a 96-well plate with 150 μL liquid medium. Purity of the isolates were ensured by repeated plating and preliminary observation by optical microscopy. Then, further amplification and sequencing of 18S rDNA (Ji et al., 2014) were used to characterise the microalgae strains. The results were searched against GenBank entries using BLAST (Altschul et al., 1990) and were manually aligned with representative sequences from microalgae strains and related taxa, according to similarities generated by the Clustal W program (Sievers et al., 2011). After successful separation, the algae suspension was cultivated in the BG11 medium prior to use. All BG11 medium and Erlenmeyer flasks were sterilized (121 °C for 20 min) before use.

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Livestock wastewater was demonstrated to provide sufficient nutrients for microalgal growth towards simultaneous treatment and biofuel production (López-Rosales et al., 2019). In this study, two different types of wastewater from the livestock farm, i.e. original piggery effluent (OPE) and anaerobically-digested piggery effluent (DPE), were collected from Beilangzhong pig farm, Shunyi District, Beijing, China. Both OPE and DPE were centrifuged (10000 r/min for 15 min) and supernatants were collected and stored at 4 °C prior to use. The concentrations of ammonium nitrogen (NH_4^+-N), total nitrogen (TN) and phosphate phosphorus (PO_4^{3-}-P) were 477 ± 3, 519 ± 7, and 31 ± 1 mg/L for OPE and 720 ± 6, 792 ± 4 and 33 ± 0.1 mg/L for DPE, respectively. The concentrations of nitrate nitrogen (NO_3^--N) and nitrite nitrogen (NO_2^--N) in both effluents were below 0.5 mg/L.

2.2 Experimental operation

High concentrations of NH_4^+-N have been demonstrated as a key factor in reduction of the microalgal vitality (Peccia et al., 2013). Here, a batch study was conducted, which indicated that EJ 8-10 would show inhibition in growth when the concentration of NH_4^+-N was more than 100 mg/L. Therefore, this study was designed around three concentration levels of NH_4^+-N (20, 40 and 80 mg/L) in both OPE and DPE. In order to achieve comparability, OPE and DPE were diluted with deionized water to the required concentration (OPE: 5%, 10% and 20%, DPE: 2.5%, 5% and 10%). EJ 8-10 biomass was then collected from the inoculated flasks and cultivated in each

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161 medium at the identical initial concentration (OD_{680} of 0.14). Moreover, the same media
162 without addition of EJ 8-10 were set as a medium control group in order to assess
163 changes in pollutant levels. Algae grown in BG11 medium were arranged as biomass
164 growth controls. The experiment was carried out for 14 days and each group was set up
165 in triplicate.

166 **2.3 Nutrient analysis**

167 During the experiment, samples of microalgal suspension (15 mL) were collected
168 daily from each flask for analysis of nutrients. The samples were initially filtered using
169 a 0.45 μm nylon membrane filter (thickness 150-187 μm; Cytiva, Marlborough, USA),
170 and then diluted prior to analysis. NH_4^+-N and PO_4^{3-}-P were measured by
171 UV/Vis-spectrophotometry (UV-2550; Shimadze Corp., Kyoto, Japan) at 425 nm and
172 880 nm, respectively (SEPA 2010, HJ 535-2009, CHN; SEPA 1990, GB 11893-89,
173 CHN). TN was determined colourimetrically as nitrate at 220 nm and 275 nm after prior
174 digestion by persulfate (SEPA 2012, HJ 636-2012). Nutrient removal efficiency (Eq. 1)
175 and removal rate (Eq. 2) were calculated as follows,

176 $$RE = (C_i - C_o) \times C_i \times 100\% \quad \text{(Eq. 1)}$$

177 $$RR = (C_i - C_o) / \mathrm{t} \quad \text{(Eq. 2)}$$

178 Where *RE* is the removal efficiency (%), C_i is the initial concentration of NH_4^+-N, TN, and
179 PO_4^3-P (mg/L), C_o is the concentration of the nutrient at sampling (mg/L). *RR* is the average
180 nutrient removal rate (mg/L/d), t is the total cultivation time (14 d).

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2.4 Determination of microalgae growth

Dry cell weight (DCW) of microalgae has been proven to be correlated with the optical density (OD) of a suspension of algal cells, measured at a wavelength of 680 nm (OD_{680}), which is associated with chlorophyll absorption, and thus represents a convenient method for the determination of the abundance of cells containing this pigment (Ji et al., 2014). Our previous study has demonstrated a linear relationship between OD and DCW (Fig. S1), according to equation (3):

$$Y = 0.3239\ x - 0.0356\ (R^2=0.99) \quad \text{(Eq. 3)}$$

Where Y is the DCW (g/L), x is the absorbance at 680 nm. The initial OD_{680} for all experimental variations was 0.14.

Suspensions of microalgae (3 mL) were taken daily from each flask, transferred to a cuvette and measured by spectrophotometer (Gold S54T, Lengguang Tech., Shanghai, China). The results were converted to DCW based on Eq. 3.

