



# Characterization of sludge reduction and bacterial community dynamics in a pilot-scale multi-stage digester system with prolonged sludge retention time

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## Abstract

Sludge reduction performance and bacterial community dynamics in a pilot-scale multi-stage digester system with prolonged sludge retention time were characterized. Throughout the operation period of 281 days, the total loading sludge and the total digested sludge were 4700 and 3300 kg-MLSS. After 114 days of operation, the residual MLSS (RMLSS) in the reactors for sludge treatment was maintained at 18–25 kg-RMLSS m<sup>-3</sup>, and the sludge reduction efficiency achieved 95% under the *F/M* ratio (kg-loading MLSS kg-RMLSS<sup>-1</sup>) of less than 0.018. Also, among the sludge components, both fixed suspended solids and volatile suspended solids were reduced. Based on the sludge reduction performance and the RNA-based bacterial community characteristics, the combined action of the maintenance metabolism, lysis–cryptic growth, and particulate inorganic matter is proposed as the sludge reduction mechanism in the multi-stage sludge treatment process.

**Keywords** Sludge reduction · Multi-stage digesters · Prolonged retention time · Maintenance metabolism · Bacterial community dynamics

## Introduction

Conventional wastewater treatment systems generate a large amount of sludge production, and various techniques to reduce excess sludge have been investigated [1–3]. Some of these technologies—including physical, chemical, and thermal methods such as ultrasound, ozone, and heat—can (a) increase energy consumption and associated operating costs

and (b) impact the environment due to the resulting chemical reagents [1, 2]. Compared to the physical, chemical, and thermal methods, biological methods for sludge reduction are desirable as cost-saving and environmentally-friendly approaches [4, 5].

The performance of full-scale sludge reduction using biological methods in wastewater treatment plants has been investigated over the past several decades. Banaei et al. observed the process stability of a full-scale industrial wastewater treatment system including two types of biological treatment units: trickling filter and activated sludge reactor [6]. Seo et al. reported a 90% stable sludge reduction in a pilot-scale sewage sludge treatment using an aerobic digestion system with endospore-forming bacteria [5]. Jiang et al. achieved effective sludge reduction and pollutant removal in a full-scale activated sludge system with a modified sequencing bioreactor [4]. In situ sludge reduction by biological processes can occur through cell lysis–cryptic growth, uncoupling metabolism, maintenance metabolism, and worm’s predation [7]. Although some biological sludge reduction processes have been published, further studies are needed to improve biological sludge reduction efficiency and to develop new processes.

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In biological sludge treatment systems, microorganisms play a key role in sludge reduction [5]. Therefore, analysis and interpretation of microbial communities are required to understand sludge treatment mechanisms. Next-generation sequencing techniques with DNA-based and RNA-based pools have been used recently to assess microbial communities and interpret key taxa contributing to the biological systems [8]. Most of the bacterial community analyses with molecular techniques have been conducted using DNA-based analysis [4, 8]. With DNA-based analysis, it is difficult to distinguish between active and dormant cells in the microbial community; furthermore, this process can only identify whether cells exist, and whether they are active. Therefore, recent studies have focused on RNA-based community analysis, which is more likely to reflect active cells that contribute to system performance [9, 10].

A multi-stage digester system using longer sludge retention time (SRT) and low food to microorganism ( $F/M$ ) ratio for sludge treatment was proposed (Global Eco Technology Co. LTD, Incheon, Republic of Korea, Fig. 1). To better understand sludge treatment mechanisms in the pilot plant using this process, it is necessary to interpret changes in sludge characteristics and active microbial cells. In this study, the performance of the pilot-scale sludge treatment process and the contributors to sludge reduction were evaluated by calculating the mass balance. RNA-based bacterial community analysis and a literature review for the functional properties of key bacteria were conducted to discuss their functional roles in contributing to sludge reduction.

## Materials and methods

### Feeding sludge source and inoculum cultivation

Excess sludge from a Hajapo wastewater treatment plant (located in Yangpyeong-gun, Gyeonggi-do, Republic of Korea) was used as influent (feeding sludge) for the pilot-scale sludge treatment system. The treatment capacity of the wastewater treatment plant is  $900 \text{ m}^3 \text{ day}^{-1}$ . The multi-stage sludge digester system in this study was inoculated with microbial agents (Global Eco Technology, Incheon, Republic of Korea) incubated in a seed bioreactor ( $1.06 \text{ m } \Phi \times 1.36 \text{ H}$ , polyethylene, cylinder type) under the following conditions: (a) the excess sludge emitted from the Hajapo wastewater treatment plant was used as a substrate for microbial incubation, (b) the excess sludge was supplied with aerobic and anoxic conditions ( $0.8\text{--}1.4 \text{ m}^3 \text{ day}^{-1}$ ) and incubated at an aeration of  $20 \text{ h day}^{-1}$  with hydraulic retention times (HRT) of 41–93 days, and (c) the seed bioreactor used a working volume of  $1 \text{ m}^3$ .

### Pilot-scale sludge treatment process description

The pilot-scale sludge treatment process consisted of multi-stage biodigesters for solid sludge treatment and a submerged fixed-film bioreactor for wastewater treatment (Fig. 1). The multi-stage biodigesters consisted of a pre-biodigester, three biodigesters, and a submerged membrane bioreactor. The

