Effect of treated sewage characteristics on duckweed biomass production and microbial communities

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ABSTRACT

Duckweed biomass production in a duckweed pond fed with three differently treated sewage (i.e. sewage treated by primary sedimentation (PS); conventional activated sludge process (CAS); and downflow hanging sponge process (DHS)) was evaluated in order to assess the effects of water quality on biomass yield. Higher and stable biomass production was observed when the duckweed pond was fed with PS or DHS-effluent than with CAS-effluent, evidently due to the difference in nutrient loads. Availability of nutrients, especially phosphorus, affected the biomass production rate: the higher the nutrient, the faster the production. Microbial community analysis revealed that the members of Rhizobiales were likely to contribute to stable and high biomass growth. From the results of the study, a sewage treatment system consisting of a primary sedimentation followed by a duckweed pond and a tertiary treatment unit can be proposed to maximize biomass production without compromising treatment objectives. Size and operational parameters of the duckweed pond should be determined primarily based on the nutrient availability in the influent water to maximize duckweed growth.

Key words | biomass production, duckweed, microbial community, phosphorus load, sewage

INTRODUCTION

In recent years, recovery and reuse of nutrients available in waste and wastewater have been gaining popularity. Nutrients such as nitrogen and phosphorus are scarce and can be sequestered in the form of bioresources rather than to remove them using energy intensive treatment systems. One such approach for the utilization of nutrients in wastewater is cultivation of aquatic plants such as duckweed in a pond system. Duckweed is generally known for fast growth and high biomass production capacity, second to algae (Xu et al. 2012). Duckweed is also easy to harvest, since it floats on water surface. Several studies have demonstrated that duckweed can efficiently utilize nutrients such as nitrogen and phosphorus from various wastewater sources including domestic sewage (Alaerts et al. 1996; Al-Nozaily et al. 2000), agricultural wastewater (Ge et al. 2012), swine wastewater (Mohedano et al. 2012), and effluents from anaerobic digesters treating sewage sludge (Toyama et al. 2018). Depending on species and cultivation conditions, duckweed is high in starch and protein contents, but low in lignin and cellulose (Ge et al. 2012). Productivity and nutritional values of duckweed can be controlled by adjusting parameters such as temperature (Lasfar et al. 2007), photoperiod (Lasfar et al. 2007; Yin et al. 2015), light intensity (Yin et al. 2015), coverage of duckweed in farm (Zhao et al. 2014a; Verma & Suthar 2015), concentration of nutrients (Caicedo et al. 2000), and selection of duckweed species (Zhao et al. 2014b). Due to these characteristics, duckweed has been an attractive source for biofuel (e.g. bioethanol (Ge et al. 2012), biomethane (Toyama et al. 2018), biohydrogen (Xu & Deshusses 2015)), livestock feed (Goopy & Murray 2003) and even human food (Appenroth et al. 2017).

Among different types of wastewater, domestic sewage has the highest potential as a feed for duckweed cultivation primarily because of its availability, abundance and presence of various essential nutrients. Several previous
studies have investigated the potential of using duckweed pond as sewage treatment system while recovering nutrients in the form of plant biomass. Alaerts et al. (1996) reported a lagoon covered with duckweed as an effective system for sewage treatment and cultivation of biomass. Likewise, Skillicom et al. (1995) demonstrated the performance of a combination of primary sedimentation and duckweed pond, which was comparable a wastewater treatment system with a tertiary treatment unit. In a typical duckweed pond, microorganisms are responsible for organic matter removal and duckweed is responsible for nutrients (nitrogen and phosphorus) removal. However, the overall process requires relatively long hydraulic retention time (HRT), usually more than 20 days. Long HRT is typically required for organics removal by microbes to satisfy effluent discharge standards. Uptake of nutrients by duckweed is much faster. Therefore, the process HRT becomes a bottleneck to maximize duckweed growth because of the limitations in nutrient supply. Available nutrients in the wastewater feed is consumed quickly by the plant, limiting its growth potential. A sewage pretreatment system for the removal of the majority of organics is usually desirable prior to a duckweed pond. El-Shafai et al. (2007) investigated an upflow anaerobic sludge blanket (UASB) as a pretreatment unit for a duckweed pond. However, there is a need of a study to evaluate the type of pretreatment that can maximize the duckweed biomass yield.

Another important aspect of creating and maintaining a healthy and high yield duckweed pond is microorganisms co-existing with the plant. It has been demonstrated that duckweed harbors diverse groups of microorganisms with unique capabilities to degrade contaminants (Appenroth et al. 2016). For example, Yamaga et al. (2010) reported presence and activity of microorganisms with capability to degrade recalcitrant contaminants such as phenol. In addition, there are microbes that promote or inhibit the growth of duckweeds (Yamaga et al. 2010; Tang et al. 2015; Ishizawa et al. 2017). Identification of microorganisms that promote duckweed growth is of particular interest. Further elucidation of microbial community residing under different duckweed growth conditions can improve our understanding of interactions between the duckweed and microorganisms and its impact on the growth of the biomass.

