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Comprehensive evaluation of nitrogen removal rate and biomass, ethanol, and methane production yields by combination of four major duckweeds and three types of wastewater effluent

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ABSTRACT

To assess the potential of duckweeds as agents for nitrogen removal and biofuel feedstocks, *Spirodela polyrhiza*, *Lemna minor*, *Lemna gibba*, and *Landoltia punctata* were cultured in effluents of municipal wastewater, swine wastewater, or anaerobic digestion for 4 days. Total dissolved inorganic nitrogen (T-DIN) of 20–50 mg/L in effluents was effectively removed by inoculating with 0.3–1.0 g/L duckweeds. *S. polyrhiza* showed the highest nitrogen removal (2.0–10.8 mg T-DIN/L/day) and biomass production (52.6–70.3 mg d.w./L/day) rates in all the three effluents. Ethanol and methane were produced from duckweed biomass grown in each effluent. *S. polyrhiza* and *L. punctata* biomass showed higher ethanol (0.168–0.191, 0.166–0.172 and 0.174–0.191 g-ethanol/g-biomass, respectively) and methane (340–413 and 343–408 NL CH_4/kg VS, respectively) production potentials than the others, which is related to their higher carbon and starch contents and calorific values.

1. Introduction

Nitrogen removal from domestic, industrial, and agricultural wastewaters is necessary to prevent the eutrophication and pollution of aquatic environments. Conventional biological nitrogen removal methods used in tertiary treatment at wastewater treatment plants, although generally reliable and effective in nitrogen removal, are energy-intensive and quite costly. On the other hand, nitrogen is an essential nutrient for plants and is used in fertilizers to increase crop production. Recently, rather than removing nitrogen from wastewater by nitrification/denitrification, nitrogen recovery from wastewater has been recognized as a desirable technology for bioresource production. Due to their rapid nitrogen uptake and strong potential as a renewable bioresource, aquatic plants have been highlighted as promising tools for a sustainable system combining energy-saving and low-cost nitrogen removal and valuable resource production from wastewaters (Soda et al., 2013; Zhao et al., 2014a). Aquatic plants have several major advantages over terrestrial energy crops: They can take up nutrients directly from wastewater, do not need extra fertilization or irrigation, can grow throughout the year, and do not compete with food crop production and agricultural land use.

Duckweeds are the aquatic plants most studied for wastewater treatment because of their rapid growth and high nutrient uptake (Cheng et al., 2002; Dalu and Ndamba, 2003; Mohedano et al., 2012; Ran et al., 2004; Xu and Shen, 2011). Duckweeds are also an ideal feedstock for production of biofuels, especially of ethanol (Chen et al., 2012; Fujita et al., 2016; Ge et al., 2012; Soda et al., 2015; Takai et al., 2014; Xu et al., 2011, 2012), due to their soft biomass and high starch content that can be easily and effectively saccharified to glucose. Therefore, duckweed culture in wastewater treatment plants offers dual benefits of low-cost nitrogen removal and biofuel production. In addition, duckweeds are superior to microalgae with regard to the cost and ease of harvesting.

Duckweeds are classified into five genera, *Lemna*, *Landoltia*, *Spirodela*, *Wolffia*, and *Wolffiella*, and comprise about 37 species (Landolt, 1986). Several duckweed species have been examined for large-scale practical cultures or lab-scale experiments for nitrogen removal and/or biofuel production. The biomass production differs depending on duckweed species (Zhao et al., 2014b; Ziegler et al., 2015) and wastewater nutrient concentrations (Soda et al., 2015). Likewise, starch contents of duckweeds differ between species (Zhao et al., 2014b) and nutrient concentrations (Li et al., 2016; Zhao et al., 2014b). Previous studies evaluated the nitrogen removal capability, biomass production, or biofuel production of single duckweed species and/or one kind of wastewater. To develop an efficient duckweed-based nitrogen removal and biofuel production system, it is necessary to

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Table 1
Initial nitrogen concentrations in effluent samples.

Effluent sample	Nitrogen concentrations (mg/L)		
	NH_4-N	NO_2-N	NO_3-N
Secondary effluent of municipal wastewater	3.9	0.1	4.1
Secondary effluent of swine wastewater	75.1	2.6	2.3
Effluent of anaerobic digestion (1:1 diluted by using tap water)	30.1	0	3.3

compare both the nitrogen removal capability and biofuel production potential of different common duckweed species in different types of wastewater.

Methane fermentation by anaerobic digestion is the most feasible and cost-effective technology to produce biofuel from organic matter, including wastewater sludge, municipal solid waste, animal manure, food waste, and plant biomass (Appels et al., 2011; Chynoweth et al., 2001; Sawatdeenarunat et al., 2016). Application of anaerobic digestion to a wastewater treatment plant can turn the treatment plant into a net energy producer (McCarty et al., 2011; Scherson and Criddle, 2014). Co-digestion of duckweed biomass with wastewater sludge would increase methane productivity in wastewater treatment plants. Some recent studies demonstrated methane production by anaerobic digestion of duckweed biomass (Cu et al., 2015; Gaur et al., 2017; Ramaraj and Unpaprom, 2016; Yadav et al., 2017). To our knowledge, however, only one study has quantified the methane production potential of duckweed, *Spirodela polyrhiza* (Cu et al., 2015). To expand the application range of these dual benefits of growing duckweeds in wastewater treatment plants, it is important to evaluate the potentials of various common duckweed species in different types of wastewater for the production of methane as well as ethanol.

This study aimed to compare the capabilities of four common duckweed species for nitrogen removal, biomass production, and ethanol and methane production. Nitrogen removal rates and biomass production rates of *S. polyrhiza*, *Lemna minor*, *Lemna gibba*, and *Landoltia punctata* were examined under three different wastewater cultures: secondary effluent of a municipal wastewater treatment plant, secondary effluent of swine wastewater, and effluent of anaerobic digestion of human fecal sludge. Productivities of ethanol and methane were determined for the duckweed biomass to assess the four species' potential use as feedstock for biofuels. Our results provide the first comprehensive dataset on the potentials of nitrogen removal, ethanol production, and methane production of common duckweeds.