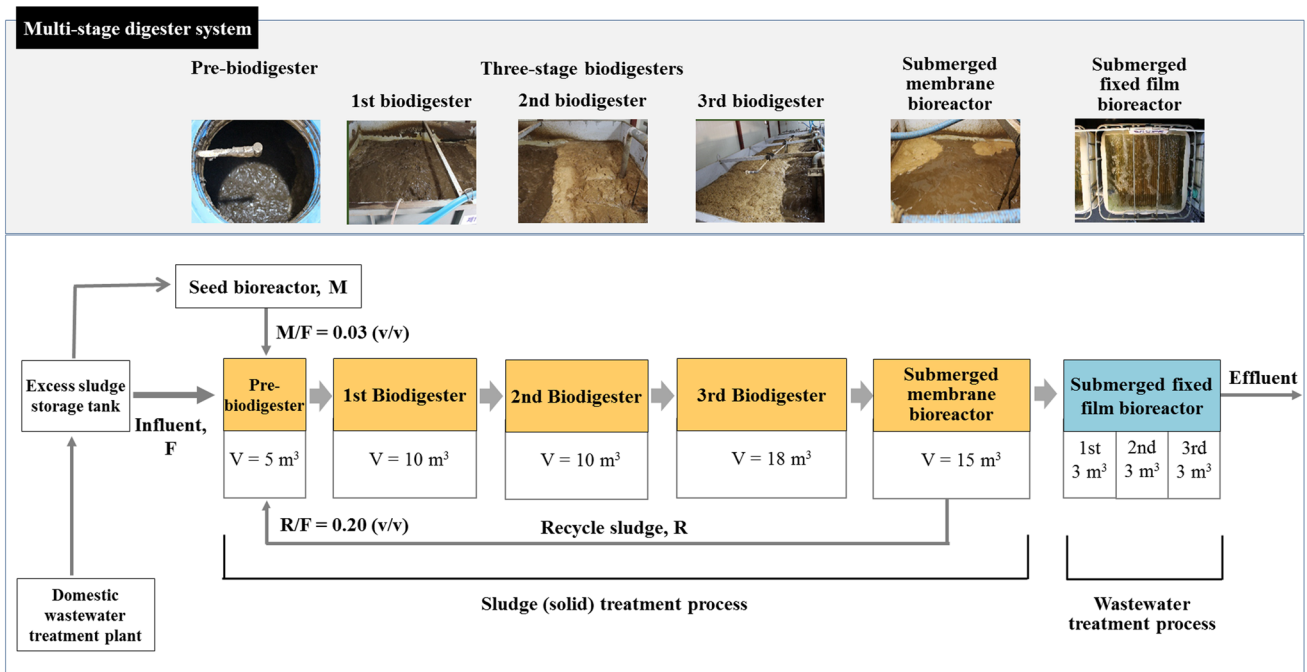


Fig. 1 Schematic diagrams of a pilot-scale multi-stage sludge treatment process

pre-biodigester was a polyethylene cylindrical tank 1.85 m in diameter ( $\Phi$ ) and 2.28 m high ( $H$ ). The three-stage biodigesters were polyethylene squire reactors: the first two were 3 m long ( $L$ )  $\times$  2 m wide ( $W$ )  $\times$  2 m high ( $H$ ), and the third was 3.6 m  $\times$  2 m  $\times$  2 m. The submerged membrane bioreactor was a polyethylene squire reactor (4.5 m  $\times$  2 m  $\times$  2 m) with a hollow fiber membrane (0.5 m  $\times$  0.5 m  $\times$  0.5 m) submerged in the bioreactor. The membrane in the membrane bioreactor was hollow fiber (Mitsubishi Rayon Co., Japan) with 0.1  $\mu$ m of effective pore size; it was used to separate sludge from the filter-treated water. The working volume of the bioreactors was 5 m<sup>3</sup> for the pre-digester, 10 m<sup>3</sup> for biodigester 1, 10 m<sup>3</sup> for biodigester 2, 18 m<sup>3</sup> for the biodigester 3, and 15 m<sup>3</sup> for the membrane bioreactor. Porous pipe (diameter 3 mm, polyethylene) was installed at the bottom of each bioreactor to supply air. The submersible pump (OP 310, Hanil pump Co., Korea) was used to transfer sludge solution from the pre-bioreactor to the post-bioreactor and recirculate sludge from the submerged membrane bioreactor to the pre-biodigester.

For the treatment of rejected wastewater from the submerged membrane bioreactor, a series of three submerged fixed-film bioreactors was installed. Each bioreactor was a polyethylene squire tank (1.6 m  $\times$  1.6 m  $\times$  1.4 m) with 25 bundles of fiber membranes installed. A porous pipe was installed at the bottom of each bioreactor, and the working volume of each bioreactor was 3 m<sup>3</sup>.

### Operating conditions of the sludge treatment process

The pilot-scale sludge treatment process was constructed in Yangpyeong-gun, Gyeonggi-do, Republic of Korea (latitude: 34.435880° N, longitude: 127.530937° E). The sludge treatment capacity of this pilot-scale plant was 2–3 m<sup>3</sup> day<sup>-1</sup>. Domestic sludge was treated in the Hajapo wastewater treatment plant, which has a treatment capacity of 900 m<sup>3</sup> day<sup>-1</sup> and produces excess sludge of 10 m<sup>3</sup> day<sup>-1</sup>. Effluent containing ejected sludge from the wastewater treatment plant was used as influent in the pilot-scale sludge treatment process. The pilot-scale sludge treatment process was operated for 281 days, from January to October 2017. The influent of 0.8–1.4 m<sup>3</sup> day<sup>-1</sup> with different MLSS was supplied into the process: 1.4 m<sup>3</sup> day<sup>-1</sup> with a low concentration of 10.5  $\pm$  2.7 g L<sup>-1</sup> for the first 114 days (January to May) and 0.8 m<sup>3</sup> day<sup>-1</sup> with a high concentration of 21.4  $\pm$  4.9 g L<sup>-1</sup> for 114–281 days (May–October).

The microbial agents of the seed bioreactor were supplied to the pre-biodigester with a flow rate of approximately 3% (volume ratio, v/v) of the influent loading. The recycle sludge ejected from the submerged membrane bioreactor was supplied to pre-biodigester with a flow rate of approximately 20% (v/v) of the influent loading ( $R/F = 0.20$ , v/v). The total sludge input supplied to the pilot system was

291 m<sup>3</sup> for 281 days. The HRT of the sludge solution in the multi-stage biodigester was 41 days for an influent flow rate of 1.4 m<sup>3</sup> days<sup>-1</sup>, and 73 days for an influent flow rate of 0.8 m<sup>3</sup> days<sup>-1</sup>. To achieve 0.08 m<sup>3</sup>-air m<sup>-3</sup>-reactor min<sup>-1</sup> of air injection in the multi-stage biodigester using a Root blower (HRS-L, Zhejiang Col, China), air was supplied at 0.4, 0.8, 0.8, 1.46, and 1.2 m<sup>3</sup> min<sup>-1</sup> to the pre-biodigester, biodigester 1, biodigester 2, biodigester 3, and the submerged membrane bioreactor. In the fixed-film bioreactors for wastewater treatment, air was supplied at 0.16 m<sup>3</sup> min<sup>-1</sup> (0.05 m<sup>3</sup>-air m<sup>-3</sup>-reactor min<sup>-1</sup>) using a linear air pump (YLP-80, YoungNam Air Pump Co., Republic of Korea). An operation cycle was conducted by aeration using an air pump for 20 h to each bioreactor, after which aeration was stopped for 4 h.

### Analysis procedure

Samples were collected for each reactor every week throughout the operation. Biological oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (T-N), total phosphorus (T-P), and suspended solids (SS) including mixed liquor suspended solids (MLSS) and fixed suspended solids (FSS) of the samples were measured according to the Korea Standard methods for Examinations of Water Quality [11]. Dissolved oxygen (DO) and pH of the samples were measured according to the Korean Standard Soil Analysis Method [12].

### Process analysis using mass balances

The mass balances for evaluating sludge reduction in the pilot plant are given in Fig. 2. The mass balances for total mass, MLSS, and FSS were calculated based on the general material balance equation: accumulation within the system = input through system boundaries – Output through system boundaries + Generation within the system – Consumption within the system. The mass balances were calculated according to Eqs. (1), (2), and (3) for total mass, MLSS, and FSS.

$$\frac{d(M_{\text{solution},j})}{dt} = \frac{d(V\rho_j)}{dt} = (F_{j-1}\rho_{j-1} - F_j\rho_j) + (A_{i,j}H_{i,j} - A_{o,j}H_{o,j}) + F_M\rho_M + F_R\rho_R, \tag{1}$$

$$\frac{d(M_{\text{MLSS},j})}{dt} = \frac{d(VS_j)}{dt} = (F_{j-1}S_{j-1} - F_jS_j) + F_M S_M + F_R S_R + r_{\text{MLSS},j}, \tag{2}$$

$$\frac{d(M_{\text{FSS},j})}{dt} = \frac{d(VI_j)}{dt} = (F_{j-1}I_{j-1} - F_jI_j) + F_M I_M + F_R I_R + r_{\text{FSS},j}, \tag{3}$$