The major objectives of this study were to: (1) investigate the effects of treated sewage characteristics on duckweed biomass production and (2) elucidate the microbial community under different duckweed growth conditions. Two different sewage treatment processes, i.e. conventional activated sludge (CAS) and downflow hanging sponge (DHS), were selected to obtain three different qualities of treated sewage: primary-treated sewage (PS-effluent), CAS-treated sewage (CAS-effluent), and DHS-treated sewage (DHS-effluent). Conventional activated sludge is the most popular sewage treatment system in developed countries. In CAS, sewage is first treated in a primary sedimentation tank, where particulate organic matters (i.e. suspended solids) are removed. The primary sedimentation tank generates primary sludge and effluent sewage, which still contains significant amount of organic matters and nutrients. Next, the primary-treated sewage is sent to an aerobic bioreactor, where microorganisms degrade organic matter and use nutrients. Therefore, a CAS-effluent contains low organic matter and nutrients. Although the CAS is a well-established and reliable process, it is often not suitable for developing countries due to huge power requirement and high operational cost. For sewage treatment in developing countries, affordable alternative processes with low power consumption is more suitable. One such technology is DHS (Kubota et al. 2014; Okubo et al. 2016). DHS is an aerobic biological treatment process that uses polyurethane sponge as growth and retention matrix for active microbial biomass. Due to natural diffusion of oxygen into the wastewater, energy required for the operation of DHS is very low (Okubo et al. 2016). Additionally, excess sludge generated from the DHS process is significantly small making it a low maintenance process (Onodera et al. 2013). In a DHS process, primary-treated sewage is used as an influent. The DHS effluent contains low organic matter (comparable to that of the CAS). However, the DHS effluent usually contains nitrate as a result of nitrification in the reactor. The duckweed biomass yield in congruence with sewage treatment performance was evaluated using three differently treated sewage: PS-effluent, CAS-effluent, and DHS-effluent. Finally, a sewage treatment system capable of producing higher duckweed biomass and better treatment performance was proposed.

MATERIALS AND METHODS

Biomass production and sewage treatment using duckweed

Two species of duckweed, Lemna sp. and Spirodea sp., were used for the study. Lemna sp. and Spirodea sp. were collected at a stream in Okinawa and at a pond in Miyagi, Japan, respectively. Sewage treated by different processes (i.e. primary sedimentation, CAS and DHS) was fed to
duckweed ponds (DP), which has a plug-flow regime with an HRT of 1 day (DP, width: 32 cm, length: 46 cm, water depth: 5 cm, water volume: 7.4 L). The tests were conducted at 25 °C under light exposure (about 8,000 l×, 16 h/day of irradiation with light emitting diode). Test duration was two weeks for DP fed with PS-effluent and four weeks for DPs fed with CAS and DHS effluents. In all DPs, duckweed was grown using treated sewage for approximately one week before the start of the study. In the beginning, approximately 30 g (wet-weight) of duckweed was supplied to each DP to cover the DP water surface. Duckweed was harvested every week for biomass monitoring and other analyses. After harvesting, 30 g (wet-weight) of the harvested biomass was returned to the DP. However, in the CAS-effluent fed DP, 50 g of biomass was returned to the pond in the second week due to slower biomass growth. Likewise, in the DHS-effluent fed DP, in the third and fourth week of the study, 15 g of the biomass was returned to the pond in order to see the effect of initial duckweed biomass on growth and productivity (Figure 1).

Figure 1 | Monitoring results of the wet-weight change of duckweed and concentrations of nitrogen and phosphorus in influent and effluent of DP cultivated with *Lemna* sp. (a) and *Spirodela* sp. (b) PS, CAS and DHS-treated sewages were fed to the DP. The numbers with the arrows correspond to the sample names for microbial community analysis (shown in Table S1 in the supplementary file).
Dry weight of the duckweed was measured by drying the biomass at 105 °C. Biomass production rate and growth rate were calculated as described in the supplementary file. Protein and carbohydrate contents were analyzed using an elemental analyzer Flash2000 (Thermo Fisher Scientific, Waltham, MA, USA). Phosphorus content was quantified using ascorbic acid method (APHA 2012).