2.5 Lipid content and fatty acid methyl ester (FAME) analysis

At the end of the experiment, algal cells were harvested by centrifugation at 10000 rpm for 10 min and freeze dried (Thermo Savant; Thermo Fisher Scientific, Waltham, USA) for further analysis. The total lipid content was measured using a modified method based on Abou-Shanab et al. (2013). Briefly, dried algae powder (0.1 g) was weighed into clean screw-top glass tubes and a 1:2 chloroform-methanol (*v/v*) mixture (10 mL) was added. After ultrasonication for 1 h, the tube was incubated overnight at 27 °C while shaking at 100 rpm. On the following day, an additional

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aliquot of chloroform (1.25 mL) was added and the extraction mixture sonicated again for 30 min. The solid algal residues were removed by passing the suspension through glass fiber filter (0.45 µm; Cytiva, Marlborough, USA). The filtrate was then transferred to another clean screw-top glass tube containing 1.25 mL of water in order to separate the chloroform and aqueous methanol layers. After centrifugation, a clean biphasic system was obtained and the lower chloroform layer was carefully removed, washed using NaCl solution (5 mL; 5% *w/w*), evaporated in a drying oven at 50 °C, and followed by gravimetric measurement of the lipid. The lipid content, lipid yield and lipid productivity were calculated by the following equations (4-6). Experiments were performed in triplicate and average values were reported.

$$C = W_l / W_b \times 100\% \quad \text{(Eq. 4)}$$

$$Py = DW \times C \quad \text{(Eq. 5)}$$

$$Pt = Py / t \quad \text{(Eq. 6)}$$

Where, C is the lipid content (%), W_l is the lipid weight (mg) and W_b the algae weight (mg), Py is the lipid yield (mg/L), DW is the DCW of algae (mg/L), Pt is the lipid productivity (mg/L/d), and t is the cultivation time (d).

Fatty acid content and compositional analysis were performed in two steps, including the preparation of FAMEs and Gas Chromatography-Mass Spectrometry (GC–MS) analysis (Luo et al., 2016). FAMEs were prepared by a one-step extraction transesterification method described by Wang et al., (2010). Briefly, samples of dried

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algae (0.1 g) were placed in a 25 mL screw-top glass bottle with a mixture of methanol, concentrated sulfuric acid, and chloroform (4.25:0.75:5; 10 mL). Transesterification was carried out in a 90 °C water bath (Cole-Parmer, Vernon Hills, USA) for 90 min. Upon completion of the reaction, the chloroform layer, containing FAMEs, was carefully collected and subjected to GC–MS analysis (QP2010; Shimadzu Corp., Kyoto, Japan). The GC was equipped with a flame ionization detector and a RTX-Wax capillary column (30 m × 0.32 mm × 0.25 μm; Restek Corp., Bellefonte, USA). The oven temperature programme was 100 °C, held for 3 min, and then raised to 200 °C at a rate of 4 °C/min. Finally, the temperature was increased to 250 °C at a rate of 3 °C/min, and held for 5 min. The injector temperature was set at 230 °C. The carrier gas (helium) was controlled at 30 mL/min. The FAME compounds were identified by comparison with spectra from the NIST Mass Spectral Database and quantified by comparing the peak areas with that of the external standard (C18:2) (Sigma–Aldrich; St Louis, USA).

2.6 Statistical Analysis

SigmaPlot software (version 12.5, Systat Software Inc., San Jose, USA) was used for plotting and data analysis (Kizito et al., 2017). One-way analysis of variance (ANOVA) was used to evaluate significant differences of the lipid content in the microalgae between different groups cultivated in different proportions of OPE and DPE ($p < 0.05$).

3. Results and Discussion

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3.1 Nutrient removal performance

In both original (OPE) and digested (DPE) piggery wastewaters, most of the nitrogen was in the form of ammonium nitrogen (NH_4^+-N). It has been demonstrated that the NH_4^+-N can be removed by several routs, including algal uptake for biomass growth (Mezzari et al., 2013), and nitrification processes by nitrifies when wastewater was induced for microalgae cultivation (González-Fernández et al., 2011). In this study, under different initial NH_4^+-N concentrations, the removal efficiencies attained above 90% in both wastewaters after 14 days' cultivation of the microalgae (Fig. 1a-c). The average removal rates of NH_4^+-N were 1.76-7.92 mg/L/d in OPE, which were clearly higher than those (1.61-4.84 mg/L/d) from the DPE (Table S2). The removal rates achieved in this study agreed with previous studies (2.2-20 mg/L/d) of using other microalgae species for piggery wastewater treatment (Luo et al., 2019). Throughout the experiment, TN removal efficiencies were 42%, 80%, and 89% in 5%, 10% and 20% OPE groups, and 83%, 82% and 84% in the 2.5%, 5%, 10% DPE groups (Fig. 1 d-f, Table S2), respectively. The removal performances for TN were lower than those for NH_4^+-N in the corresponding groups, which may have been because some organic nitrogen could not be converted to NH_4^+-N in the wastewater and assimilated by microalgae (Hu et al., 2012).