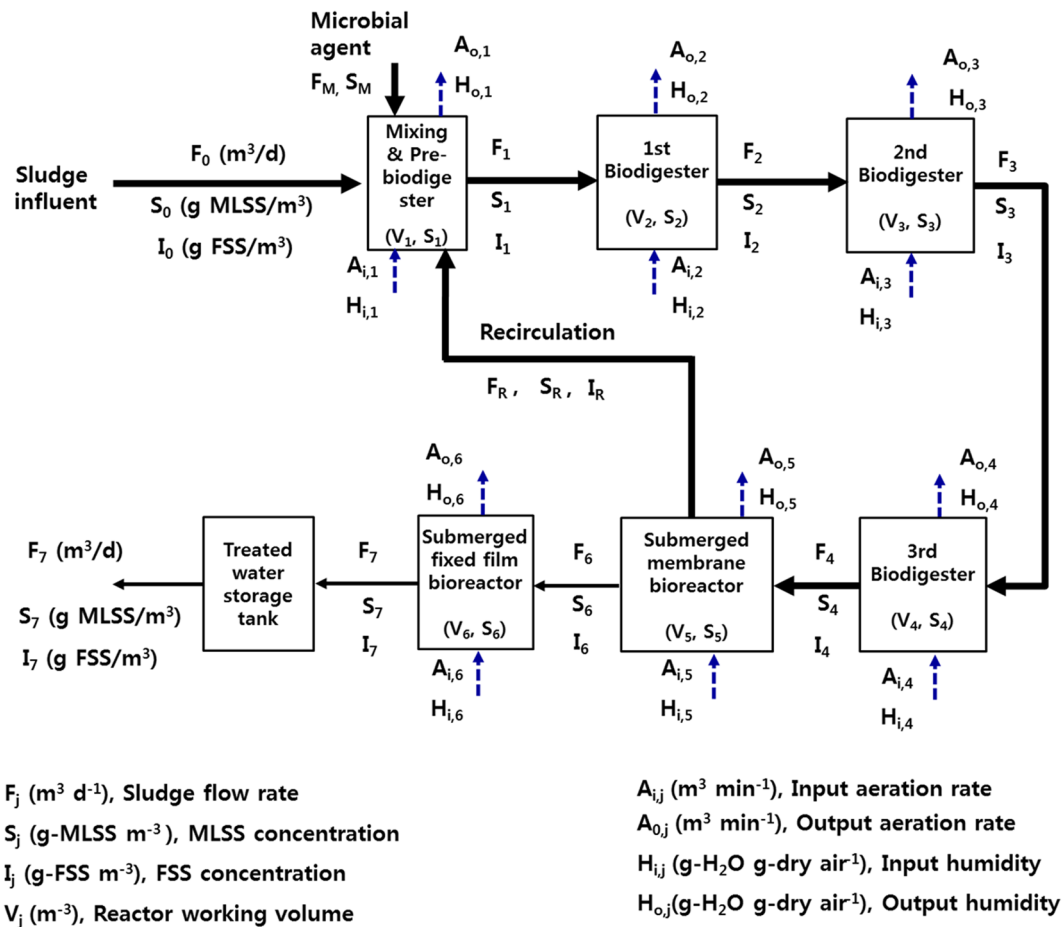


Fig. 2 Flow chart of MLSS and FSS mass balances

where  $F$ , flow rates of sludge solution in stream of  $j$ ,  $M$ , and  $R$  ( $\text{m}^3 \text{day}^{-1}$ );  $S$ , MLSS concentration in stream of  $j$ ,  $M$ , and  $R$  ( $\text{kg-MLSS m}^{-3}$ );  $I$ , FSS concentration in stream of  $j$ ,  $M$ , and  $R$  ( $\text{kg-FSS m}^{-3}$ );  $V_j$ , working volume of reactor ( $\text{m}^3$ );  $\rho$ , density of sludge solution in stream of  $j$ ,  $M$ , and  $R$  ( $\text{kg m}^{-3}$ );  $M$ , mass of sludge solution, MLSS and FSS ( $\text{kg}$ );  $A_{ij}$  and  $A_{oj}$ , aeration rate in stream of inlet and outlet ( $\text{kg-dry air day}^{-1}$ );  $H_{ij}$  and  $H_{oj}$ , humidity in stream of inlet and outlet ( $\text{kg-H}_2\text{O kg-dry air}^{-1}$ ); and  $r_{\text{MLSS},j}$  and  $r_{\text{FSS},j}$ , reaction rate of MLSS and FSS ( $\text{kg m}^{-3} \text{day}^{-1}$ ).

### RNA-based bacterial community analysis

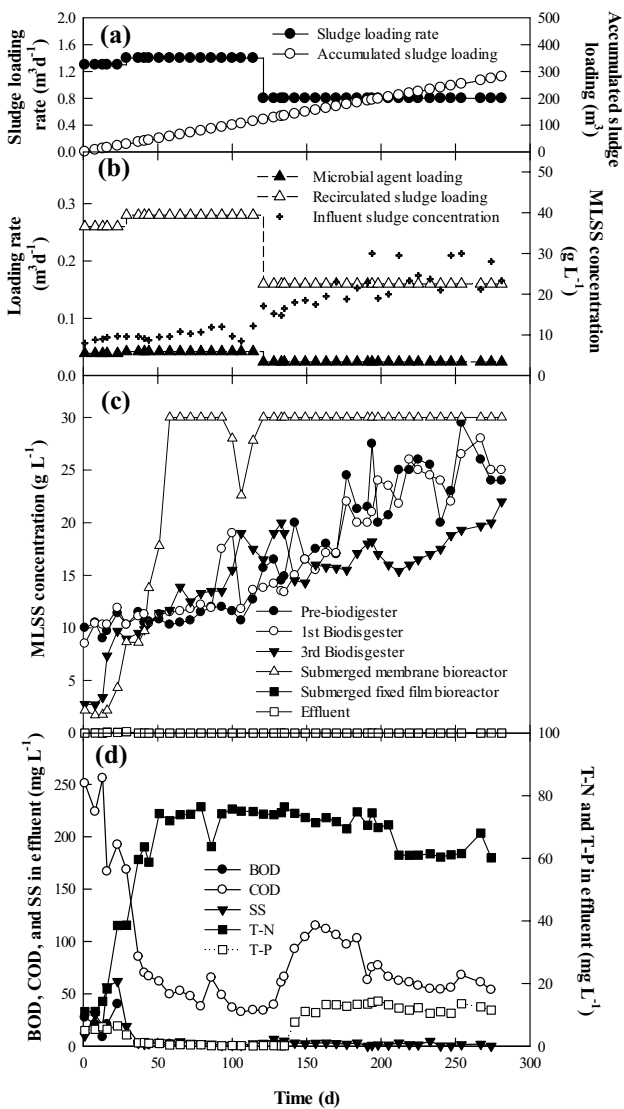
After completion of the pilot-scale sludge treatment process operation, the sludge solutions in each bioreactor—except for the submerged fixed-film bioreactor—were sampled. In the submerged fixed-film bioreactor, the biofilms on the fiber membranes were collected using a sterilized spatula. 1.5 mL of each sample was centrifuged at  $16,000\times g$  for 5 min, and the recovered pellet was immediately frozen at  $-70^\circ\text{C}$  before RNA extraction. RNA was extracted manually as

described in a previous study [8]. The detailed procedure for the RNA-based bacterial community analysis is shown in the supplementary material. Obtained sequence data were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/>) under accession number SRP182733. The similarity of bacterial communities between the processes in the sludge reduction system was analyzed via principal component analysis (PCA) using CANOCO 4.5 software (Microcomputer Power, Ithaca, USA).