The sewage treatment performance of the DP was evaluated by analyzing influent and effluent concentrations of NH$_4$-N, NO$_2$-N, NO$_3$-N, PO$_4^{3-}$-P and coliforms. Ammonia (NH$_4$-N) was analyzed using Ion Chromatography DX-120 (Dionex, Sunnyvale, CA, USA). Nitrite (NO$_2$-N) and nitrate (NO$_3$-N) were analyzed using Agilent 7100 CE system (Agilent Technologies, Santa Clara, CA, USA). Coliforms were monitored using CompactDry EC (Nissui, Tokyo, Japan).

**Microbial community structure analysis**

Microbial community structure analysis of duckweed plant and DP water were performed. Duckweed samples were collected 7.5 cm away from the inlet after seven days of cultivation. Sampling schedule and sample names are summarized in Figure 1 and Table S1 in the supplementary file.

DNA extraction was conducted using ISOIL for Bacteria Beating Kit (NIPPON GENE, Tokyo, Japan). The V3-V4 regions of the 16S rRNA gene were amplified using forward primer 341F and mixed reverse primer 806R/806R-P (30:1) (Matsubayashi et al. 2017). The polymerase chain reaction (PCR) condition was as follows: 25 cycles of 94 °C for 5 s, 50 °C for 5 s, and 68 °C for 10 s, and a final extension at 68 °C for 7 min using TaKaRa Taq HS LowDNA (TaKaRa, Kusatsu, Japan). Amplified products were purified with Agencourt® AMPure® XP (Beckman Coulter, Brea, CA, USA) following the manufacturer’s instruction. Purified DNA was used as template for the second PCR with overhang adapter primers for MiSeq analysis. After purification, a third PCR was conducted with Nextera XT Index Kit v2 Set A. The PCR products were sequenced by Illumina MiSeq platform. Sequences were analyzed by using QIIME software (version 1.8.0). In brief, the quality of obtained sequences was checked by Trimomatic software. After chimera check with usearch61, operational taxonomic units (OTU) were generated with 97% as sequence identity threshold. For taxonomic assignment, Silva 128 database was used. Due to the overabundance of cyanobacterial sequences in the duckweed samples, microbial community analysis was conducted after eliminating the sequences belonging to Cyanobacteria by QIIME script ‘filter_taxa_from_ott_table.py’. The nucleotide sequence data are available in the DDBJ Sequence Read Archive (DRA) accession number DRA009068.

**RESULTS AND DISCUSSION**

**Duckweed biomass production**

Duckweed biomass production was evaluated using three different treated sewage as feed (i.e. PS, CAS and DHS-effluent). Characteristics of the treated sewage in terms of NH$_4$-N, NO$_2$-N, NO$_3$-N and PO$_4^{3-}$-P concentrations are presented in Table 1. Monitoring results of the wet-weight changes of duckweed in the DP during the study are presented in Figure 1. High and stable biomass production was observed when PS-effluent was used as DP feed. The biomass production rates were 19.7 ton-dry-weight/hectar (ha)/year for *Lemna* sp. and 14.6 ton-dry-weight/ha/year for *Spirodela* sp. Doubling time was 3.1 days and 4.0 days for *Lemna* sp. and *Spirodela* sp., respectively. These growth rates and doubling times fall within the normal range observed and reported in several studies (El-Shafai et al. 2007; Yu et al. 2014; Ziegler et al. 2014; Zhao et al. 2015). The results indicated that nutrients supplied by the PS-effluent was sufficient for duckweeds to maintain high and stable growth rate compared to those by CAS and DHS-effluent.

With CAS-effluent as feed, biomass production was lower. The biomass production rate of *Lemna* sp. decreased from 7.3 ton-dry-weight/ha/year in the first week to 0.2 ton-dry-weight/ha/year in the third week. In the fourth week, the biomass in the DP decreased after one-week of cultivation, indicating higher decay-rate than the growth-rate. In the case of *Spirodela* sp., biomass production rate decreased significantly from 12.0 ton-dry-weight/ha/year in the first week to 2.0 ton-dry-weight/ha/year in the third week. These reductions in growth rates are presumably due to insufficient phosphorus in the system. Compared to the PS-effluent, phosphate concentration in CAS-treated sewage was much lower (3.7 ± 0.1 mg PO$_4^{3-}$-P/L in PS-effluent vs 14.6 ± 5.2 mg PO$_4^{3-}$-P/L in CAS-effluent).