2. Materials and methods

2.1. Plant materials

Bacteria-free *S. polyrhiza*, *L. minor*, *L. gibba*, and *L. punctata* were prepared by washing in 0.5% sodium hypochlorite for 3 min, then in 70% ethanol for 1 min, and finally in sterilized water three times for 1 min. Duckweeds were aseptically and routinely cultured in flasks

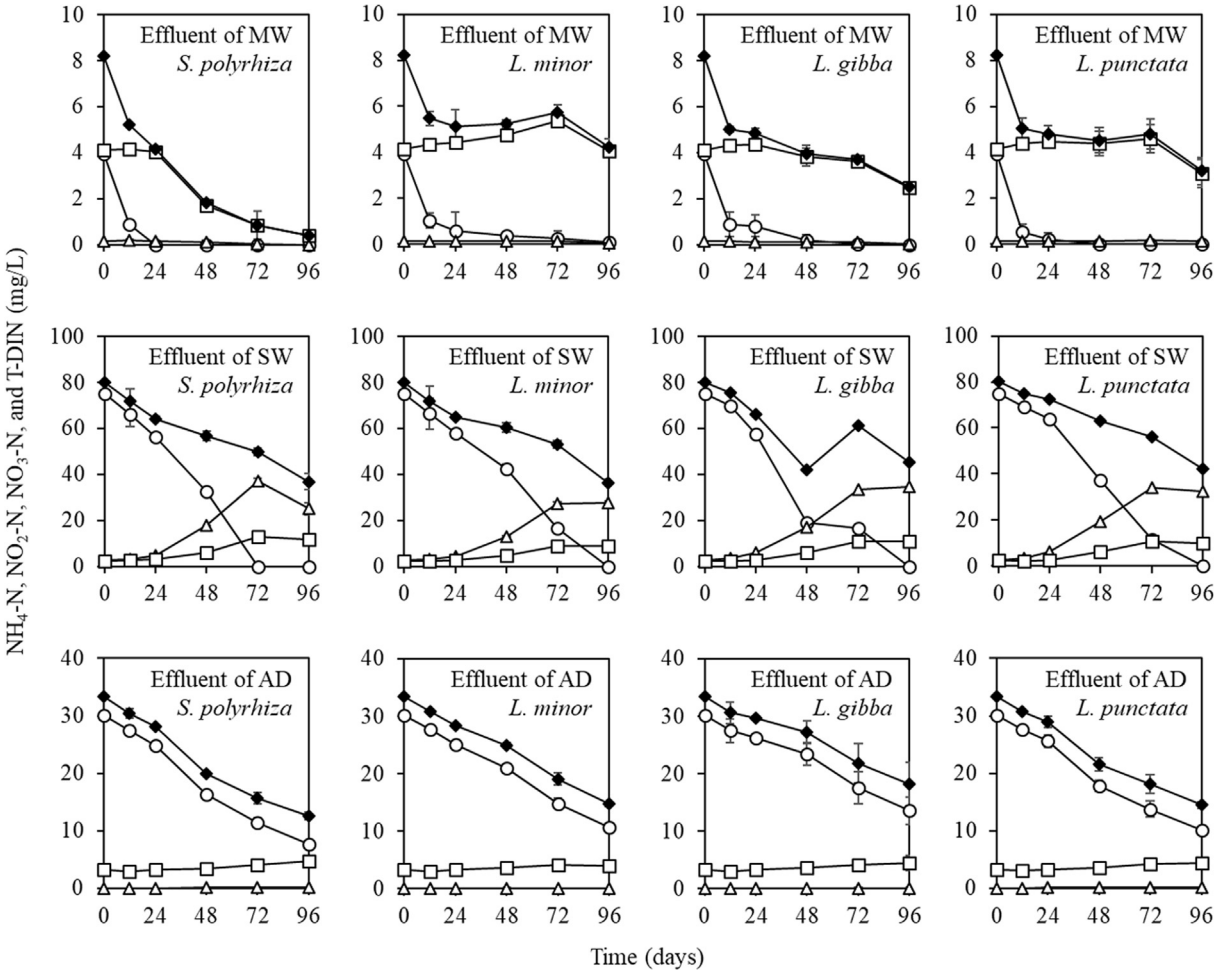

Fig. 1. Changes in NH_4-N (open circles), NO_2-N (open triangles), NO_3-N (open squares), and T-DIN (closed diamonds) concentrations in three effluent samples (MW: municipal wastewater; SW: swine wastewater; AD, anaerobic digestion) during 4 days of duckweed cultivation. Values are mean ± SD (n = 2).

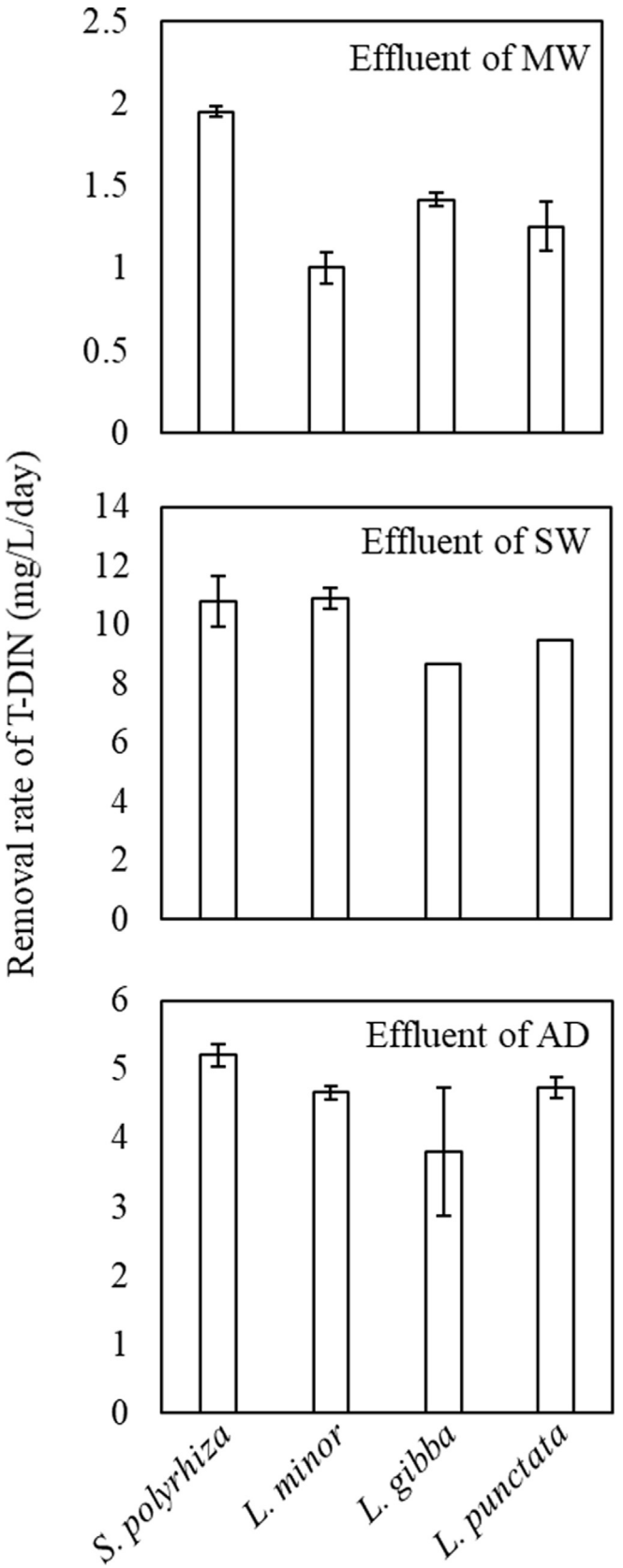

Fig. 2. Removal rates of T-DIN from the three effluent samples (MW: municipal wastewater; SW: swine wastewater; AD, anaerobic digestion) by four duckweed species during 4 days of cultivation. Values are mean ± SD (n = 2).