Phosphorus is an important element in microalgal cell metabolism and takes part in several key processes such as cell proliferation, nucleic acid and ATP synthesis (Peccia

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et al., 2013). Although many countries have imposed strict regulations on the discharge of phosphorus in wastewater, the effects of eutrophication caused by phosphorus still leads to huge environmental and economic losses every year (Maroušek et al., 2020b). Orthophosphate is essential macro-nutrient for nucleic acids, phospholipids, proteins, and the intermediates of carbohydrate metabolism, along with microalgal growth. Microalgae tend to store large amounts of phosphorus inside the cells, and high concentrations of phosphorus in the external environment can further promote intracellular absorption (Maroušek et al., 2019). Previous studies have demonstrated the rapid absorption of phosphorus by microalgae in the early stage of growth (Shen et al., 2020). In this study, we also found sharply decreasing PO_4^{3-}-P concentrations in the first 7 days from all the wastewater groups (Fig. 2g-i). After 14 days' operation, the removal efficiencies of PO_4^{3-}-P (Fig. 1g-i) in all groups reached nearly 100%, except for the 20% OPE group (87.5 ± 6.8%). Generally, this performance is supported by previous studies (Franchino et al., 2013). The average PO_4^{3-}-P removal rates attained 0.21-0.36 mg/L/d in OPE groups (Table S2), which was similar to those found in a previous study with a removal rate of 0.22-0.38 mg/L/d (Cheng & Tian, 2013). Moreover, these values are significantly higher than the removal rates (0.06-0.24 mg/L/d) achieved in those groups cultured in DPE. In addition, a previous investigation demonstrated that the initial N/P ratio could affect nutrient uptake and algae growth, and the most appropriate N/P was demonstrated to be 16:1 (Kim et al., 2013). In this study, the initial N/P ratio of OPE was 15.4:1 (based on the initial concentrations of NH_4^+-N and PO_4^{3-}-P), which was

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283 close to the optimum N/P ratio, compared with the value determined from analysis of
284 the DPE (21.8:1). This may have led to the higher removal rates of both NH_4^+-N and
285 PO_4^{3-}-P observed the experiment.

286 **3.2 Biomass production and lipid accumulation**

287 The algal biomass growth did not exhibit obvious stagnation and adjustment stages
288 under cultivation in both OPE and DPE media (Fig. 2a), which indicated that
289 *Desmodesmus* sp. EJ 8-10 might readily adapt to varying concentrations of effluent
290 wastewater from pig farms. After day 7, the biomass concentrations were 0.25, 0.26 and
291 0.24 g/L in 5%, 10%, and 20% OPE groups, and 0.21, 0.27, and 0.16 g/L in 2.5%, 5%,
292 10% DPE groups, respectively. However, reduced biomass growth was observed after
293 day 7 in all groups (Fig. 1). The average biomass of EJ 8-10 after 14 days of culture
294 achieved to 0.33-0.39 g/L and 0.15-0.35 g/L from OPE and DPE cultures, respectively.
295 These values were significantly lower than those from the control group where algae
296 were cultivated in the optimal sufficient BG11 medium. The main reason for this
297 behaviour would be that rapid adsorption of nutrients in the early stage of processing
298 led to an insufficient nutrient supply in the later stages, which limited the microalgal
299 growth (Wang et al., 2010). Moreover, Jiang et al., (2018) has reported that turbidity in
300 the real wastewater may lead to low light transmittance and inhibit photosynthesis and
301 thus the growth of microalgae. Future studies through adding BG11 medium at similar
302 levels of turbidity into the culture medium should be conducted in order to clarify the

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303 key cause and effects. Nevertheless, it is feasible to consider supplementing wastewater
304 to replenish necessary nutrients in order to obtain higher accumulation of microalgal
305 biomass in a future study.

306 When algal growth was compared under the same initial NH_4^+-N concentrations in
307 the different media, the biomass growth was always observed to be higher, at all
308 dilutions, in OPE than that in the corresponding DPE (Fig. 2a). It can be postulated that
309 the more optimal N/P ration (close to 16:1) (Kim et al., 2013) and presence of other
310 micronutrients (e.g. amino acids and vitamins) in the OPE would have benefited the
311 growth of the microalgae (Uggetti et al., 2014). In contrast, the lipid content (dry
312 weight %) of the microalgae exhibited a negative relationship with biomass production,
313 where the algae cultivated in the DPE medium (19.4-28%) showed significantly higher
314 lipid contents than those in OPE medium (18.7-22.3%) under the same initial levels of
315 NH_4^+-N (Fig. 2b). Under conditions of sufficient P supply from the medium, nitrogen
316 inhibition has been deemed as one of the common triggers for lipid accumulation in
317 microalgae (Shen et al., 2020). Thus, the OPE contained lower N/P ratio compare with
318 the DPE, which could theoretically have contributed to a higher lipid content in the
319 microalgae. Thus, the current observed lower levels of lipid accumulation (Fig. 2b) may
320 be attributed to insufficient P nutrients for lipid synthesis in the algal cells (Merzlyak et
321 al., 2007). The results from this study indicated that piggery wastewater without
322 anaerobic digestion treatment could enhance the growth of algal biomass, however, lead

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to lower lipid accumulation compared with the medium of anaerobically-digested wastewater.