**Table 1 | Characteristics of the treated sewage used in this study**

|          | NH$_4$-N (mg/L) | NO$_2$-N (mg/L) | NO$_3$-N (mg/L) | TIN (mg/L) | PO$_4^{3-}$-P (mg/L) |
|----------|----------------|----------------|----------------|------------|---------------------|
| PS-Effluent | 34.9 ± 1.5 | 1.1 ± 0.6 | 0.0 ± 0.0 | 36.4 ± 1.2 | 3.7 ± 0.1 |
| CAS-Effluent | 18.6 ± 3.2 | 1.2 ± 1.1 | 0.4 ± 0.3 | 20.2 ± 3.8 | 0.2 ± 0.1 |
| DHS-Effluent | 5.5 ± 2.7 | 1.2 ± 0.7 | 12.6 ± 1.3 | 19.3 ± 3.5 | 1.6 ± 0.3 |
0.2 ± 0.1 mg PO$_4^{3-}$-P/L in CAS-effluent, Table 1). Supplementing CAS-effluent with phosphate (2 mg PO$_4^{3-}$-P/L) resulted in stable biomass production (data not shown), confirming that phosphorus was the growth limiting nutrient.

In the DP with DHS-effluent as feed, duckweed growth was stable and the biomass production rate was 11.7 ton-dry-weight/ha/year (doubling time: 3.8 days) for *Lemna* sp. and 10.6 ton-dry-weight/ha/year (doubling time: 4.6 days) for *Spirodela* sp. These values are lower than those obtained with the PS-effluent. The lower growth rate could be because of the presence of more nitrate than ammonia in the DHS-effluent due to nitrification in the DHS process and lower phosphate concentration than in the PS-effluent (3.7 ± 0.1 mg PO$_4^{3-}$-P/L in PS-effluent vs 1.6 ± 0.3 mg PO$_4^{3-}$-P/L in DHS-effluent, Table 1). The results of water quality analysis showed almost complete ammonia utilization and a little utilization of nitrate in the DS (Figure 1). Duckweed is known to preferentially use ammonium nitrogen over nitrate (Fang et al. 2007), suggesting that duckweed initially utilized ammonia and then started utilizing nitrate after ammonia was exhausted.

In the third and fourth week of the operation, duckweed biomass returned to the DP was reduced to approximately 15 g wet-weight, covering only approximately half of the water surface. Soon, the biomass production rates increased (12.9 ton-dry-weight/ha/year for *Lemna* sp. and 13.4 ton-dry-weight/ha/year for *Spirodela* sp.) and the doubling times shortened (2.8 days for *Lemna* sp. and 3.1 days for *Spirodela* sp.). However, a significant algae growth was also observed and the DP effluent color turned greenish. Eventually, duckweed completely covered the water surface. Presence of algae in duckweed pond interferes with wastewater treatment as well as duckweed biomass production. Szabó et al. (1998) observed reduced chlorophyll content in the duckweed due to the presence of algae. Therefore, it is recommended to keep the DP water surface completely covered with duckweed in order to maintain stable biomass production and treatment performance.

These results indicated that PS-effluent is the more favorable feed for higher biomass yield. The biomass production rates with PS-effluent were 1.5 times faster for *Lemna* sp. and 1.3 times faster for *Spirodela* sp. compared to those with DHS-effluent. The *Lemna* sp. showed higher biomass production rate than *Spirodela* sp.

**Nutrient and coliform removal**

Sewage treatment efficiency of the duckweed pond was evaluated in terms of nutrient (nitrogen and phosphorus) and coliform removals. Results of NH$_4^+$-N, NO$_2^-$-N, NO$_3^-$-N and PO$_4^{3-}$-P concentrations in DP effluents are shown in Figure 1. Sufficient removal of total ionic nitrogen (TIN: the sum of NH$_4^+$-N, NO$_2^-$-N and NO$_3^-$-N) and phosphorus were observed throughout the DP operation. Nutrient removal was commensurate with biomass production (Figure 2). In the PS-effluent fed DP with *Lemna* sp., mean removal of TIN and PO$_4^{3-}$-P was 25% and 54%, respectively. These equaled to 0.50 g-N/m$^2$/day and 0.11 g-P/m$^2$/day of nitrogen and phosphorus removals, respectively. Likewise, with *Spirodela* sp., mean removal of TIN and PO$_4^{3-}$-P was 39% and 57%, respectively. These equaled to 0.79 g-N/m$^2$/day and 0.12 g-P/m$^2$/day of nitrogen and phosphorus removals, respectively.

In DHS-effluent fed DP with *Lemna* sp., mean removal of TIN and PO$_4^{3-}$-P was 26% and 58%, respectively. These equaled to 0.24 g-N/m$^2$/day and 0.04 g-P/m$^2$/day of nitrogen and phosphorus uptake, respectively. Likewise, with *Spirodela* sp., mean removal of TIN and PO$_4^{3-}$-P was 35% and 59%, respectively. These equaled to 0.33 g-N/m$^2$/day and 0.04 g-P/m$^2$/day of nitrogen and phosphorus uptake, respectively. When the biomass growth rates were higher (3rd and 4th week), TIN and PO$_4^{3-}$-P removal rates
increased as well. For *Lemma* sp., TIN and PO$_4^3-$P removal rates increased by approximately 1.6 times (0.37 g-N/m$^2$/day) and 1.3 times (0.05 g-P/m$^2$/day), respectively. For *Spirodela* sp., TIN and PO$_4^3-$P removal rates increased by approximately 1.4 times (0.46 g-N/m$^2$/day) and 1.2 times (0.05 g-P/m$^2$/day), respectively.