containing sterilized Hoagland solution (36.1 mg/L KNO_3, 293 mg/L K_2SO_4, 3.87 mg/L NaH_2PO_4, 103 mg/L $MgSO_4{\cdot}7H_2O$, 147 mg/L $CaCl_2{\cdot}H_2O$, 3.33 mg/L $FeSO_4{\cdot}7H_2O$, 0.95 mg/L H_3BO_3, 0.39 mg/L $MnCl_2{\cdot}4H_2O$, 0.03 mg/L $CuSO_4{\cdot}5H_2O$, 0.08 mg/L $ZnSO_4{\cdot}7H_2O$, and 0.254 mg/L $H_2MoO_4{\cdot}4H_2O$; pH 7.0) in a growth chamber (28 ± 1 °C, fluorescent lamps at a photosynthetic photon flux density of 80 μmol/m^2/s, 16L:8D photoperiod).

2.2. Wastewater samples

Three types of wastewater were used in this study: (1) a secondary effluent sample of municipal wastewater treatment was collected during the activated sludge process of a municipal wastewater treatment plant in Kofu city, Yamanashi, Japan; (2) a sample of secondary effluent of swine wastewater was collected during the activated sludge process of a swine wastewater treatment plant in Chuo city, Yamanashi, Japan; and (3) an effluent sample was collected during the anaerobic digestion process of a human fecal sludge treatment plant in Fuefuki city, Yamanashi, Japan. Anaerobic digestion effluent was diluted 1:1 by using tap water in this study. Water quality characteristics of the three effluent samples are listed in Table 1.

2.3. Experimental design

This study is composed of two experiments. In experiment 1, we tested nitrogen removal and biomass production of the four duckweeds in the three different effluents, and in experiment 2, we assessed the production of ethanol and methane from biomass of the four duckweeds grown in the three effluents.

In experiment 1, each duckweed species was first pre-cultured separately in 1 L of each effluent sample in a plastic container (160 mm × 125 mm × 80 mm) for 7 days to acclimate duckweed samples to effluent sample conditions. Then 0.3–1.0 g of the freshly acclimated duckweed—a quantity sufficient to cover about 50% of the water surface with a single layer of fronds—was transferred to another plastic container (of the same dimensions) containing 1 L of each effluent sample. The duckweeds were cultivated in a growth chamber for 4 days. During the cultivation period, a water sample was collected at 0, 12, 24, 48, 72, and 96 h for water quality analysis (for details, see Section 2.4). After 4 days, all vegetative duckweed fronds were collected from each container, washed gently using tap water, and dried at 90 °C for 3 h.

For experiment 2, about 50 g of each acclimated duckweed species was inoculated into 60 L (20 L × three cultivation tanks [380 mm × 280 mm × 300 mm]) of each effluent sample and harvested after growing for 4 days in an open-air greenhouse. For the ethanol production assay, the vegetative duckweed biomass was dried and powdered, and the resultant dried biomass powder was used as feedstock for ethanol fermentation (see Section 2.6). For the methane production assay, fresh duckweed biomass was directly used as feedstock for methane fermentation with no pretreatment (see Section 2.7).

2.4. Water quality analyses

Dissolved NH_4-N, NO_2-N, and NO_3-N were measured. Each collected water sample was passed through a glass fiber filter (pore size, 10 μm) to remove suspended materials. For NH_4-N, the indophenol method was used; for NO_2-N, the N-(1-naphthyl) ethylenediamine method; and for NO_3-N, the reduction–N-(1-naphthyl) ethylenediamine method and UV adsorption (at 220 and 275 nm) method. Total dissolved inorganic nitrogen (T-DIN) was calculated by summing NH_4-N, NO_2-N, and NO_3-N.

2.5. Duckweed biomass analyses

The collected biomass samples were dried and weighed, and the increase in dry weight biomass during the 4-day cultivation (Δd.w. = final value − initial value) was calculated. Then, the biomass