## Results and discussion

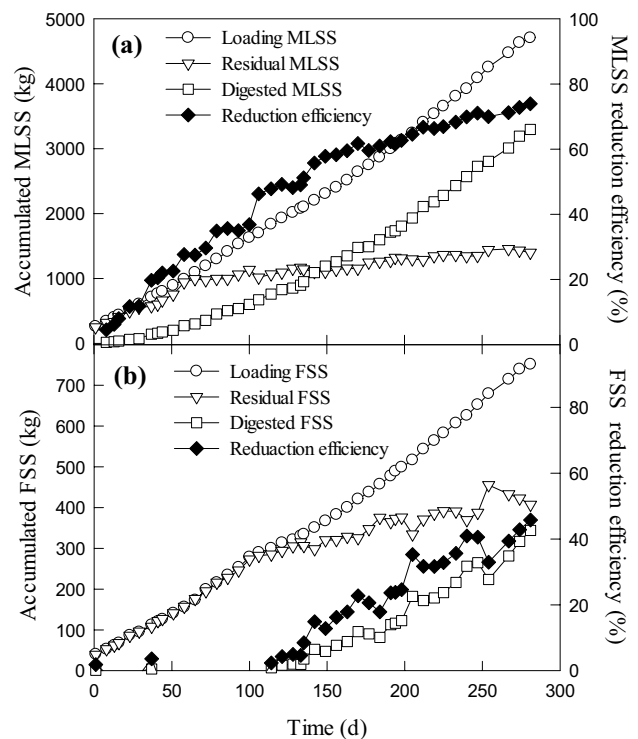
### Performance of the pilot-scale sludge treatment process

Figure 3 shows the performance of the pilot-scale sludge treatment process. Relatively low sludge solution ( $10.5\pm 2.7 \text{ kg-MLSS m}^{-3}$ ) was fed into the process at a loading rate of  $1.4 \text{ m}^3 \text{day}^{-1}$  from 0 to 114 days (Fig. 3a,



**Fig. 3** Time profiles of sludge treatment performance: **a** sludge loading rate and accumulated sludge loading; **b** influent sludge concentration, microbial agent loading rate, and recirculated sludge loading rate; **c** MLSS concentrations in the pre-bioidigester, bioidigester 1, bioidigester 2, bioidigester 3, submerged membrane bioreactor, and submerged fixed-film bioreactor, and effluent; and **d** BOD, COD, SS, T-N, and T-P concentrations in effluent

b). After 114 day, a relatively high sludge solution ( $21.4 \pm 4.9 \text{ kg-MLSS m}^{-3}$ ) was fed at  $0.8 \text{ m}^3 \text{ d}^{-1}$  (Fig. 3a, b). Due to the differences in MLSS concentrations of the sludge solution between the two periods, the recirculation sludge loading rate and microbial agents loading rate after 114 days were controlled lower than the rates before 114 days (Fig. 3b). The accumulated sludge loading amount was  $291 \text{ m}^3$  for 281 days (Fig. 3a). The MLSS concentrations in the pre-bioidigester and the bioidigesters increased from  $2.75\text{--}10.00 \text{ kg-MLSS m}^{-3}$  on day 1 to  $20.0\text{--}28.0 \text{ kg-MLSS m}^{-3}$  at day 281 (Fig. 3c). The



**Fig. 4** Time profiles of sludge amounts and sludge reduction efficiencies in the overall process: **a** accumulated MLSS and MLSS reduction efficiency and **b** accumulated FSS and FSS reduction efficiency

MLSS in the membrane bioreactor increased sharply during 50 days of operation and after that maintained constant at a level of approximately  $30.0 \text{ kg-MLSS m}^{-3}$  during the remaining time of operation (Fig. 3c).

The BOD, COD, T-N, and T-P concentrations of the influent were  $3009 \pm 1850$ ,  $3938 \pm 1486$ ,  $547 \pm 128$ , and  $356 \pm 96 \text{ mg L}^{-1}$  (data not shown). However, after an acclimation period of 40 d, the BOD and COD concentrations of the effluent (discharged water from the process) were reduced to  $1\text{--}2 \text{ mg L}^{-1}$  and  $55\text{--}75 \text{ mg L}^{-1}$  (Fig. 3d). The BOD and COD removal efficiencies were 99.9% and 98.0%. The T-N and T-P concentrations in the effluent were  $60\text{--}75 \text{ mg L}^{-1}$  and  $10\text{--}14 \text{ mg L}^{-1}$ , indicating the removal efficiencies of 86–89% for T-N and 96–97% for T-P (Fig. 3d). The MLSS concentrations of the influent were ranged from 6 to  $30 \text{ g L}^{-1}$  (Fig. 3b), but the SS concentrations of the effluent were below  $5 \text{ g L}^{-1}$  (Fig. 3d). The SS removal efficiency was over 99%.

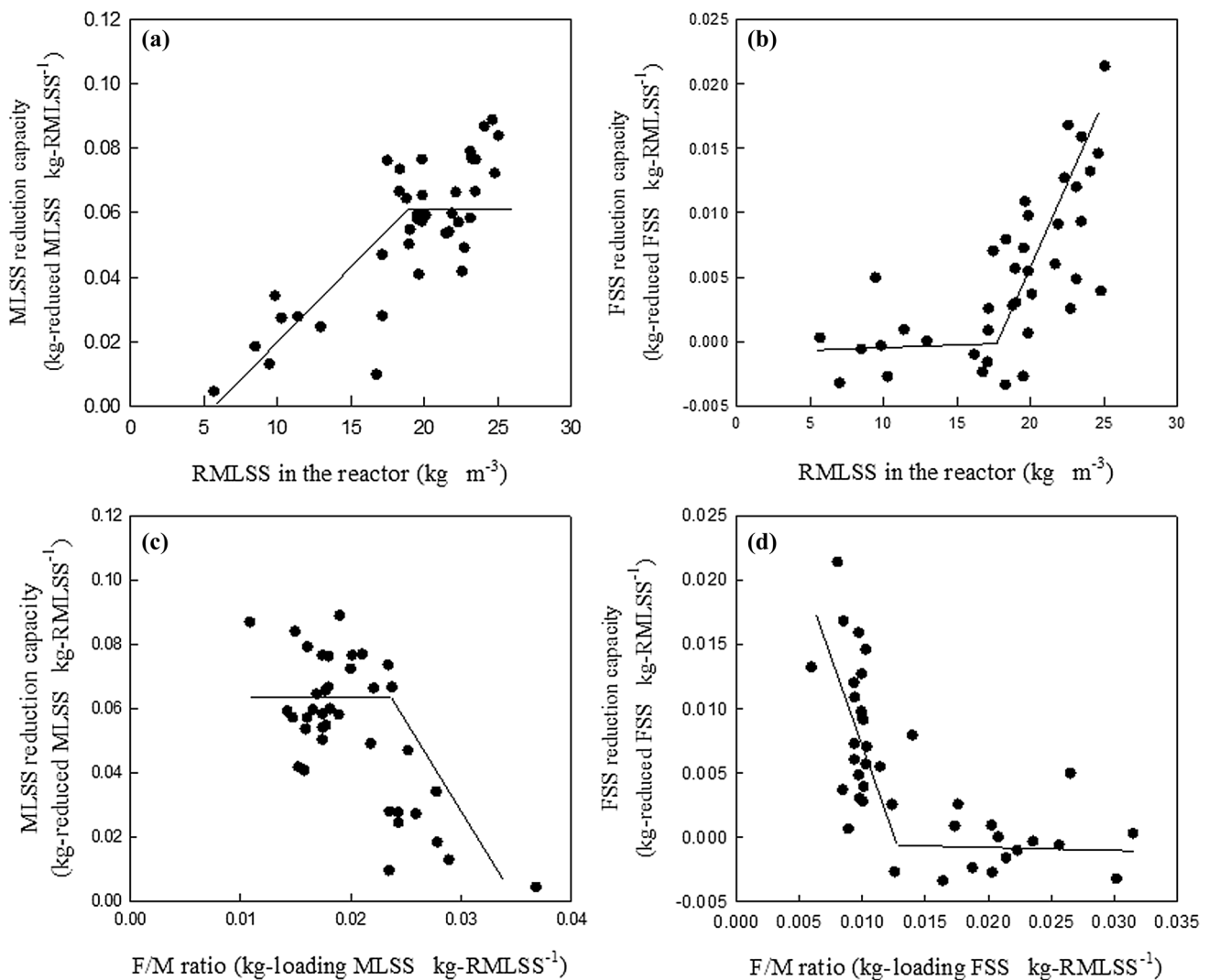
### Sludge reduction throughout the operation

Time profiles of the loading, residual, and digested sludge amounts during the overall process are shown in Fig. 4. The digested MLSS amount increased remarkably after 65 days, and the residual MLSS (RMLSS) amount increased very

slowly (Fig. 4a). The system achieved a total MLSS reduction efficiency of 74% via digestion of 3296 kg from a loading of 4706 kg across the entire operation period. The FSS amount increased over time without any reduction during the initial 114 days, but it began to decrease after 114 days (Fig. 4b). The residual FSS (RFSS) was maintained at a level of 280–420 kg (avg. 6.2 kg-FSS m<sup>-3</sup>) after 114 days. The system achieved total FSS reduction efficiency of 46% (reduction to 344 kg from a loading FSS of 751 kg) for 281 days.