It was observed that with the decrease in biomass production, the TIN removal efficiency of CAS-effluent fed DP also decreased. The nitrogen removal rate of *Lemma* sp. decreased from 0.54 g-N/m$^2$/day (1st week, 45% removal) to 0.24 g-N/m$^2$/day (4th week, 30% removal). Similarly, nitrogen removal rate of *Spirodela* sp. decreased from 0.48 g-N/m$^2$/day (1st week, 40% removal) to 0.16 g-N/m$^2$/day (4th week, 19% removal). In the CAS-effluent fed DP, the PO$_4^3-$P concentration in the CAS-effluent was very low (average 0.2 mg/L) and phosphate in the treated water was below the detection limit.

Pathogen removal efficiency of a sewage treatment system is an important objective. Removal of coliforms by duckweed has been reported in previous studies (Papadopoulos et al. 2011; Tavares et al. 2010). The coliform reduction was from $7.7 \times 10^5$ to $5.1 \times 10^4$ cfu/100 mL in the PS-effluent fed DP and from $2.5 \times 10^6$ to $1.6 \times 10^5$ cfu/100 mL in the DHS-effluent fed DP. It is important to note that the DHS effluent already has a lower count of coliforms, because of the removal by the DHS process. In both cases, the coliform removal was approximately one order of magnitude, which is an important contribution by the DP. To confirm the effect of duckweed on coliform removal, a batch test was conducted using a freshly sampled PS-effluent (coliform count: $1.4 \times 10^7$ cfu/mL). A fresh PS-effluent was incubated with and without duckweed at 25°C under the same light exposure used in the DP test setups. In the presence of duckweed, the coliforms in the PS-effluent were reduced by 81% after one-day cultivation, as compared to 22% reduction in the test batch without duckweed. The result from the batch test demonstrated that the presence of duckweed accelerates the removal of coliforms.

### Nutritional value of duckweed

Starch and protein are the two major nutritional components of interest in duckweed. The nutritional contents in the duckweed depend on the operational conditions of the pond (Xu et al. 2011; Cui & Cheng 2015). In this study, protein, carbohydrate, C, N and P contents of the duckweed were analyzed (Table 2). Duckweed grown with PS and DHS-effluent showed similar composition. Protein and carbohydrate contents were approximately 29% and 22%, respectively. On the other hand, duckweed grown with CAS-effluent showed comparatively low protein content but high carbohydrate content. The results indicated that the duckweed cultivated with nutrient-rich feed contains relatively more protein and less carbohydrate. Nutrient scarcity produces duckweed with relatively higher carbohydrate content. These observations are consistent with those of the previous studies, which showed decrease in protein content due to low nutrient availability (Xu et al. 2011; Tao et al. 2013).

There was no significant difference in the elemental composition of carbon (C) in the duckweed. However, nitrogen (N) content varied for different species although the cultivation conditions were similar. *Spirodela* sp. showed greater nitrogen content than *Lemma* sp., which is consistent with the corresponding protein contents. This observation was also consistent with nitrogen removal and uptake rates by each species. A significant difference was observed in phosphorus (P) content. The P content in the duckweed cultivated using CAS-effluent was much lower than that cultivated using PS or DHS-effluent. This observation is also consistent with the low phosphorus content in the CAS-effluent and low phosphorus uptake.

### Nitrogen and phosphorus loads for biomass production

Nitrogen and phosphorus are the key growth elements for duckweed. Variation in organic matter concentration did
not seem to affect duckweed biomass growth and growth rates. Theoretical amounts of nitrogen and phosphorus required for the highest biomass growth rate (i.e. with PS-effluent) and those supplied by PS, DHS and CAS-effluent in this study are shown in Table 3. The amount of phosphorus supplied by DHS or CAS-effluent was lower than the amount required to achieve a similar biomass production as that by the PS-effluent. Obviously, DHS and CAS effluents are secondary treated wastewater.

The minimum amount of nutrient required to maintain duckweed growth was calculated. Phosphorus load was calculated using the values of dry duckweed biomass weight, phosphate concentration, and daily hydraulic loading (see supplementary file for calculation). Average phosphorus load on the first day of the week for CAS-effluent fed DP was 0.7 mg-P/g-dry-duckweed/day. Notably, biomass growth was low in the first week (see Figure 1). The minimum phosphorus loading required for stable biomass growth was 2.2 mg-P/g-dry-duckweed/day (with DHS-effluent fed DP after biomass growth). Hence, the minimum phosphorus load for stable biomass production is estimated to be between 0.7 and 2.2 mg-P/g-dry-duckweed/day.