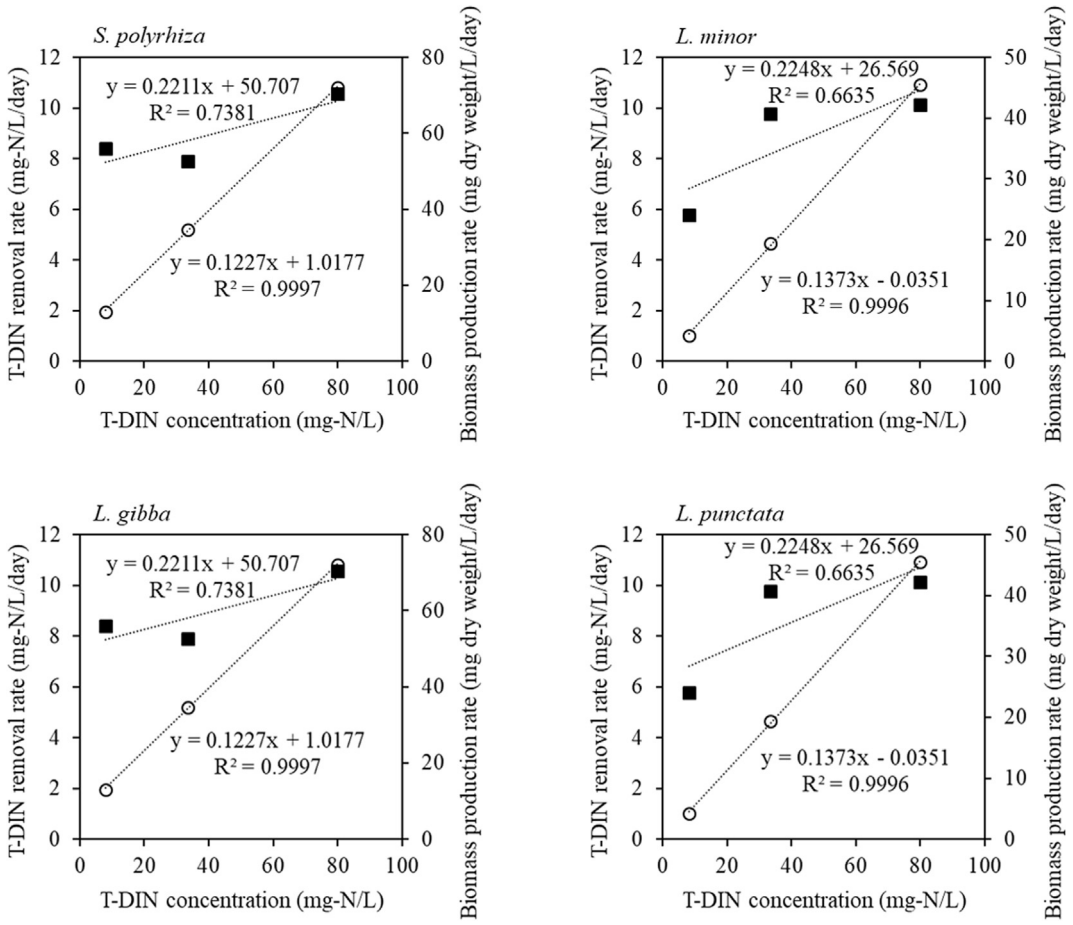

Fig. 3. Relationships between initial T-DIN concentration in the effluent sample and T-DIN removal rate by duckweed cultivation (open circles) and biomass production rate of duckweed (closed squares).

production rate per 1 L of effluent (mg d.w./L/day) was calculated.

In addition, dried biomass was powdered and used for analyses. The starch content was measured by using a total starch assay kit (Megazyme International, Wicklow, Ireland). The total carbon and nitrogen contents in plant biomass were measured by using a stable isotope analysis system (ANCA-GSL, Hydra 20-20; Sercon Ltd., Crewe, UK). Gross energy value (gross calorific value) of duckweed biomass was measured by using an auto-calculating bomb calorimeter (CA-4AJ; Shimadzu Co. Ltd., Kyoto, Japan).

2.6. Ethanol production assay

The ethanol production assay of duckweed biomass was conducted in a duplicate batch test using simultaneous saccharification and fermentation (SSF). Pretreatment and SSF were conducted according to the protocol developed by Soda et al. (2015). For pretreatment, 1 g of dried biomass powder was added to 5 mL of distilled water in a 50-mL vial and autoclaved at 121 °C for 20 min. Then, 20 mL of 10 mM citrate buffer (pH 5.0), 45 mg of α-amylase (165 units/mg, Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2 mg of amyloglucosidase (> 20 units/mg, Oriental Yeast Co., Ltd., Tokyo, Japan), and 50 mg of dry yeast (*Saccharomyces cerevisiae*; Super Camellia, Nisshin Seifun Group Inc., Tokyo, Japan) were added to each vial containing pretreated biomass. Each vial was closed with a butyl rubber stopper and aluminum crimp attached to a gas-vent syringe and incubated at 37 °C with 150 rpm shaking under dark conditions for 24 h. A solution sample was collected periodically from each SSF vial by syringe, centrifuged (11,000 × *g*, 5 min), and filtered (pore size, 0.2 μm). The amount of ethanol was measured by using a Shimadzu high-performance liquid chromatography system with a refractive index detector and an Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad Laboratories Inc., Hercules, CA, USA). The mobile phase was 5 mM sulfuric acid buffer, and the column was maintained at 65 °C.

2.7. Methane production assay

Methane fermentation was conducted in a duplicate batch anaerobic digestion test. Anaerobic digestion was performed in a 500-mL glass vessel containing 300 mL of anaerobic digestion sludge and 30 g of fresh duckweed biomass without any pretreatment (e.g., crushing). The inoculum sludge was collected from the mesophilic anaerobic digestion reactor of a sewage sludge treatment plant. To acclimate the sludge to each duckweed biomass, the sludge was fed with each duckweed biomass for 2 weeks before inoculation. Water content and volatile solids (VS) content of the acclimated sludge sample were about 98% (w/w) and 68% (w/d.w.), respectively. Each vessel was sealed and flushed with N_2 gas to maintain anaerobic conditions. An anaerobic digestion assay was conducted statically at 38 °C for 28 days. Anaerobic digestion with inoculum sludge alone (i.e., without duckweed biomass) was also conducted as a blank experiment. The biogas emitted from the sludge was collected in a wet gas meter, and the volume was determined daily. The biogas composition including methane and carbon dioxide was analyzed using a Shimadzu gas chromatography system with a thermal conductivity detector and a SHINCARBON ST column (2 m × 3 mm; Shimadzu GLC Ltd., Tokyo, Japan). The net volume of biogas produced from duckweed biomass was corrected based on the blank experiments and normalized to standard conditions (standard atmosphere pressure, 1013 hPa; 0 °C). Finally, the productivity of biogas was standardized to

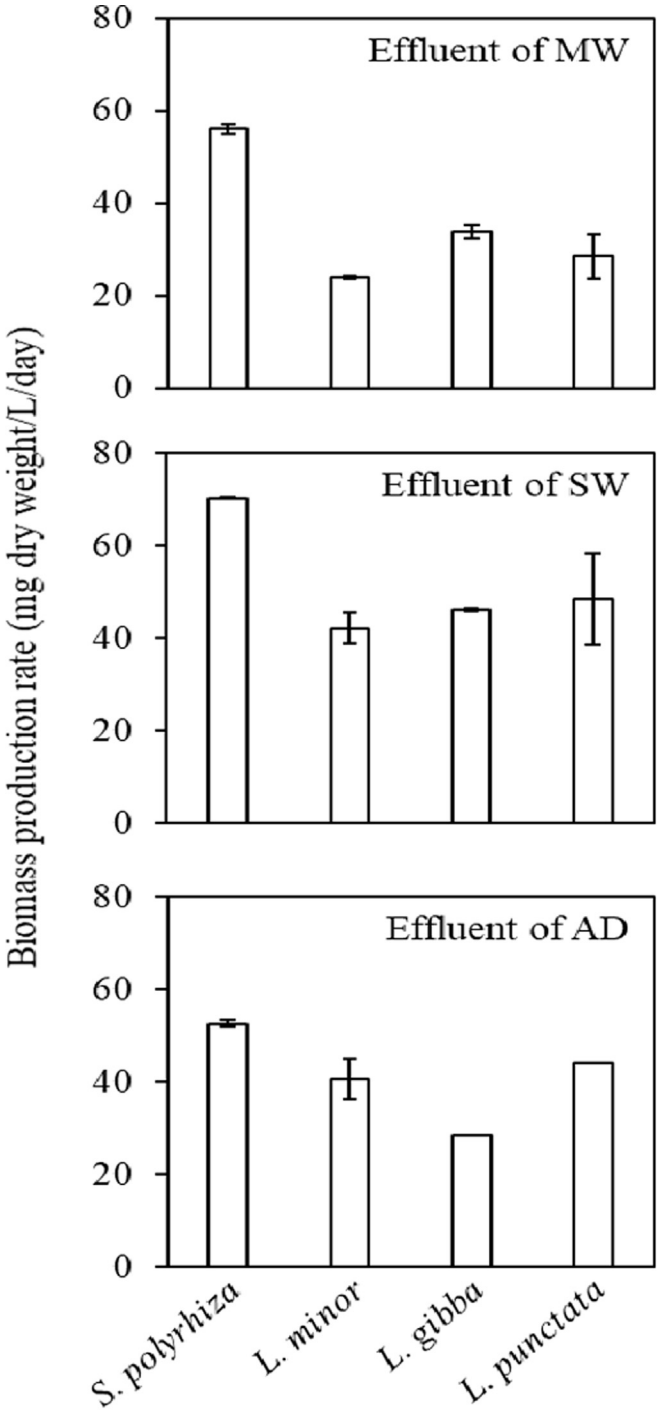

Fig. 4. Biomass production rates of four duckweed species during 4 days of cultivation in three effluents (MW: municipal wastewater; SW: swine wastewater; AD, anaerobic digestion). Values are mean ± SD (n = 2).