In the multi-stage systems, sludge (MLSS) entering each digester was decomposed by RMLSS in each digester. Therefore, the removability of sludge by RMLSS, which played the role of decomposer, was defined as sludge reduction capacity (SRC). To interpret a sludge reduction pattern

during the process operation, SRC was determined by the total amount of reduced MLSS (kg-reduced MLSS) relative to the total amount of RMLSS (kg-RMLSS) in the entire system (Fig. 5). By increasing the RMLSS in the reactor, the MLSS reduction capacity increased linearly, and then stayed at 0.05–0.09 kg-MLSS kg-RMLSS<sup>-1</sup> when RMLSS was maintained between 18 and 25 kg-RMLSS m<sup>-3</sup> (Fig. 5a). This result suggests that the sludge reduction occurred under steady-state conditions when the RMLSS in the reactor reached 18 kg-RMLSS m<sup>-3</sup>. The FSS reduction capacity also showed positive values under conditions of 18 kg-RMLSS m<sup>-3</sup> or more (Fig. 5b), indicating that the FSS began to decrease at steady state. The relationship between the *F/M* ratio and SRC is shown in Fig. 5c, d. The MLSS reduction capacity was 0.05–0.08 kg-MLSS kg-RMLSS



**Fig. 5** Relationship between sludge reduction capacity (SRC) and residual MLSS in the reactor (RMLSS), and between SRC and *F/M* ratio: **a** SRC of MLSS and RMLSS; **b** SRC of FSS and RMLSS; **c** SRC of MLSS and *F/M* ratio; and **d** SRC of FSS and *F/M* ratio

under an  $F/M$  ratio of less than 0.022 (Fig. 5c). Also, the FSS reduction capacity increased with a decreasing  $F/M$  ratio, and it showed positive values at an  $F/M$  ratio of less than 0.012 kg-loading MLSS kg-RMLSS<sup>-1</sup> (Fig. 5d).

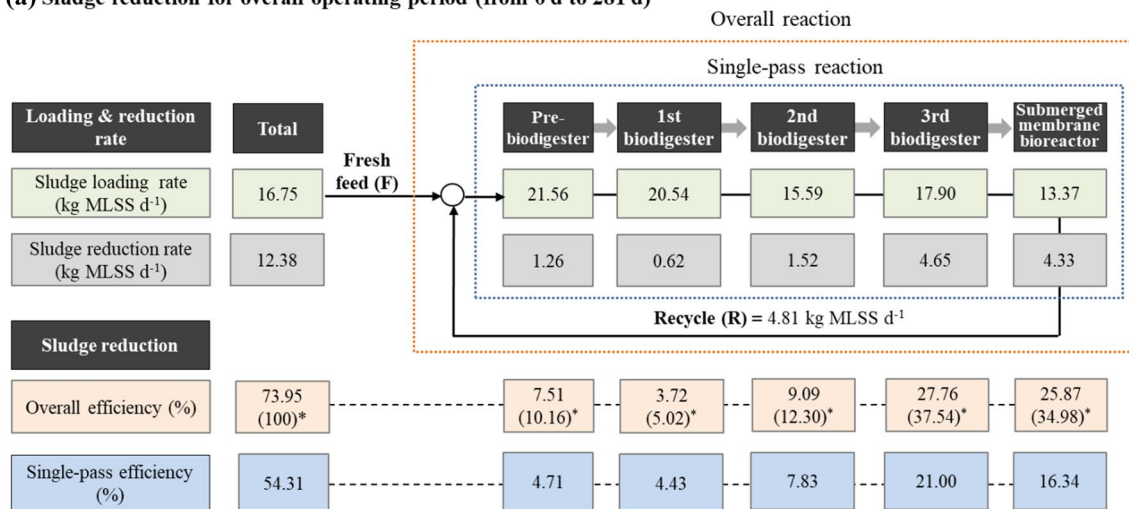
The change in RMLSS and SRCs in the overall system over time was calculated (Fig. S1a). The average RMLSS in overall bioreactors increased linearly to 18 kg-RMLSS m<sup>-3</sup> over the first 114 days of operation, after which it was maintained within the range of 18–25 kg-R MLSS m<sup>-3</sup>. After 114 days, the MLSS reduction capacity ranged from 0.05 to 0.09 kg-MLSS kg-RMLSS<sup>-1</sup>, and FSS reduction capacity was positive (Fig. S1b, c). These results indicate that the pilot-scale sludge treatment process operated at steady state after 114 days.

### Evaluation of sludge reduction in individual reactor

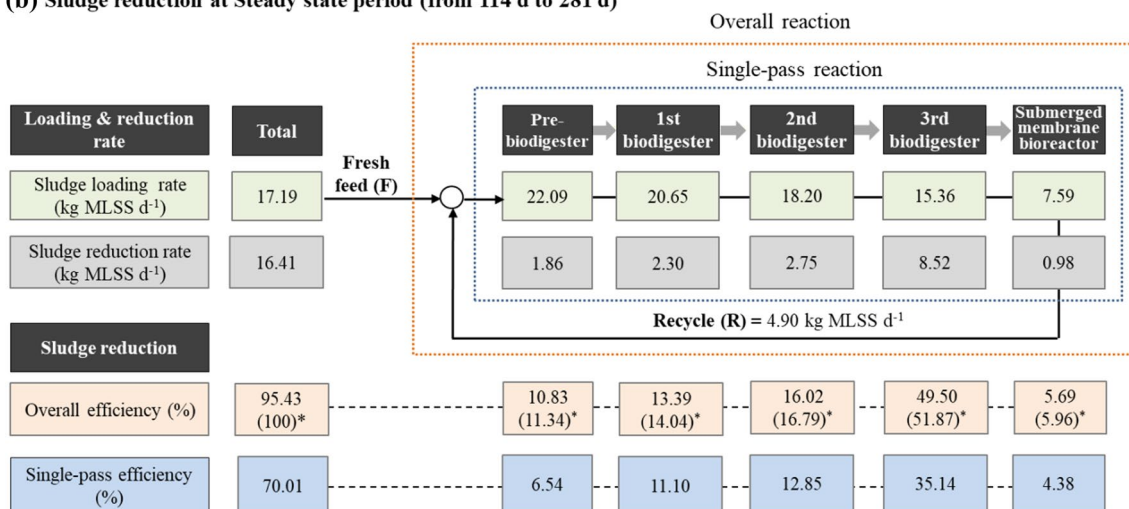
To evaluate the contribution of each reactor to sludge reduction, the sludge loading rates (kg-loading MLSS day<sup>-1</sup>) and sludge reduction rates (kg-digested MLSS day<sup>-1</sup>) in each reactor were calculated separately for two operating periods: overall (from 0 to 281 days, HRT = 41 days) and steady state (from 114 to 281 days, HRT = 73 days) (Fig. 6).