3.3 Lipid yield and productivity

Lipid productivity, which considers both the intracellular lipid content and the biomass growth, was calculated in order to provide an accurate comparison of the biofuel production potential of the microalgae (Brennan & Owende, 2010). Although algae biomass growth in all DPE cultivation systems was lower (Fig. 2a), lipid productivities from the 5% and 10% DPE groups yielded higher values (4.8-5.7 mg/L/d) than those (4.4-5.2 mg/L/d) in the corresponding 10% and 20% OPE groups (Table 1). The only lower lipid productivity (2.9 ± 0.4 mg/L/d) found in the 2.5% DPE group when compared with that (5.4 ± 0.8 mg/L/d) from the corresponding 5% OPE group, occurred because of insufficient nutrients available for algal growth (Fig. 2a). The lipid productivity of EJ 8-10 in this experiment was approximately the same as that found by Chinnasamy et al., (2010), around 4 mg/L/d. However, these values were lower than the productivity (up to 77 mg/L/d) found by some other researches (Cai et al., 2013; Hu et al., 2013; Singh et al., 2011). This may be due to the different algae species studied and that the cultivation media employed in these other investigations did not provide a nutrient shortage during the algae growth period. Nevertheless, the results supported the premise that DPE could enhance the quantity of the lipid accumulated by microalgae

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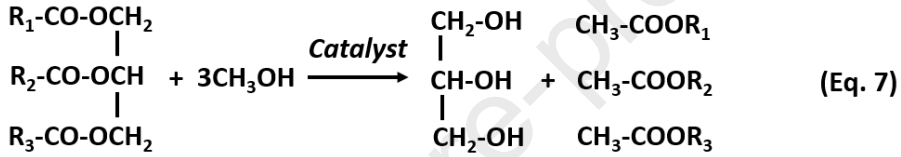
342 and it is further expected that even higher lipid production levels could be achieved with
343 sufficient nutrient supply (less dilution) from the wastewater.

344 **3.4 Fatty acid composition and quantity**

345 The fatty acids extracted from the microalgae can be converted into biodiesel
346 through a transesterification process (Demirbas, 2010). According to the following
347 specific biochemical reaction (Equation 7), the algal fatty acids could be mixed with
348 alcohol and an acid or a base to produce the methylesters that makes up the biodiesel.



(Eq. 7)

349

350 To evaluate the quality of the potential biodiesel extracted from the microalgae
351 biomass, fatty acid compositions and their quantities present were assessed at the end of
352 the experiment. As shown in Table 2, fatty acid speciation from the algae cultivated in
353 both OPE and DPE media were similar, including dodecanoic acid (lauric acid: C12:0),
354 tetradecanoic acid (myristic acid: C14:0), (9Z)-Tetradec-9-enoic acid (myristoleic acid:
355 C14:1), hexadecanoic acid (palmitic acid: C16:0), octadecanoic acid (stearic acid;
356 C18:0), (9Z)-Octadec-9-enoic acid (oleic acid; C18:1) and
357 (9*Z*,12*Z*)-octadeca-9,12-dienoic acid (linoleic acid; C18:2). Among them, the content of
358 the latter (C18:2) acid exhibited the highest value, which has previously been
359 demonstrated as the most common fatty acid in microalgae (Wang et al., 2010).
360 C16-C18 acids have been recognised as the most appropriate biofuel sources derived

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361 from microalgae (Marjakangas et al., 2015). These generally showed significantly
362 higher concentrations (50.3-60.7 mg/g) in the algae cultivated in the DPE medium
363 compared with the levels (41.1-46.3 mg/g) obtained from algae cultivated in OPE (Fig.
364 3).

365 Currently, attention to fatty acid composition has been concentrated on the
366 reduction of saturated fatty acids (SFAs) and polyunsaturated fatty acids (PUFAs) and
367 increase in monounsaturated fatty acids (MUFAs), in order to promote higher-quality
368 biofuel/biodiesel sources (Brennan & Owende, 2010). Reductions in SFAs and PUFAs
369 have been identified as a priority since they could potentially lead to instability of the
370 biodiesel (Deng et al., 2018). Enrichment of MUFAs is an effective approach by which
371 to increase the combustion performance of the synthesised biodiesel (Kumar et al.,
372 2019). The contents of the categorised fatty acids from Table 2 is visualised in Fig. 3,
373 where microalgae cultivated in DPE yielded a higher proportion of MUFAs and lower
374 proportions of SFAs and PUFAs compared with those cultured in OPE. The results
375 indicated that DPE could not only enhance total lipid accumulation but also provide an
376 increased quality of biodiesel due to the superior fatty acid compositions.