Based on the outcomes of the study, a sewage treatment system targeting higher duckweed biomass yield and high organics and nutrient removal efficiency can be proposed. The proposed system consists of a primary sedimentation unit followed by a duckweed pond, and then a tertiary treatment unit (e.g. DHS process) (Figure 3). The system can be designed to provide an optimum nutrient loading, especially phosphorus, for high biomass yield. A tertiary biological polishing unit after duckweed pond is essential to remove remaining organics and leftover nutrients.

**Microbial community structure**

Duckweed can be anatomically divided into two parts, leaves and roots. In this study, the whole plant (both leaves and roots) was subjected to DNA extraction and prokaryotic community analysis. Furthermore, microbial community of the pond water was also analyzed to see an overall microbiome of the pond. Prokaryotic community structures after analyzing the V3-V4 region of 16S rRNA gene are shown in Figure S1 in the supplementary file. Most of the sequences obtained from the duckweed samples were chloroplast-derived. The ratio of sequences belonging to chloroplast declined when the duckweed growth was unfavorable (e.g. CAS3LR and CAS4LR in Figure S1). To eliminate the effects of these sequences, analysis was carried out after removing sequences belonging to *Cyanobacteria* using QIIME software. In addition, 1,000 sequences were randomly selected and used for further analysis to prevent the bias caused by the difference of sequence depth among the samples. The alpha diversity indices were similar in all samples: average OTU number was 190 (152–235) and Shannon index was 6.0 (5.4–6.5) (Table S2). Since only 1,000 sequences were picked, the coverage was not as high as generally reported, but was still 0.9 on average (0.87–0.93). The microbial community structure at order level after removing *Cyanobacteria* is shown in Figure 4. Those with an average relative abundance less than 1% were classified as ‘Others’. Comparative sequence analysis showed that class *Alphaproteobacteria* (*Rhizobiales*, *Rhodospirillales*, *Sphingomonadales*), *Betaproteobacteria* (*Burkholderiales*, *Methylophilales*) and *Gammaproteobacteria* (*Pseudomonadales*) were dominant in the samples of

| Nitrogen and phosphorus load for each effluent | (g/m²/day) | (g/m³/day) | PO₄³⁻·P |
|-----------------------------------------------|-----------|------------|---------|
| Supplied                                      |           |            |         |
| PS-Effluent                                   | 1.82 ± 0.06 | 0.19 ± 0.00 |
| DHS-Effluent                                   | 0.96 ± 0.17 | 0.08 ± 0.02 |
| CAS-Effluent                                   | 1.01 ± 0.19 | 0.01 ± 0.00 |
| Removed                                       |           |            |         |
| *Lemna* sp.                                   | 0.50 ± 0.01 | 0.11 ± 0.00 |
| *Spirodela* sp.                               | 0.79 ± 0.02 | 0.12 ± 0.00 |

Nitrogen and phosphorus removal rates under the highest duckweed growth rate (i.e., with PS-effluent).
duckweed. Principal coordinate analysis (PCoA) (weighted unifrac) indicated that the microbial communities of duckweed plant were not strongly related to the ones in the DP water (Figure 5). This indicated that a specific group of microbial community grew on the duckweed plant. The PCoA with weighted unifrac did not show any significant difference between the microbial community structures on duckweed cultivated with different feeds (weighted unifrac significance test: \( p > 0.05 \)).

In order to evaluate the effects of microbial community on duckweed growth, duckweed samples were divided into four groups: CAS-good; good growth with CAS-effluent (i.e. CAS1LR and CAS1SR), CAS-bad; bad growth with CAS-effluent (i.e. CAS3LR, CAS4LR and CAS3SR), PS-effluent, and DHS-effluent. The OTUs with more than 1.5% average abundance in one of the groups are listed in Table 4. Most of the OTUs were common in all four groups. Some OTUs were found in high abundances in all groups (e.g. OTUs 4259, 3659, 5779, 5619, 3265 and 6306). On the other hand, there were OTUs that showed high relative abundances in certain groups. We found that the presence of OTUs belonging to *Rhizobiales* were more prominent in duckweed cultivated in PS or DHS effluents compared to the duckweed cultivated in CAS-effluent. The members of *Rhizobiales* are reported as endophytes producing indole related compounds (Gilbert et al. 2018). Therefore, it is presumed that this group was contributing to stable and high duckweed biomass production. The OTUs 3265 and 6306 were close to *Rhizobium*, known to promote plant growth through nitrogen fixation (Kittiwongwattana & Thawai 2013). These members could be responsible for providing ammonia to duckweed. These OTUs were more abundant in DHS-effluent fed duckweed (OTU 3265: 6.0% and OTU 6306: 7.3%). This suggested that the duckweeds received ammonia supplement through nitrogen fixation especially after consuming available ammonia in the feed water.