During the overall operating period (based on overall reaction), total sludge loading rate, total sludge reduction rate, and overall sludge reduction efficiency were 16.75 kg-loading MLSS day<sup>-1</sup>, 12.38 kg-digested MLSS day<sup>-1</sup>, and 73.95% (Fig. 6a). The contribution of each reactor to the overall sludge removal efficiency was 37.54% for the biodigester 3, 34.98% for the submerged membrane bioreactor,

(a) Sludge reduction for overall operating period (from 0 d to 281 d)



(b) Sludge reduction at Steady state period (from 114 d to 281 d)



**Fig. 6** Sludge reduction rates and efficiencies of the individual bioreactor: **a** sludge reduction during the overall operating period (from 0 to 281 days) and **b** sludge reduction during the steady-state period (from 114 to 281 days)

12.30% for biodigester 2, 10.16% for the pre-biodigester, and 5.02% for biodigester 1. On the other hand, based on the single-pass reaction, the single-pass sludge reduction efficiency was 54.31%.

During the steady-state period from 114 to 281 days (based on overall reaction), total sludge loading rate, total sludge reduction rate, and overall sludge reduction efficiency were 17.19 kg-loading MLSS day<sup>-1</sup>, 16.41 kg-digested MLSS day<sup>-1</sup>, and 95.43% (Fig. 6b). The sludge reduction rate for biodigester 3 was 8.52 kg-digested MLSS day<sup>-1</sup>, which corresponded to 51.87% of the overall sludge reduction. The total sludge reduction rate of the three biodigesters was 13.57 kg-digested MLSS day<sup>-1</sup>, which corresponded to 82.70% of the overall sludge reduction, indicating the biodigesters were key reactors for sludge reduction. The sludge reduction rates for the submerged membrane bioreactor was 0.98 kg-digested MLSS day<sup>-1</sup>, which suggested that the role of this reactor was the separation of solids (sludge) and liquids (wastewater) rather than sludge digestion during this period. Based on the single-pass reaction, the single-pass sludge reduction efficiency at the steady-state period was 70.01%.

Several researchers report sludge reduction using maintenance energy with prolonged SRT and low *F/M* ratio without pretreatment of sludge (Table S1). Sludge reduction efficiencies are over 90% under *F/M* ratios of 0.07–0.04 [13–17], but they are 54–90% under *F/M* ratios of 0.11–0.26 [18–20]. The sludge treatment process proposed in this study is an environmentally-friendly process that does not require additional chemicals or energy.

This is achieved by operating at an ambient temperature of 15–30 °C, resulting in a sludge reduction efficiency of 95% at steady state.

### Active bacterial communities in each reactor

The active bacterial communities present in sludge reduction during each process were analyzed using Illumina MiSeq sequencing. Metatranscriptome analysis results of active bacterial communities in each process are shown in Table 1. The biomixer, biodigesters, and membrane bioreactor showed a relatively large operational taxonomic unit (OUT) of 1201–1466, and the proliferation solution showed the smallest OUT of 198. The Chao1 index indicated that the major reactors, such as the biomixer, biodigesters, and membrane bioreactor exhibited greater richness than the other reactors. These major reactors also showed higher Shannon and Simpson indices, which suggests that active bacterial communities in these major reactors were more diverse—distributed more evenly—than in other reactors. Lozupone et al. assert that diversity of the microbial community is a crucial indicator of system resilience and functional stability [21]. Briones and Raskin indicate that biodiversity and system stability exhibit a positive correlation with functional complementation [22].

Phylogenetic classification in the active bacterial community at each process is shown in Fig. 7a. The predominant phylum in the overall process was Proteobacteria, except for Firmicutes, at a relative abundance of over 60% in the proliferation solution. The film bioreactors were dominated

**Table 1** Metatranscriptome analysis results of active microbial communities at each bioreactor in the pilot plant system

| Sampling site                            | No. of reads <sup>a</sup> | No. of OTUs | Chao1 <sup>b</sup> | Diversity index      |                      | Good's coverage <sup>e</sup> |
|--|---------------------------|-------------|--------------------|----------------------|----------------------|------------------------------|
|  |                           |             |                    | Shannon <sup>c</sup> | Simpson <sup>d</sup> |                              |
| Inoculum (IC)                            | 97,930 ± 36,815           | 758 ± 98    | 898 ± 54           | 4.98 ± 0.16          | 0.90 ± 0.01          | 0.998 ± 0.001                |
| Proliferation solution (PS)              | 48,684 ± 11,367           | 198 ± 15    | 307 ± 45           | 3.52 ± 0.34          | 0.81 ± 0.04          | 0.999 ± 0.001                |
| Excess sludge (ES)                       | 55,598 ± 28,650           | 1201 ± 162  | 1498 ± 122         | 7.04 ± 0.09          | 0.97 ± 0.00          | 0.993 ± 0.005                |
| Pre-biodigester (BM)                     | 47,118 ± 48,275           | 1289 ± 341  | 1564 ± 272         | 7.56 ± 0.14          | 0.98 ± 0.01          | 0.986 ± 0.014                |
| Biodigester 1 (BD1)                      | 68,886 ± 27,957           | 1466 ± 182  | 1759 ± 194         | 6.88 ± 0.46          | 0.95 ± 0.02          | 0.995 ± 0.002                |
| Biodigester 2 (BD2)                      | 73,811 ± 25,104           | 1330 ± 316  | 1552 ± 444         | 6.79 ± 0.53          | 0.95 ± 0.02          | 0.996 ± 0.002                |
| Biodigester 3 (BD3)                      | 78,911 ± 40,868           | 1308 ± 258  | 1587 ± 242         | 6.26 ± 0.65          | 0.92 ± 0.05          | 0.995 ± 0.002                |
| Submerged membrane bioreactor (SMB)      | 72,096 ± 22,377           | 1332 ± 100  | 1640 ± 82          | 6.63 ± 0.03          | 0.95 ± 0.00          | 0.995 ± 0.002                |
| Submerged fixed-film bioreactor 1 (SFB1) | 62,719 ± 16,252           | 579 ± 43    | 737 ± 76           | 6.23 ± 0.05          | 0.97 ± 0.00          | 0.998 ± 0.000                |
| Submerged fixed-film bioreactor 2 (SFB2) | 68,304 ± 14,835           | 567 ± 18    | 751 ± 17           | 5.89 ± 0.13          | 0.95 ± 0.01          | 0.998 ± 0.001                |
| Submerged fixed-film bioreactor 3 (SFB3) | 56,156 ± 17,126           | 485 ± 48    | 612 ± 78           | 6.05 ± 0.13          | 0.97 ± 0.01          | 0.998 ± 0.000                |