377 **3.5 Insights into the livestock waste treatment flowsheet**

378 Cultivation of microalgae could be integrated into the livestock wastewater
379 processing currently recommended for anaerobically-digested effluent treatment. The
380 potential for superior fatty acids composition for production of high-quality biodiesel

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potential could be achieved by culturing microalgae in anaerobically-digested effluent (Fig. 3). However, the nutrient composition, e.g. high N/P ratio and availability of fewer micronutrients, in the wastewater after anaerobic digestion has been shown to significantly hinder growth of algal biomass (Fig. 2a) and further limit the potential for biofuel generation (Table 1). Previous studies have demonstrated that, for efficient growth of microalgae, micronutrients such as iron and manganese, are required at levels of 2.5–30 ppm and that trace elements, such as cobalt, zinc, and molybdenum, are essential in very low concentrations (2.5–4.5 ppm) (Juneja et al., 2013). The original livestock wastewater, without treatment by anaerobic digestion, is deemed to contain such micronutrients (Uggetti et al., 2014), beneficial for microalgae growth. Moreover, the addition of such micronutrients (Ghafari et al., 2015), and trace metals (Han et al., 2019) to culture media could also lead to higher (4-39%) lipid content accumulation by different microalgae, including *Chlorella sorokiniana*, *Chlorella vulgaris, Dunaliella tertiolecta, Tetraselmis suecica*, and *Scenedesmus obliquus*.

Therefore, following this concept, the original piggery effluent with more macro- and micro-nutrients and the effluent from other secondary treatments could be introduced as a cost-effective measure in order to adjust the composition of the digested wastewater. After achieving the optimum culture medium, the proposed flowsheet (Fig. 4) could contribute to sustainable livestock waste management through simultaneously treating the waste and producing high-quality biofuel. Many developing countries,

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401 located in the tropical and subtropical areas, could receive sufficient annual solar
402 irradiance (Shahsavari and Akabari, 2018), which could benefit the algae cultivation
403 and further biodiesel generation. Moreover, the swift livestock industries growth in
404 developing countries has triggered serious environmental pollutions due to the discharge
405 of livestock wastes. Therefore, the proposed livestock management approach could
406 offer sustainable energy production, wastewater treatment, and an economic boost to the
407 developing countries. Nevertheless, evaluation of the implicated costs of the proposed
408 process needs to be further studied before it could be commercialised and applied ay an
409 industrial level.

410 **4. Conclusions**

411 This study investigated the potential for the production of high quality biofuel
412 from microalgae cultured in both original (OPE) and anaerobically-digested (DPE)
413 piggery effluent at different nutrient concentrations. After 14 days' cultivation, the
414 microalgae achieved higher values of biomass (0.33-0.39 g/L) when cultured in OPE
415 compared with those (0.15-0.35 g/L) from DPE cultures. However, higher lipid
416 productivity (5.7 mg/L/d) and more optimal lipid compositions were observed in the
417 microalgal cells from DPE cultures, which supported the superior potential of DEP for
418 high quantity and high quality biofuel generation. Based on these results, we proposed
419 that using original livestock effluent and/or final effluent from the secondary treatment
420 to manipulate the digested piggery effluent towards upgrading the sustainable livestock

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waste management flowsheet. It is recommended that further studies should focus on demonstrating the process at scale and on detailed cost-benefit analysis and relevant cost analysis of the proposed flowsheet.

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Table 1 Biomass productivity, lipid yield and lipid productivity of the microalgae cultivated in original piggery effluent (OPE) and digested piggery effluent (DPE) at different dilutions.

Wastewater	Biomass productivity (mg/L/d)	Lipid yield (mg/L)	Lipid productivity (mg/L/d)
5% OPE	24.4 ± 1.4^{b}	76.3 ± 11.9^{ab}	5.4 ± 0.8^{ab}
10% OPE	27.6 ± 1.1^{a}	72.4 ± 6.9^{ab}	5.2 ± 0.5^{ab}
20% OPE	23.5 ± 0.8^{b}	61.9 ± 0.3^{c}	4.4 ± 0.1^{c}
2.5% DPE	10.4 ± 0.7^{d}	40.9 ± 4.7^{d}	2.9 ± 0.4^{d}
5% DPE	21.8 ± 1.7^{c}	80.2 ± 5.8^{a}	5.7 ± 0.5^{a}
10% DPE	24.7 ± 0.1^{b}	67.1 ± 3.8^{b}	4.8 ± 0.3^{b}

Different letters besides the values in the same column represent significant differences.

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Table 2 Compositions of fatty acids, including saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), of microalgae cultivated in original piggery effluent (OPE) and digested piggery effluent (DPE) at different dilutions.