Presence of an interesting OTU (OUT1471) was observed in the samples. This OTU had higher presence in the CAS-bad group compared to the other groups (7.5% vs 0.5–1.5%). The
Table 4 | List of OTUs having greater than 1.5% abundance in one of the groups.

| OTU     | PS-effluent | CAS-good | CAS-bad | DHS-effluent | taxonomy                              |
|---------|-------------|-----------|----------|--------------|---------------------------------------|
| OTU4259 | 2.9 ± 3.6a  | 6.8 ± 4.7 | 6.5 ± 9.7| 4.5 ± 4.7    | Betaproteobacteria; Methylophilus      |
| OTU3659 | 5.9 ± 1.5   | 4.5 ± 0.6 | 4.7 ± 4.2| 2.8 ± 2.1    | Alphaproteobacteria; Rhizobaceaeb      |
| OTU5779 | 4.3 ± 4.8   | 5.1 ± 3.5 | 3.1 ± 5.4| 2.2 ± 3.0    | Gammaproteobacteria; Moraxellaceae     |
| OTU5619 | 3.4 ± 0.7   | 1.9 ± 0.7 | 3.4 ± 2.1| 1.9 ± 1.7    | Betaproteobacteria; Comamonadaceae     |
| OTU3265 | 3.1 ± 1.0   | 0.6 ± 0.1 | 1.8 ± 1.8| 6.0 ± 7.8    | Alphaproteobacteria; Rhizobium         |
| OTU6306 | 2.4 ± 2.3   | 1.6 ± 1.4 | 1.5 ± 2.3| 7.3 ± 9.8    | Alphaproteobacteria; Rhizobium         |
| OTU1724 | 5.6 ± 2.5   | 0.0 ± 0.0 | 0.1 ± 0.1| 0.0 ± 0.1    | Alphaproteobacteria; Rhodobacteraceae  |
| OTU4486 | 3.9 ± 1.6   | 0.4 ± 0.1 | 0.3 ± 0.3| 1.1 ± 0.6    | Alphaproteobacteria; Rhizobiales       |
| OTU4197 | 3.8 ± 0.5   | 0.4 ± 0.1 | 2.5 ± 2.6| 0.3 ± 0.2    | Alphaproteobacteria; Bosea             |
| OTU3823 | 3.0 ± 3.5   | 0.0 ± 0.0 | 0.0 ± 0.1| 0.2 ± 0.3    | Firmicutes; Clostridia; Peptostreptococcaceae |
| OTU2078 | 2.4 ± 3.0   | 0.0 ± 0.0 | 0.1 ± 0.2| 2.0 ± 2.4    | Alphaproteobacteria; Rhizobiales       |
| OTU1977 | 2.3 ± 2.1   | 0.0 ± 0.0 | 0.2 ± 0.2| 0.7 ± 0.5    | Alphaproteobacteria; Bradyrhizobiaceae  |
| OTU3951 | 2.2 ± 3.3   | 0.5 ± 0.6 | 0.1 ± 0.2| 0.9 ± 1.1    | Alphaproteobacteria; Kaistia           |
| OTU4222 | 1.7 ± 1.1   | 0.3 ± 0.4 | 0.2 ± 0.1| 0.5 ± 0.5    | Alphaproteobacteria; Rhodobacteraceae  |
| OTU2273 | 1.6 ± 0.7   | 0.1 ± 0.1 | 0.3 ± 0.2| 0.1 ± 0.1    | Alphaproteobacteria; Rhizobiales       |
| OTU3579 | 0.6 ± 1.2   | 4.2 ± 5.1 | 1.0 ± 0.7| 0.1 ± 0.2    | Alphaproteobacteria; Niveispirillum    |
| OTU7040 | 0.2 ± 0.1   | 4.1 ± 2.3 | 1.9 ± 1.6| 0.9 ± 0.6    | Betaproteobacteria; Comamonadaceae     |
| OTU2067 | 0.0 ± 0.0   | 3.6 ± 4.7 | 4.7 ± 5.5| 0.1 ± 0.1    | Betaproteobacteria; Comamonadaceae     |
| OTU2362 | 0.3 ± 0.3   | 2.9 ± 2.3 | 2.2 ± 1.5| 1.5 ± 2.5    | Gammaproteobacteria; Pseudomonas       |
| OTU5783 | 1.4 ± 0.5   | 2.1 ± 0.3 | 2.0 ± 1.5| 1.6 ± 1.9    | Alphaproteobacteria; Caulobacter       |
| OTU2652 | 0.1 ± 0.1   | 2.1 ± 1.0 | 1.5 ± 1.6| 0.2 ± 0.2    | Alphaproteobacteria; Azospirillum      |
| OTU560 | 0.5 ± 0.5   | 1.9 ± 1.6 | 0.9 ± 0.6| 1.0 ± 0.8    | Betaproteobacteria; Comamonadaceae     |
| OTU7112 | 0.1 ± 0.1   | 1.9 ± 0.8 | 0.5 ± 0.2| 0.6 ± 0.4    | Alphaproteobacteria; Asticcaaulis      |
| OTU4082 | 1.3 ± 1.8   | 1.9 ± 2.1 | 1.5 ± 2.5| 1.1 ± 1.7    | Gammaproteobacteria; Acinetobacter     |
| OTU6338 | 0.0 ± 0.0   | 1.8 ± 2.3 | 2.3 ± 2.4| 1.7 ± 2.7    | Alphaproteobacteria; Azospirillum      |
| OTU1471 | 0.5 ± 0.5   | 0.8 ± 0.5 | 7.5 ± 3.6| 1.5 ± 1.3    | Alphaproteobacteria; Sphingomonadaceae |
| OTU6841 | 0.1 ± 0.1   | 0.0 ± 0.0 | 1.9 ± 1.6| 0.0 ± 0.0    | Bacteroidetes; Sphingobacteria; Sediminibacterium |
| OTU1737 | 0.0 ± 0.0   | 0.0 ± 0.0 | 1.6 ± 1.4| 0.0 ± 0.0    | Gammaproteobacteria; Lysobacter        |
| OTU344  | 0.4 ± 0.5   | 0.7 ± 0.2 | 1.3 ± 0.3| 5.4 ± 6.7    | Betaproteobacteria; Comamonadaceae     |
| OTU6824 | 0.0 ± 0.0   | 0.2 ± 0.3 | 0.0 ± 0.1| 2.1 ± 4.7    | Betaproteobacteria; Comamonadaceae     |
| OTU6210 | 0.7 ± 0.9   | 1.0 ± 0.6 | 0.5 ± 0.4| 1.5 ± 2.2    | Betaproteobacteria; Comamonadaceae     |