<sup>a</sup>No. of reads is the number of sequences after the trimming

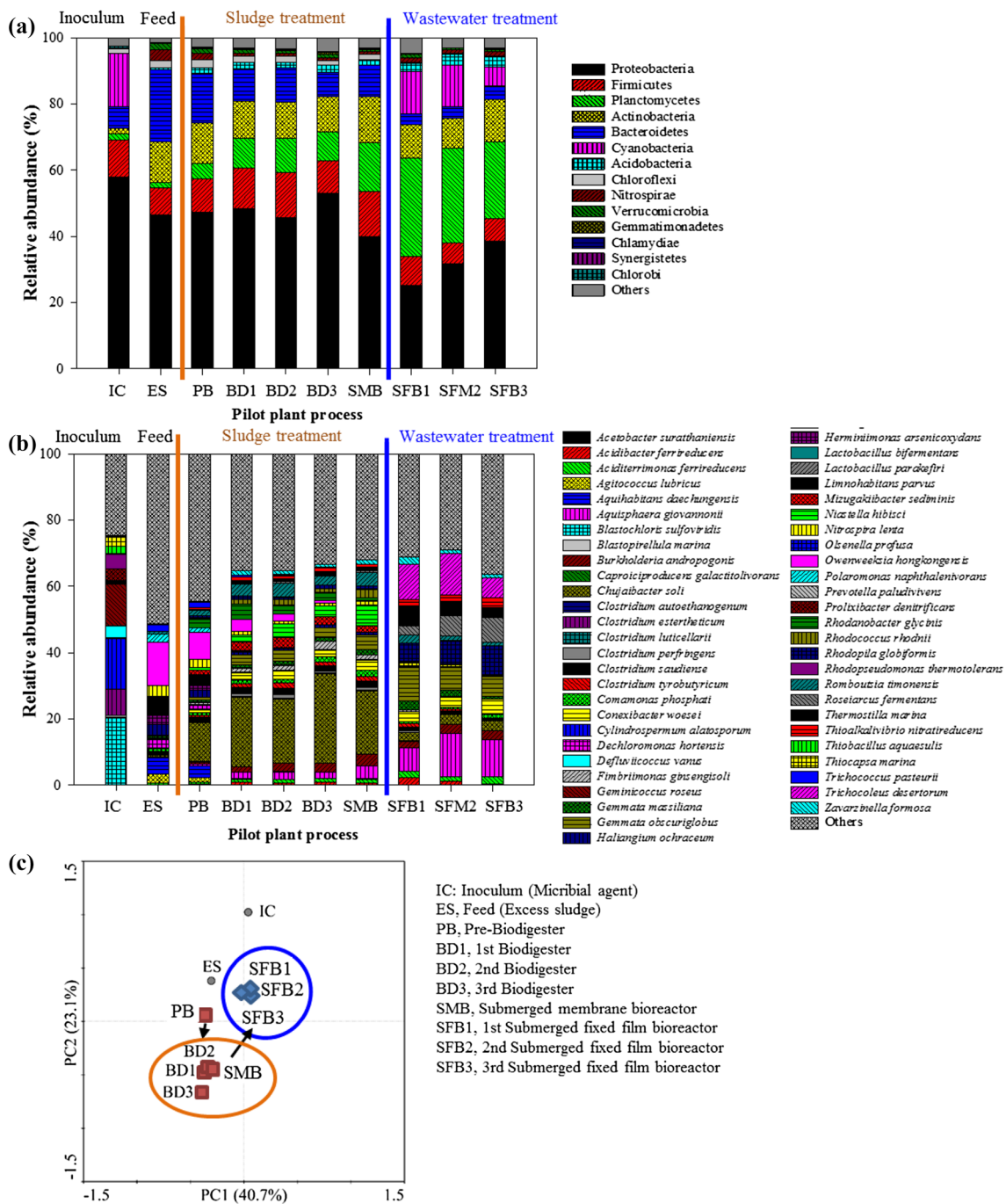
<sup>b</sup>Chao1 is the index used to evaluate the bacterial population richness

<sup>c</sup>Shannon index is used to evaluate diversity within the bacterial population

<sup>d</sup>The Simpson index represents the probability that two randomly selected individuals in the habitat will belong to the same species

<sup>e</sup>Good's coverage is calculated as  $C = 1 - (s/n)$ , where  $s$  is the number of unique OTUs and  $n$  is the number of individuals in the sample. This index provides a relative measure of how well the sample represents the larger environment





**Fig. 7** Comparison of active bacterial communities in the inoculum (microbial agent), excess sludge, and individual bioreactor: **a** phylum level; **b** species level; and **c** principal components analysis of the bacterial communities

by Proteobacteria and Planctomycetes with a relative abundance of over 50%. At the species level, *Blasochloris sulfoviridis* (20.3%), *Cylindrospermum alatosporum* (15.1%), and *Geminicoccus roseus* (12.7%) were more abundant in the inoculum, as shown in Fig. 7b. In the excess sludge as a feed, *Owenweeksia hongkongensis* was predominant with a relative abundance of 13.1%, followed by *Limnohabitans*

*parvus* (5.5%) and *Aquihabitans dae chungensis* (5.0%). The dominants in the pre-biodigester were *Chujaibacter soli* and *O. hongkongensis* with a relative abundance of 11.8% and 8.2%. In the three biodigesters, *C. soli* was the most abundant with a relative abundance range of 19.5–26.9% (avg. 22.5%), followed by *Romboutsia timonensis* (avg. 3.5%), *Rhodanobacter glycinis* (avg. 3.3%), *Niastella hibiscis* (avg.

3.0%), and *Gemmata obscuriglobus* (avg. 2.9%). In the submerged membrane bioreactor, *C. soli* was predominant with a relative abundance of 18.9%, followed by *N. hibisci* (6.4%), *G. obscuriglobus* (4.4%), and *R. timonensis* (4.2%). In the submerged fixed-film bioreactors, *Aquisphaera giovannonii*, *G. obscuriglobus*, *Trichocoleus desertorum*, *Rhodopila globiformis*, *Thermostilla marina*, and *Roseiarcus fermentans* had average relative abundance ranges of 6.9–12.9%, 6.0–10.3%, 5.8–12.6%, 5.4–8.7%, 3.1–6.1%, and 2.6–7.3%.

The PCA results based on the species level revealed the variety of clustered active bacterial communities among the bioreactors (Fig. 7c). In the major reactors for sludge degradation, the biodigesters and submerged membrane bioreactor showed similar active bacterial communities, which in turn were similar to those in the pre-biodigester. However, the active bacterial communities in the submerged fixed-film bioreactors as a post-process were clustered differently, which may be due to process characteristics using the microbial cell-attached film as well as wastewater treatment as opposed to sludge treatment. These results indicated the formation of similar bacterial communities in response to bioreactor function.

### Functional bacteria contributed to sludge reduction

Table S2 shows the characteristics of the dominant bacteria in the biodigesters and submerged membrane bioreactor, which had the highest rate of contribution to sludge degradation. Most of the bacteria were heterotrophs, which use organics as their carbon source, indicating these bacteria participated in sludge degradation. Also, some bacteria had hydrolytic enzyme activities. *A. giovannonii*, an obligately aerobic heterotroph, hydrolyzes starch, tween 20, tween 80, and polysaccharides [23]. *Mizugakiibacter sedimins* degrades gelatin by protease [24]. *N. hibisci* and *Rudaea cellulositytica* degrades cellulose by cellulose [25, 26]. *O. hongkongensis* uses high-molecular organic compounds [27], and *R. timonensis* degrades oil and petroleum hydrocarbons [28].