the amount of duckweed biomass VS and expressed as normal liters (NL) of CH_4/kg VS.

2.8. Statistical analysis

Each value used in the statistical analysis represents the results from two samples (n = 2 replicates) per experiment. All results are expressed as mean ± SD. Significance (P < .05) was analyzed by using the t-test in SPSS Statistics v. 22.0 (IBM, Armonk, NY, USA).

3. Results and discussion

3.1. Nitrogen removal

We grew four duckweed species in the three different effluent samples for 4 days. Changes in NH_4-N, NO_2-N, NO_3-N, and T-DIN during the 4-day cultivation are shown in Fig. 1. NH_4-N was removed quickly and effectively from all three effluent samples. On the other hand, the amount of NO_2-N and NO_3-N was not reduced or increased in effluent samples. NO_2-N was not detectable level in effluents of municipal wastewater and anaerobic digestion during the 4-day cultivation.

Nitrogen can be removed by both uptake/assimilation of duckweeds and bacterial transformation (nitrification/denitrification) (Mohedano et al., 2012), and some studies suggest that aquatic plants promote both nitrification and denitrification in the rhizosphere (Reddy et al., 1989; Risgaard-Petersen and Jensen, 1997). We observed increases in NO_3-N and/or NO_2-N, indicating that microbial nitrification prevailed over denitrification in the effluent of swine wastewater. This likely occurred because aerobic conditions were formed in the rhizosphere by duckweed photosynthesis, and anaerobic nitrate or nitrite respiration by denitrifying bacteria was inhibited. Previous studies showed that duckweeds easily and effectively take up NH_4-N and prefer this form over NO_3-N when both nitrogen sources are available (Cedergreen and Madsen, 2002; Fang et al., 2007). Therefore, nitrogen uptake by duckweed and microbial nitrogen transformation likely played significant roles in nitrogen removal in the experimental duckweed cultures.

The four duckweed cultures significantly and rapidly removed T-DIN from all effluent samples. In secondary effluent of municipal wastewater, secondary effluent of swine wastewater, and effluent of anaerobic digestion, 49–95%, 43–55%, and 46–62% of T-DIN was removed, respectively. Rates of T-DIN removal by duckweed species are summarized in Fig. 2, which shows that *S. polyrhiza* tended to have higher removal rates (2.0–10.8 mg T-DIN/L/day) in all effluent experiments. The T-DIN removal rates in duckweed–effluent cultures had a strong correlation with the initial T-DIN concentration in effluents (Fig. 3). T-DIN removal rates in effluent of swine wastewater (initial T-DIN, 80 mg/L) by the four duckweeds were highest, followed by those of anaerobic digestion (initial T-DIN, 33.4 mg/L) and municipal wastewater (initial T-DIN, 8.1 mg/L).

The results suggest that all four duckweed species can remove nitrogen from effluent samples over a wide range of T-DIN concentrations (8.1–80 mg/L). Although all the duckweeds tested are promising agents for nitrogen removal from wastewater effluent, *S. polyrhiza* showed the strongest ability to remove nitrogen from effluents.

3.2. Duckweed growth and biomass characteristics

All four duckweeds grew well in the effluent samples. *S. polyrhiza* showed the highest growth rate among the four duckweeds (52.6–70.3 mg d.w./L/day), whereas *L. minor*, *L. gibba*, and *L. punctata* showed similar growth rates of 24.1–42.2, 28.6–46.1, and 28.6–48.6 mg d.w./L/day, respectively (Fig. 4). Growth rates of the four duckweed species were generally higher in effluents of swine wastewater and anaerobic digestion, which are rich in nitrogen, than in effluent of municipal wastewater. Growth rates of duckweeds in effluents were correlated with the initial nitrogen concentration in effluent (Fig. 3), and a higher nitrogen concentration in effluent seems to be favorable for duckweed growth (Mohedano et al., 2012; Soda et al., 2015). Our findings indicate that all the duckweeds tested are promising agents for biomass production from effluents. Among them, *S. polyrhiza* showed relatively higher biomass production.

Table 2 lists the characteristics of vegetative frond biomass of the four duckweeds grown in each effluent for 4 days. Duckweeds showed similar carbon and nitrogen contents (%) in their frond biomass, with ranges of 39.2–44.0% and 5.3–6.6%, respectively. The carbon and

Table 2
Characteristics of frond biomass of duckweed grown in the three effluent samples.

Effluent sample and duckweed species	Carbon content (%)	Nitrogen content (%)	Starch content (%)	Calorific value (MJ/kg)
Secondary effluent of municipal wastewater				
S. polyrhiza	40.3 ± 0.2	5.3 ± 0.2	9.5 ± 0.4	12.1[a]
L. minor	40.3 ± 0.2	5.8 ± 0.0	8.2 ± 0.1	12.5[a]
L. gibba	39.2 ± 0.0	5.5 ± 0.0	8.7 ± 0.1	11.8[a]
L. punctata	41.4 ± 0.1	5.6 ± 0.2	10.3 ± 0.1	12.9[a]
Secondary effluent of swine wastewater				
S. polyrhiza	40.5 ± 0.1	6.5 ± 0.0	9.2 ± 0.1	14.1[a]
L. minor	40.9 ± 0.3	6.4 ± 0.0	9.4 ± 0.4	14.0[a]
L. gibba	40.0[a]	6.2[a]	8.5[a]	11.2[a]
L. punctata	41.8[a]	6.6[a]	8.5[a]	12.8[a]
Effluent of anaerobic digestion				
S. polyrhiza	43.8 ± 0.2	6.4 ± 0.1	17.3 ± 0.2	15.4[a]
L. minor	43.5 ± 0.0	6.2 ± 0.1	11.7 ± 0.3	15.4[a]
L. gibba	43.0 ± 0.1	6.2 ± 0.1	12.9 ± 1.3	15.2[a]
L. punctata	44.0 ± 0.1	6.2 ± 0.2	14.7 ± 0.5	15.2[a]

Values are mean ± SD (n = 2).
[a] The values were assayed in a single test.

nitrogen contents were generally higher in duckweed biomass grown in effluents of swine or anaerobic digestion, with relatively higher nitrogen concentrations than in effluent of municipal wastewater. Starch contents of frond biomass ranged from 8.2% to 17.3% across the four species and three effluents. Starch content of duckweed grown in anaerobic digestion effluent and that of *S. polyrhiza* were relatively higher. Calorific values of frond biomass ranged from 11.2 to 15.4 MJ/kg and were similar in all four species. Calorific values of duckweed grown in anaerobic digestion effluent were higher than that grown in other effluents. Characteristics associated with biofuel productively, namely the carbon content, starch content, and calorific value, of frond biomass were relatively higher in duckweeds grown in anaerobic digestion effluent and were relatively higher in *S. polyrhiza* and *L. punctata* biomass.