Fatty acids		5% OPE		10% OPE		20% OPE	
		mg/g	%	mg/g	%	mg/g	%
SFA	C10:0	0.195±0.002	0.31	0.121±0.000	0.25	0.153±0.000	0.25
	C11:0	0.410±0.003	0.65	1.040±0.066	2.15	1.081±0.004	2.98
	C12:0	2.245±0.067	3.56	2.158±0.032	4.46	2.940±0.002	4.87
	C13:0	0.048±0.000	0.08	0.246±0.000	0.51	0.579±0.000	0.96
	C14:0	2.359±0.516	3.74	1.806±0.048	3.73	3.248±0.015	5.38
	C15:0	0.054±0.001	0.09	0.532±0.005	1.10	0.911±0.013	1.51
	C16:0	1.631±0.025	2.58	1.699±0.004	3.51	0.500±0.001	0.83
	C18:0	6.976±0.500	11.05	1.756±0.049	3.63	1.465±0.001	2.43
	C21:0	0.311±0.001	0.49	0.062±0.000	0.13	0.440±0.005	0.73
MUFA	C14:1	4.687±0.639	7.43	0.543±0.000	1.12	0.938±0.002	1.55
	C15:1	0.186±0.000	0.29	0.185±0.001	0.38	0.409±0.000	0.68
	C16 : 1	0.897±0.011	1.42	0.947±0.008	1.96	2.009±0.000	3.33
	C18:1n9c	1.003±0.000	1.59	1.009±0.007	2.09	1.415±0.000	2.34
	C20:1	6.394±0.644	10.13	0.371±0.003	0.77	0.934±0.001	1.55
PUFA	C18:2n6t	34.191±3.294	54.17	32.853±0.238	67.92	38.202±0.322	63.27
	C18:2n6c	0.050±0.000	0.08	0.483±0.002	1.00	1.097±0.001	1.82
	C18:3n6	0.350±0.003	0.55	2.172±0.031	4.49	0.947±0.001	1.57
	C18:3n3	0.137±0.001	0.22	0.129±0.001	0.27	0.745±0.007	1.23
	C20:2	0.691±0.009	1.09	0.069±0.000	0.14	1.064±0.127	1.76
	C20:3n6	0.205±0.020	0.32	0.052±0.000	0.11	0.298±0.001	0.49
	C20:4n6	0.099±0.000	0.16	0.136±0.000	0.28	0.282±0.001	0.47

Fatty acids		2.5% DPE		5% DPE		10% DPE	
		mg/g	%	mg/g	%	mg/g	%
SFA	C10:0	0.098±0.000	0.16	0.142±0.001	0.19	0.098±0.000	0.14
	C11:0	0.403±0.002	0.64	0.438±0.001	0.57	0.441±0.010	0.63
	C12:0	2.442±0.029	3.90	2.493±0.022	3.27	2.192±0.066	3.15
	C13:0	0.072±0.000	0.11	0.051±0.000	0.07	0.048±0.000	0.07
	C14:0	2.197±0.002	3.51	2.714±0.000	3.56	2.851±0.070	4.10
	C15:0	0.573±0.015	0.92	0.084±0.003	0.11	0.076±0.000	0.11
	C16:0	1.017±0.007	1.63	0.960±0.001	1.26	0.888±0.007	1.28
	C18:0	1.290±0.148	2.06	1.341±0.001	1.76	1.471±0.016	2.11
	C21:0	0.541±0.024	0.87	0.302±0.001	0.40	0.288±0.000	0.41
MUFA	C14:1	4.861±0.063	7.77	6.022±0.025	7.91	6.460±0.497	9.29

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	C15:1	0.335±0.040	0.53	0.478±0.006	0.63	0.394±0.003	0.57
	C16:1	0.709±0.009	1.13	0.850±0.009	1.12	1.078±0.038	1.55
	C18:1n9c	5.727±0.061	9.15	5.917±0.035	7.77	6.069±0.357	8.72
	C20:1	0.367±0.000	0.59	1.716±0.013	2.25	0.612±0.004	0.88
PUFA	C18:2n6t	32.312±1.701	51.65	38.542±1.452	50.61	41.559±0.177	59.74
	C18:2n6c	0.546±0.054	0.87	0.557±0.001	0.73	0.044±0.000	0.06
	C18:3n6	8.451±0.128	13.51	9.051±0.380	11.88	3.923±0.131	5.64
	C18:3n3	0.239±0.003	0.38	0.480±0.009	4.57	0.407±0.000	0.58
	C20:2	0.237±0.005	0.38	0.585±0.002	0.77	0.302±0.001	0.43
	C20:3n6	0.098±0.002	0.16	0.349±0.007	0.46	0.228±0.002	0.33
	C20:4n6	0.051±0.000	0.08	0.084±0.001	0.11	0.140±0.001	0.20