Some bacteria including *A. giovannonii*, *Bacillus wiedmannii*, *Clostridium autoethanogenum*, *Clostridium tyrobutyricum*, *Intestinibacter bartlettii*, *Paludibaculum fermentans*, and *Rhizomicrobium electricum* produce organic acids by fermentative metabolism [23, 29–33]. Also, *B. wiedmannii* has been reported as a phosphate-solubilizing bacterium [34]. Organic acids and fermentative intermediates can solubilize particulate inorganic matters (PIM) [35], suggesting that these bacteria are associated with the reduction of FSS contents during sludge treatment. The solubilization of inorganic matters during the pilot-scale sludge treatment process operation in this study was observed in Table S3. The dissolved amount of many inorganic elements, such as Mg, Si, K, Ca, Zn, Ba was increased in stages through reactors from

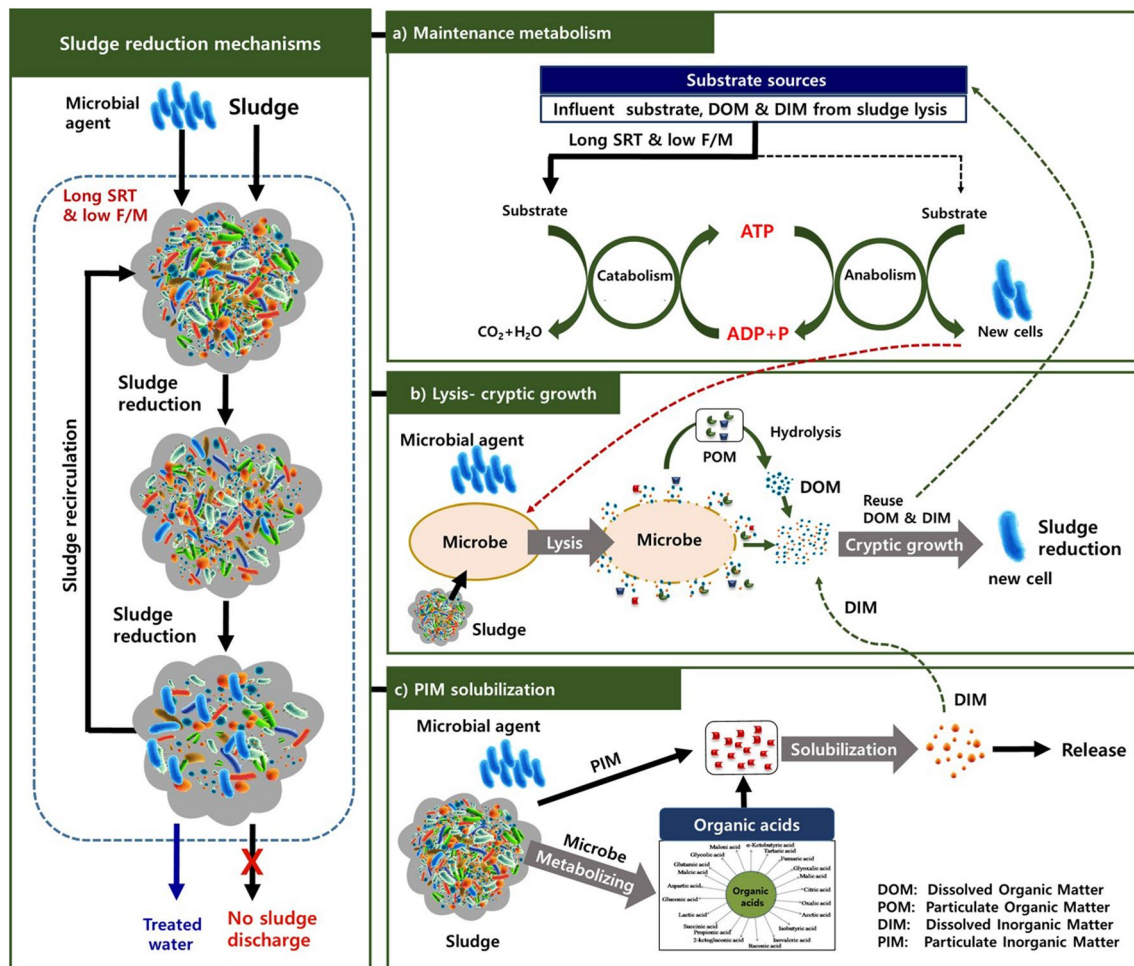
the influent excess sludge. Especially, the great increase of Si (from 8.73 mg L<sup>-1</sup> at the influent excess sludge to 21.9 mg L<sup>-1</sup> at the fixed-film bioreactors) can be interpreted as direct evidence for solubilization of inorganic matters.

*Chujaibacter soli*, an aerobic heterotroph, is among the bacterial agents that can simultaneously remove methane and odors [36, 37]. *Solirubrobacter phytolaccae* has been detected in the biocover for odor removal [38]. *N. hibisci*, *Burkholderia andropogonis*, and *R. cellulositytica* have reported that they could degrade malodorous aromatic compounds, such as phenol, benzene, and toluene [26, 39, 40]. *R. glycinis* degrades ammonia and volatile organic compounds [41]. *Rhodococcus rhodnii* and *R. globiformis* oxidize reduced sulfide, such as hydrogen sulfide [42–44]. Considering their properties, these bacteria might play important roles in decomposing odors generated during sludge degradation. Further studies are required to clarify the roles of bacteria contributing to sludge reduction.

### Hypothesis for sludge reduction mechanisms in the process

Based on the results of sludge reduction performance and bacterial community characteristics, the hypothesis for sludge reduction mechanisms in the pilot plant process is shown in Fig. 8. The hypothetical mechanism for sludge reduction can be explained by the combined actions of the maintenance metabolism, lysis–cryptic growth, and PIM solubilization [5, 7]. Under the conditions of long sludge retention time and low *F/M* ratio, the bioreactors (pre-biodigester, biodigesters, and submerged membrane bioreactor) are operated using maintenance metabolism. In maintenance metabolism, microorganisms use dissolved organic and inorganic matters, which are released from the hydrolysis of nutrients contained in excess sludge and lysis of microorganisms, to generate maintenance energy [3, 4]. When the biomass in the reactor is saturated, the amount of maintenance energy is maximized. This study demonstrated that excess sludge can be efficiently reduced by keeping the biomass in the bioreactors in a saturated state; thus, maximizing maintenance energy.

Sludge reduction can be achieved by lysis–cryptic growth, where microorganisms release and grow on lysates [3, 7, 45]. The microbial cells are disrupted and dissolved by hydrolytic enzymes, such as lysozyme, protease, lipase, and cellulase [45]. The microorganisms reuse the lysates and dissolved inorganic matters (DIM) for new cell growth. In this study, it is possible the lysis–cryptic growth in the bioreactors might be enhanced by hydrolytic enzyme-producing bacteria, including *Aquisphaera*, *Mizugakiibacter*, *Niastella*, *Rudaea*, *Owenweeksia*, and *Romboutsia* [23–28]. PIM solubilization is a process that



**Fig. 8** Hypothetical mechanism for sludge reduction in the pilot-scale sludge treatment process: **a** maintenance metabolism; **b** lysis–cryptic growth; and **c** particulate inorganic matter (PIM) solubilization

releases DIM from PIM. In this process, PIM is dissolved by metabolic intermediates that produce fermentative bacteria, which the resulting DIM uses for metabolism maintenance and lysis–cryptic growth.

### Conclusion

As an environmentally-friendly excess sludge treatment method, a multi-stage process with prolonged solid retention time was proposed. In this study, sludge reduction performance and bacterial community dynamics in the process were characterized using mass balance analysis and an RNA-based pyrosequencing method. The process achieved no sludge discharge except sampling at a total loading sludge of 4700 kg-MLSS throughout the operation period of 281 days. Under conditions of high RMLSS in the reactors (18–25 kg-RMLSS m<sup>-3</sup>) and low *F/M* ratio (less than

0.018 kg-loading MLSS kg-RMLSS<sup>-1</sup>), sludge reduction efficiency was 95%. This result suggests that excess sludge can be efficiently reduced through a strategy that maximizes maintenance energy by keeping biomass (sludge) in the bio-reactors in a saturated state. Functional bacteria contributing to reduced sludge were confirmed via RNA-based bacterial community analysis: (a) aerobic and anaerobic heterotrophs for sludge decomposition, (b) heterotrophs with hydrolytic activities for sludge lysis, and (c) fermentative bacteria for PIM solubilization. Hydrolysis, PIM solubilization, and maximizing maintenance energy are proposed as sludge reduction mechanisms in the sludge treatment process. Comprehensive studies using proteomics and metabolomics will be necessary to clarify the mechanism for sludge reduction in this process.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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