3.3. Ethanol production from frond biomass

The ethanol production potential of duckweed biomass grown in three effluent samples was examined by SSF batch experiments (Fig. 5). Frond biomass of all duckweeds was effectively converted to ethanol, and the fermentation was nearly completed within 12 h. The ethanol production potential (ethanol yield per dry weight biomass) was similar across species and ranged from 0.165 to 0.191 g-ethanol/g-biomass (Table 3). Frond biomass of *S. polyrhiza* and *L. punctata* showed relatively higher ethanol production potentials compared to those of *L. minor* and *L. gibba*. In addition, the ethanol production potentials of duckweeds grown in effluent of anaerobic digestion were higher than those of the other effluents. These results likely reflect the higher carbon content, starch content, and calorific value of duckweeds grown in anaerobic digestion effluent.

To better evaluate the potential of duckweeds as feedstock for the production of ethanol, the results obtained in this study were compared with those of previous reports (Table 4). Ethanol production potentials of the four duckweed species grown in three effluents were comparable to those of waste biomass (sugarcane bagasse), catch crop (alfalfa fiber), aquatic plants (water hyacinth and water lettuce), and fronds of duckweeds (*L. minor* and *Wolffia globosa*) and were slightly lower than those of starch-rich turions of duckweed (*Wolffia arrhiza*) and microalgae (*Chlamydomonas reinhardtii* and *Chlorella vulgaris*). Therefore, duckweed frond biomass seems to be suitable as an ethanol feedstock.

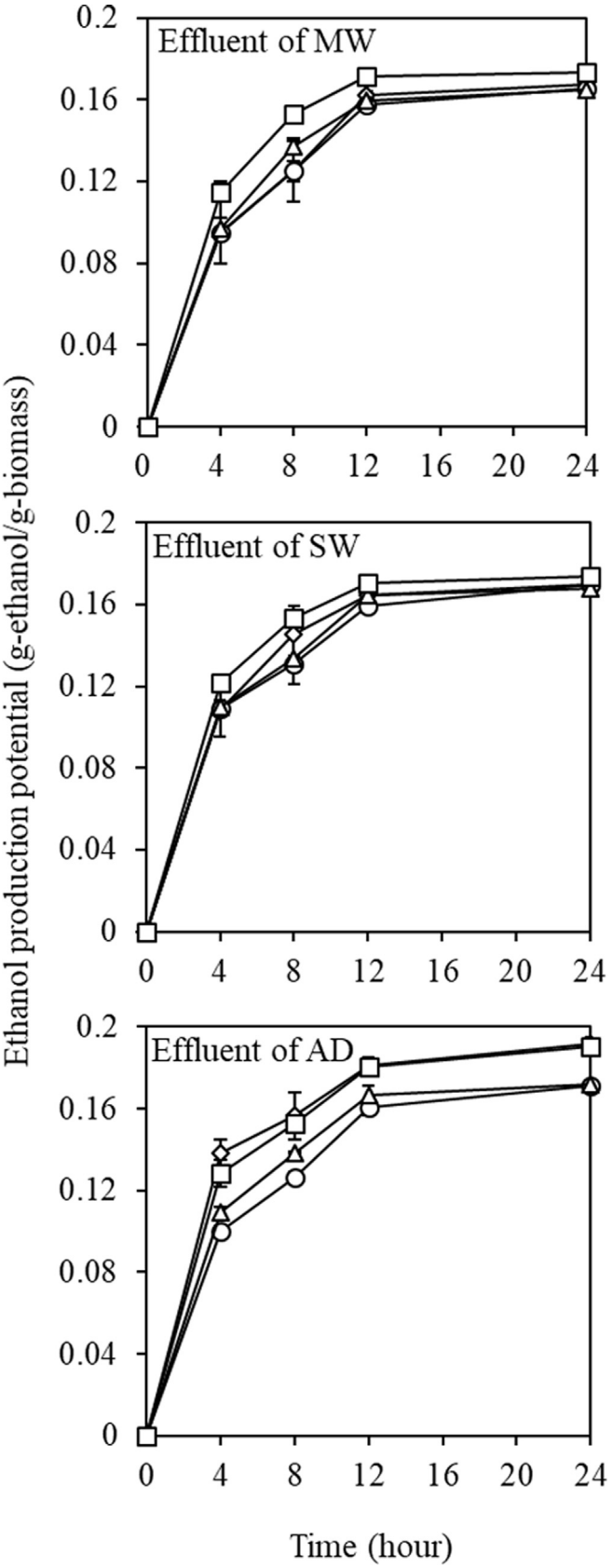

Fig. 5. Curves of ethanol production from frond biomass of four duckweed species grown in the three effluents (MW: municipal wastewater; SW: swine wastewater; AD, anaerobic digestion; Diamonds: *S. polyrhiza*; Circles: *L. minor*; Triangles: *L. gibba*; Squares: *L. punctata*). Values are mean ± SD (n = 2).

Table 3
Summary of ethanol production potential of four duckweeds grown in the three effluent samples.

Effluent sample and duckweed species	Ethanol production potential (g-ethanol/g-biomass)
Secondary effluent of municipal wastewater	
S. polyrhiza	0.168 ± 0.001
L. minor	0.166 ± 0.001
L. gibba	0.165 ± 0.003
L. punctata	0.174 ± 0.001
Secondary effluent of swine wastewater	
S. polyrhiza	0.170 ± 0.001
L. minor	0.170 ± 0.002
L. gibba	0.168 ± 0.001
L. punctata	0.174 ± 0.000
Effluent of anaerobic digestion	
S. polyrhiza	0.191 ± 0.002
L. minor	0.172 ± 0.001
L. gibba	0.172 ± 0.001
L. punctata	0.191 ± 0.003

Values are mean ± SD (n = 2).