aFrequencies ≥1.5% are in bold font.

bUnderlined taxonomies belong to Rhizobiales.

OTU1471 is close to the members of the genus Sphingobaldus, family Sphingomonadaceae. It is difficult to conclude whether the presence of this OTU affected the growth of duckweed or the poor growth of the duckweed promoted the propagation of this OTU. There are some studies investigating bacteria which promote or inhibit the growth of duckweeds (Yamaga et al. 2010; Ishizawa et al. 2017). In this study, we presumed that the members of Rhizobiales could be the ones maintaining the healthy growth of duckweed. It is possible that PS or DHS-effluent contained some specific substrates promoting the growth of Rhizobiales. Nevertheless, clarifying the relationship between duckweed growth and microorganisms is deceptively difficult.

Microbial community structures suggested competition between duckweed and nitrifiers for nutrient use. Nitrosomonas, known as ammonia oxidizing bacteria (AOB) and Nitrospira and Nitrospira, known as nitrite oxidizing bacteria (NOB) were found in DPs with good growth conditions of...
the duckweed. The average abundance of AOB and NOB are shown in Table S3. Higher abundance of AOB was observed in CAS-good and PS-effluent duckweed microbial community in comparison to the DHS-effluent duckweed microbial community. This is because the CAS and PS effluents contained high NH$_4^-$-N than in the DHS-effluent. In addition, more AOB and NOB were found associated with Spirodela sp.

CONCLUSION

Duckweed biomass production largely depends on the availability of nutrients, especially phosphorus. Presence of organics had a negligible effect on the growth and growth rates of the plant. In this study, duckweed fed with PS or DHS effluent had higher and stable growth, whereas duckweed growth was unstable in DP fed with CAS-effluent, especially due to phosphorus deficiency. Use of secondary treated sewage, such as CAS-effluent, as duckweed pond feed, is not beneficial in terms of higher biomass production. Microbial community analysis showed that the presence of the members of Rhizobiales contributed to stable and high duckweed biomass production. For better biomass yield and additional treatment of wastewater, duckweed pond as a secondary treatment unit is recommended. A system consisting of a primary sedimentation unit followed by a duckweed pond and a biological process is recommended for a complete sewage treatment and nutrient recovery in the form of plant biomass.

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SUPPLEMENTARY MATERIAL

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