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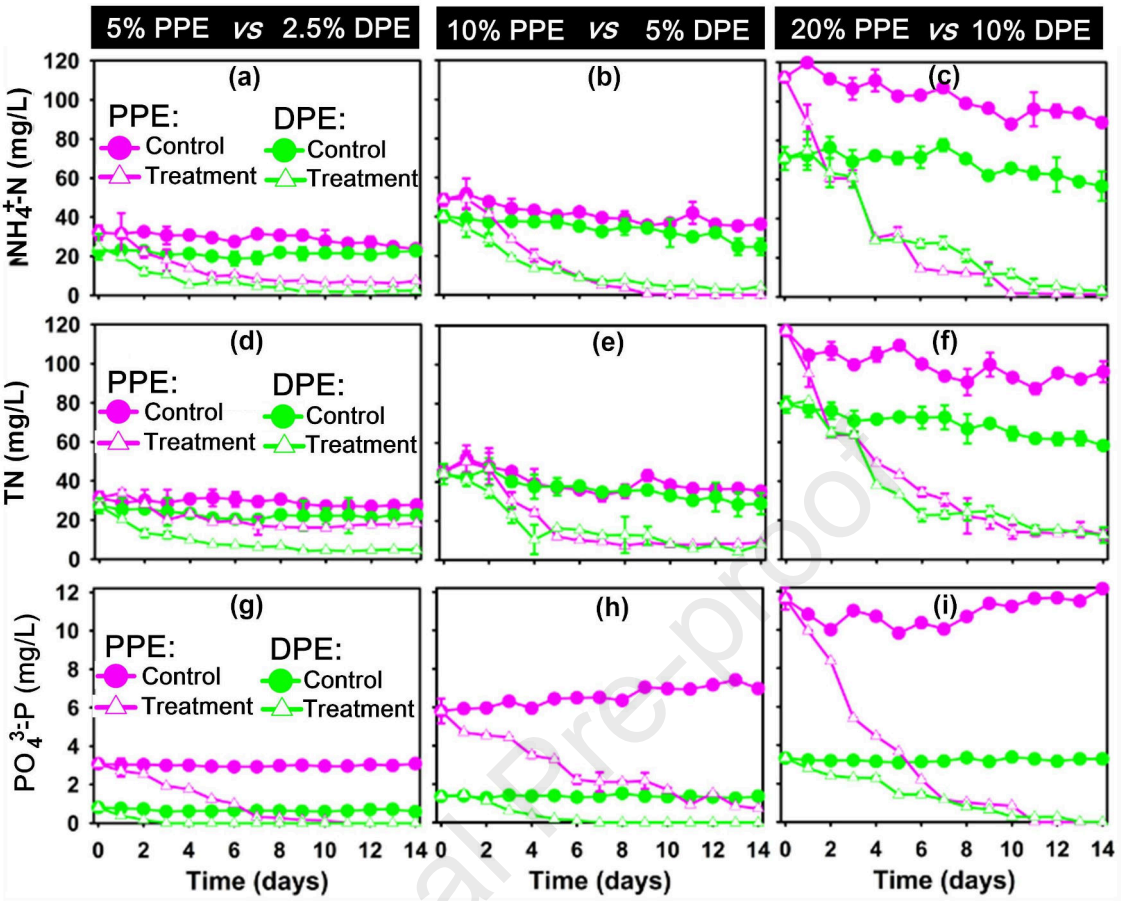

Fig. 1. Dynamics of NH_4^+-N (a-c), TN (d-f) and PO_4^{3-}-P (g-i) in the water through microalgae cultivation in original piggery effluent (OPE) and digested piggery effluent (DPE) at different dilutions

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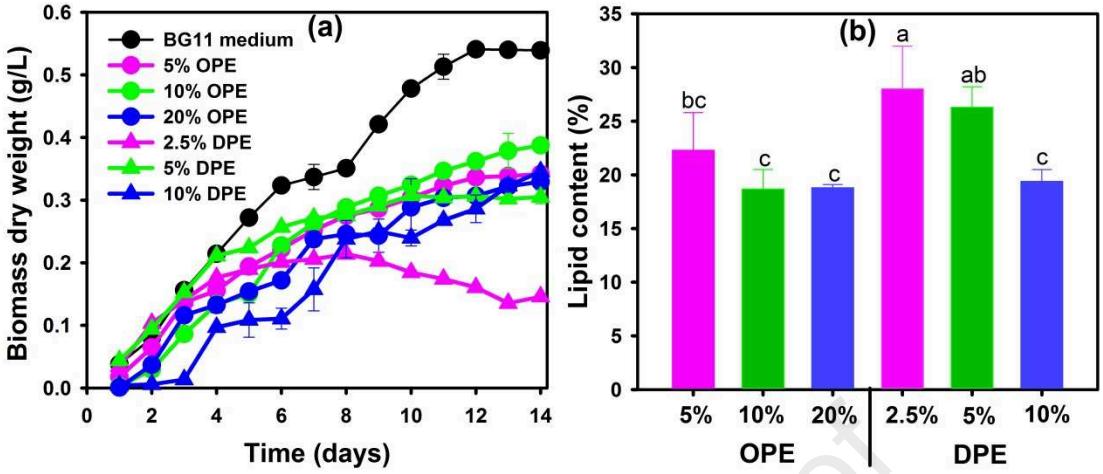

Fig. 2. Dynamics of microalgal biomass production (a) and lipid content of the microalgae at the conclusion of the experiment (b) under cultivation in original piggery effluent (OPE) and digested piggery effluent (DPE). Different letters above the bars in Fig. 2b represent significant differences between the groups.