3.4. Methane production from frond biomass

The methane production potential of duckweed biomass grown in the three effluents was examined. Methane production from frond biomass of all four duckweeds was effective and nearly completed within 3 weeks (Fig. 6). The final methane contents in biogas and the methane production potential (methane yield per unit organic matter of duckweed, NL CH_4/kg VS) of all duckweed species are summarized in Table 5. Frond biomass of *S. polyrhiza* and *L. punctata* showed higher methane production potentials compared to those of *L. minor* and *L. gibba*. Also, methane production potentials of duckweed grown in effluent of anaerobic digestion were higher than those of the other effluents. Again, the differences might be related to the higher carbon content, starch content, and calorific value of duckweed grown in anaerobic digestion effluent.

Methane production potentials of the four duckweeds grown in effluents were similar to or higher than those of main crops, catch crops, and perennial crops and were similar to those of microalgae (Table 6). Duckweed fronds have soft biomass with low lignin content, which should increase their methane production potential. Only one study has shown the methane production potential (340 NL CH_4/kg VS) of one duckweed species, *S. polyrhiza* (Cu et al., 2015). However, little information is yet available on the capacity of duckweeds on methane production. Influence of different duckweed species and different types of wastewater for duckweed culture have not studied. Our results clearly show that the methane productively of duckweeds differs depending on duckweed species and wastewater for duckweed culture. In this study, *S. polyrhiza* (413 NL CH_4/kg VS) and *L. punctata* (408 NL CH_4/kg VS) grown in anaerobic digestion effluent biomass showed the higher methane production potentials compared to the previous report (Cu et al., 2015). Our results reveal the new value of duckweed biomass as feedstock for methane production.

3.5. Comparison of nitrogen removal, biomass production, and suitability of frond biomass for biofuel production among duckweed species

Cultivation of the duckweed species *S. polyrhiza*, *L. minor*, *L. gibba*, and *L. punctata* in the municipal wastewater, swine wastewater, and anaerobic digestion effluents significantly removed T-DIN from the effluents and produced suitable biomass for ethanol and methane

Table 4
Comparison of the ethanol production potentials of various feedstocks.

Feedstock	Pretreatment	Fermentation mode	Fermentation strain	Ethanol production potential (g-ethanol/g-biomass)	Reference
Catch crop					
Alfalfa fiber	Liquid hot water pretreatment	SSF	*Saccharomyces cerevisiae*	0.18	Sreenath et al. (2001)
Waste biomass					
Sugarcane bagasse	Steam pretreatment	SHF	Recombinant *S. cerevisiae* TMB3001	0.18	Martín et al. (2002)
Aquatic plants					
Water hyacinth leaves	Alkaline/oxidative pretreatment	SSF	*S. cerevisiae* NBRC 2346	0.14	Mishima et al. (2008)
			Escherichia coli KO11	0.17	Mishima et al. (2008)
Water lettuce leaves	Alkaline/oxidative pretreatment	SSF	*S. cerevisiae* NBRC 2346	0.15	Mishima et al. (2008)
			E. coli KO11	0.16	Mishima et al. (2008)
Duckweeds					
L. minor	Alkaline and heating (100 °C for 10 min)	SHF	Yeast SPSC01 and *S. cerevisiae* ATCC 24859	0.086	Ge et al. (2012)
Wolffia globosa (vegetative fronds)	121 °C for 20 min	SSF	*S. cerevisiae*	0.17	Soda et al. (2015)
Wolffia arrhiza (starch-rich turions)	Alkaline/oxidative pretreatment	SSF	*S. cerevisiae* NBRC 2346	0.28	Takai et al. (2014)
Microalgae					
Chlamydomonas reinhardtii	Liquefaction by 0.005% α-amylase at 90 °C for 30 min	SHF	*S. cerevisiae*	0.235	Choi et al. (2010)
Chlorella vulgaris FSP-E	Hydrolysis by sulfuric acid	SHF	*Zymomonas mobilis*	0.233	Ho et al. (2013)
Duckweeds					
S. polyrhiza	121 °C for 20 min	SSF	*S. cerevisiae*	0.168–0.191	This study
L. minor	121 °C for 20 min	SSF	*S. cerevisiae*	0.166–0.172	This study
L. gibba	121 °C for 20 min	SSF	*S. cerevisiae*	0.165–0.172	This study
L. punctata	121 °C for 20 min	SSF	*S. cerevisiae*	0.174–0.191	This study

SSF: simultaneous saccharification and fermentation, SHF: separate hydrolysis and fermentation.

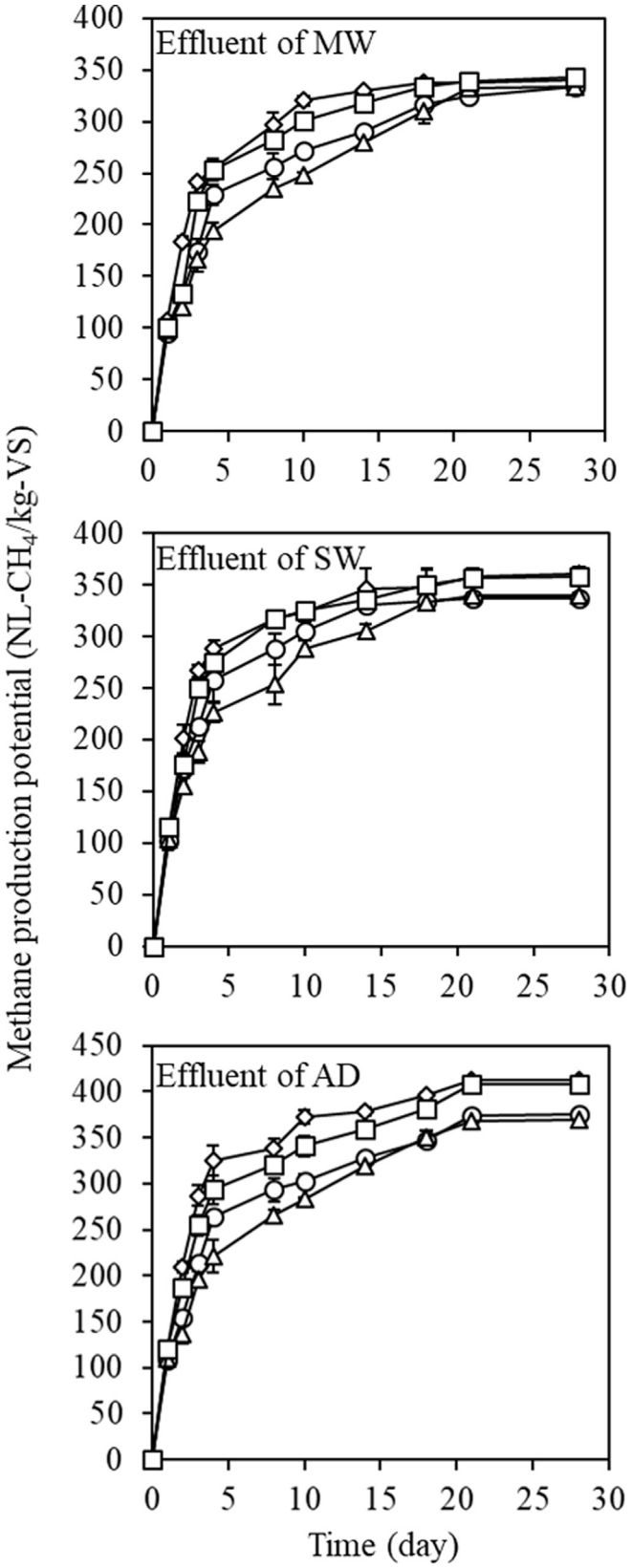

Fig. 6. Curves of methane production from frond biomass of four duckweed species grown in the three effluents (MW: municipal wastewater; SW: swine wastewater; AD, anaerobic digestion; Diamonds: *S. polyrhiza*; Circles: *L. minor*; Triangles: *L. gibba*; Squares: *L. punctata*). Values are mean ± SD (n = 2).

Table 5
Summary of methane production potentials of four duckweeds grown in the three effluent samples.

Effluent sample and duckweed species	CH_4 content in biogas (%)	Methane production potential (NL CH_4/kg VS)
Secondary effluent of municipal wastewater		
S. polyrhiza	63.8 ± 1.7	340 ± 4
L. minor	62.3 ± 1.9	334 ± 4
L. gibba	62.6 ± 1.4	334 ± 2
L. punctata	63.0 ± 1.9	343 ± 1
Secondary effluent of swine wastewater		
S. polyrhiza	64.9 ± 1.8	361 ± 7
L. minor	63.4 ± 1.3	337 ± 2
L. gibba	61.5 ± 0.6	340 ± 3
L. punctata	63.1 ± 0.9	358 ± 8
Effluent of anaerobic digestion		
S. polyrhiza	64.5 ± 1.3	413 ± 3
L. minor	63.7 ± 0.8	375 ± 3
L. gibba	64.0 ± 2.0	370 ± 2
L. punctata	64.5 ± 2.2	408 ± 3

Values are mean ± SD (n = 2).

production. Among the four duckweeds, *S. polyrhiza* showed a higher T-DIN removal rate and biomass production. Ethanol and methane production potentials of the effluent-grown duckweed frond biomass were higher than or similar to those of other plant or microalgal feedstocks. Among the four species, *S. polyrhiza* and *L. punctata* showed higher ethanol and methane production potentials compared to the other duckweeds.

Based on the experimental conditions in this study (28 ± 1 °C, photoperiod of 16 h light [80 μmol/m^2/s] and 8 h dark, 1 L effluent in a polypropylene container [160 mm × 125 mm × 80 mm, 0.02 m^2 surface area]), the dry biomass yield of *S. polyrhiza* grown in anaerobic digestion effluent was estimated as 3.5 g/m^2/day, 1.3 kg/m^2/year, and 13 t/ha/year. *Lemna japonica* 0223, *S. polyrhiza*, and *W. globosa* also have high biomass yields of 6.1 g/m^2/day (2.2 kg/m^2/year; Zhao et al., 2015), 12.4 g/m^2/day (4.5 kg/m^2/year; Xu et al., 2011), and 9.29 g/m^2/day (3.39 kg/m^2/year; Soda et al., 2015), respectively. Our results were comparable to these previous data. Based on ethanol (0.191 g-ethanol/g-biomass) and methane (413 NL CH_4/kg VS, 0.86 kg VS/kg-biomass) productivities, ethanol yield per *S. polyrhiza* culture area in anaerobic digestion effluent was estimated as 0.67 g/m^2/day, 0.25 kg/m^2/year, and 2.5 t/ha/year, and methane yield per *S. polyrhiza* culture area in anaerobic digestion effluent was estimated as 1.7 NL CH_4/m^2/day, 0.62 kNL CH_4/m^2/year, and 6.2 MNL CH_4/ha/year. This newly obtained data set from this study should encourage the development of duckweed-based biofuel production in wastewater treatment plants.

Methane production from duckweed biomass can be reasonably coupled with anaerobic digestion in the wastewater treatment process. Anaerobic digestion can remove organic matter from wastewater and wastewater sludge and produce methane gas, but it cannot remove nitrogen. After this process is completed, duckweed cultivation can remove nitrogen from the nitrogen-rich effluent and produce suitable biomass for additional methane production. The duckweed biomass can be added to the anaerobic digestion system to increase methane production. The idea of coupling anaerobic digestion with cultivation of duckweed, especially *S. polyrhiza*, is a promising strategy for sustainable wastewater treatment and biofuel production.

4. Conclusion

S. polyrhiza, *L. minor*, *L. gibba*, and *L. punctata* effectively removed nitrogen from effluents of municipal wastewater, swine wastewater, and anaerobic digestion. The biomass of effluent-grown duckweeds could be converted to ethanol by heat pretreatment and SSF using enzymes and dry yeast and to methane by anaerobic digestion. Ethanol

Table 6
Comparison of the methane production potentials of various feedstocks.

Feedstock	Temperature (°C)	Methane production potential (NL CH_4/kg VS or NL CH_4/kg organic dry matter)	References
Main crops			
Sugar beet	35	374.9	Herrmann et al. (2016)
Maize	35	328.2	Herrmann et al. (2016)
Potatoes	35	330.6	Herrmann et al. (2016)
Winter wheat	35	300.8	Herrmann et al. (2016)
Catch crops			
Forage sorghum	35	305.5	Herrmann et al. (2016)
Annual ryegrass	35	300.1	Herrmann et al. (2016)
Alfalfa clover grass mix	35	288.4	Herrmann et al. (2016)
Perennial crop			
Tall wheatgrass	35	257.9	Herrmann et al. (2016)
Duckweed			
S. polyrhiza	37	340	Cu et al. (2015)
Microalgae			
Euglena gracilis	39	380.6–503.2	Grimm et al. (2015)
Chlorella vulgaris	35	361	Frigon et al. (20133
Scenedesmus sp. AMDD Jul-201	35	410	Frigon et al. (2013)
Duckweeds			
S. polyrhiza	38	340–413	This study
L. minor	38	334–375	This study
L. gibba	38	334–370	This study
L. punctata	38	343–408	This study

and methane production potentials were higher than or similar to those of crops and microalgae. *S. polyrhiza* showed higher nitrogen removal and biomass production rates, and *S. polyrhiza* and *L. punctata* showed higher ethanol and methane production potentials. Duckweeds are promising agents for nitrogen removal and biofuel production in wastewater treatment plants.

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