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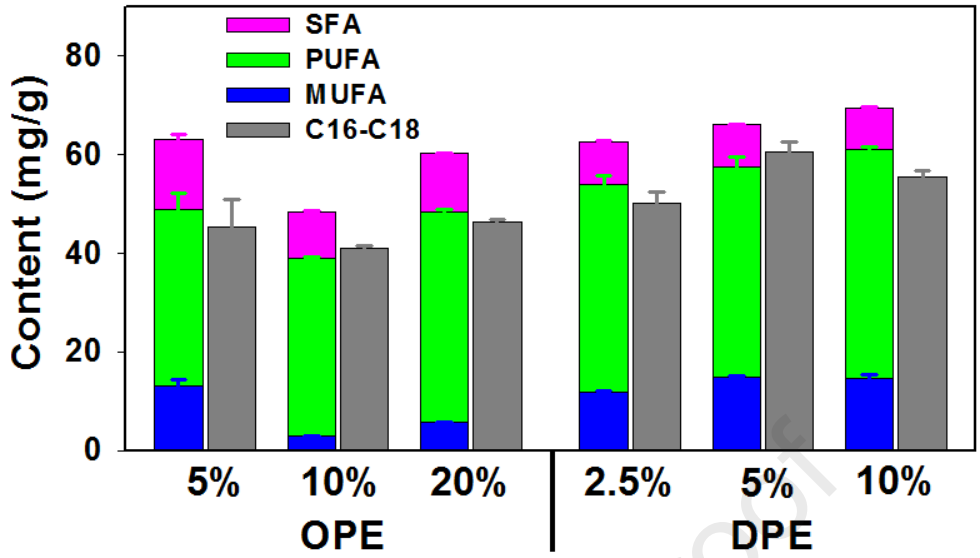

Fig. 3. The contents of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and total C16-C18 acids in microalgae cultivated in original piggery effluent (OPE) and digested piggery effluent (DPE) at different dilutions

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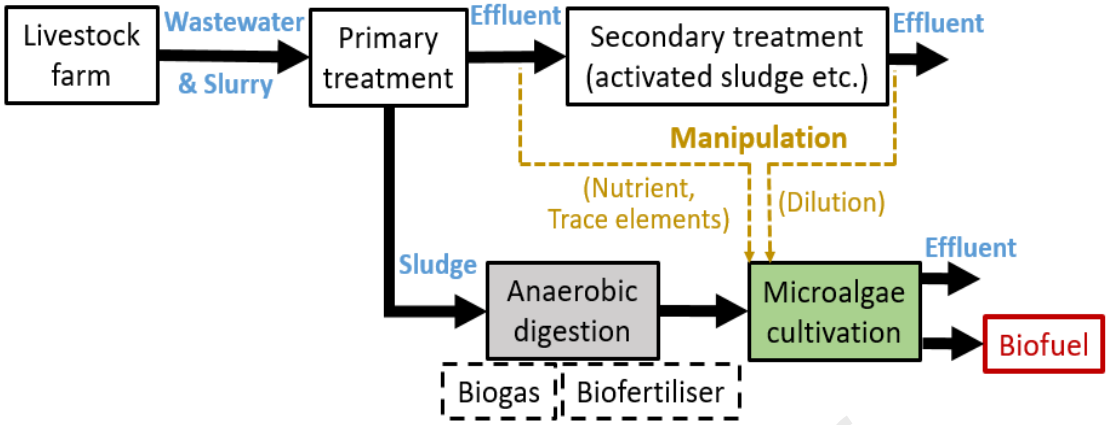

Fig. 4. Proposed integrated livestock waste treatment flowsheet based on anaerobic digestion and microalgal technologies towards waste treatment and high-quality biofuel generation

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CRediT author statement

Li Gang: Conceptualization, Methodology, Validation, Writing- Original draft preparation. **Zhang Jiang:** Writing- Original draft preparation. **Li Huan:** Writing- Original draft preparation. **Hu Ruichen:** Writing- Reviewing and Editing. **Yao Xiaolong:** Writing- Reviewing and Editing. **Liu Ying:** Methodology, Validation; Visualization. **Zhou Yuguang:** Conceptualization, Supervision, Funding acquisition, Writing- Reviewing and Editing. **Lyu Tao**: Data curation, Writing- Original draft preparation, Writing- Reviewing and Editing.

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Highlights:

- Microalgal biomass growth was hindered in digested piggery effluent (DPE) culture
- Microalgae removed >90% of N and P from both DPE and original piggery effluent (OPE)
- DPE could increase the lipid content and productivity from microalgae
- Microalgae cultured in DPE had better quality biofuel potential than that from OPE
- A new flowsheet was proposed for livestock waste treatment and biofuel recovery

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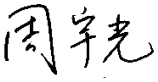
Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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