Original Article

Excess Sludge Reduction Using a High-pressure Jet Device via a Modified Ludzack-Ettinger Process: Performance and Microbial Communities

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ABSTRACT

Excess sludge reduction is a central challenge in wastewater treatment, requiring a cost-effective technology. Here, we report the pilot-scale implementation of two Modified Ludzack-Ettinger (MLE) processes with and without a high-pressure jet device (HPJD) as a sludge reduction method based on physically decomposing bacterial cells in activated sludge. The MLE process with an HPJD (MLE-HPJD) bifurcated the activated sludge return lines and directed them to anoxic and oxic tanks; an HPJD was incorporated in the latter. The operation of both processes by continuous municipal wastewater supply for 111 days demonstrated that HPJD application reduced the cumulative waste activated sludge amount by 56.6% without an increase in the effluent suspended solids (SS) concentration. The two MLE processes displayed comparable removal performances for organic carbon and nitrogen, indicating that sludge solubilization by HPJD does not hinder bacterial activity in activated sludge. A combination of the 16S rRNA gene amplicon sequencing and quantitative fluorescence *in situ* hybridization (FISH) revealed that microbial community compositions were distinct in the two processes. While still effecting excess sludge reduction, HPJD selectively lowered the relative abundance of filamentous bacteria, potentially causing sludge bulking. Further, the relative abundance and cluster structure of ammonia-oxidizing bacteria (AOB) in the MLE-HPJD and MLE processes were comparable, indicating that the application of HPJD did not impair nitrification performance. Monitoring the eukaryote community by microscopy showed that activated sludge in the MLE-HPJD had a much higher abundance of Protomastigida. Therefore, the MLE-HPJD process is an efficient sludge reduction technology that does not compromise organic carbon and nitrogen removal.

Keywords: activated sludge system, MLE process, waste activated sludge, high pressure jet device, microbial community composition

INTRODUCTION

A conventional activated sludge system (CAS) has been broadly implemented in wastewater treatment plants (WWTPs). Despite the broad implementation worldwide, a CAS has a primary challenge in excess sludge production, which requires energy- and labor- intensiveness for treatment and management. For this reason, sludge treatment costs account for up to 60% of the total operating expenditure of

a WWTP [1]. Therefore, the reduction of waste activated sludge is of importance as a countermeasure towards cost effectiveness. A lysis-cryptic process could troubleshoot the typical CAS challenge. The integration of a unit for the reduction of excess activated sludge into a CAS has been proposed, and its feasibility widely studied [2,3]. As a means of cell disintegration, a lysis-cryptic process installs a reaction tank or device enhancing cell solubilization by a chemical, mechanical, or biochemical method. Despite its promise

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for excess sludge reduction, a lysis-cryptic process still has downsides. These include high capital/operating costs and a reduction in wastewater treatment performance, *e.g.*, organic matter remaining in treated water, which are obstacles for widespread dissemination [4].

As an alternative lysis-cryptic process, an excess sludge reduction technology using a high-pressure jet device (HPJD) holds potential promise as it is more high-throughput and cost-effective than the other lysis-cryptic methods [5]. An HPJD, consisting of a high-pressure pump and pipe with one small and two large diameters in the middle and at both edges, respectively, disrupts cell walls and membranes of bacteria in activated sludge. A CAS incorporating an HPJD in the concentrated activated sludge return line reduces excess sludge reduction by 65%, compared with a conventional CAS, without impairing removal of organic matter, nitrogen and phosphorus [6]. Furthermore, an HPJD, intensively mixing activated sludge with aspirated air, generates fine bubbles, which exert supportive aeration [6]. Despite a promising and successful case of an HPJD installed in a CAS, an HPJD has not been applied to an advanced activated sludge system for both organic matter and nitrogen removal, *e.g.*, Modified Ludzack-Ettinger (MLE) process [7]. A thorough evaluation of the HPJD applicability to an MLE is deemed necessary since the effect of a lysis-cryptic process on nitrogen removal performance in an MLE has been found to be contradictory. According to the previous works, nitrogen performance was maintained [8,9] or reduced [10] by installing a sludge reduction unit at a nitrogen loading rate ranging from 21 to 124 mg-N/(L·day). The application of an HPJD to an MLE process, therefore, warrants intensive investigation.

In light of the potential link between microbial community composition and process performance in an engineered system [11,12], the effect of a lysis-cryptic method on the transitions of MLE process performance and the concomitant microbial community composition is a key issue. The introduction of a lysis-cryptic method to activated sludge systems shaped a unique microbial community composition, distinct from those devoid of a lysis-cryptic method [13–15]. Our previous work testing a pilot-scale CAS-installing HPJD confirmed a dynamic shift of the microbial community composition in activated sludge [6]. However, the findings are still only at the observation stage, necessitating in-depth analysis to reveal the nature of the change in the microbial community and the relationship with treated water quality.

A potential downside of an HPJD stems from disaggregation of activated sludge flocs, likely leading to an increase in the suspended solids (SS) concentration in treated water as

previously observed [6]. The downside may also jeopardize the growth and retention of slow-growing nitrifying guilds due to excessive disaggregation and cell damage when subjected to an HPJD. Although running time or frequency of the HPJD operation positively correlates with the degree of bacterial cell destruction up to a certain level [5], excessive HPJD treatment does not necessarily improve bacterial cell destruction [16]. As a method to adjust the amount of activated sludge treated by an HPJD, the return concentrated activated sludge line in an MLE process was bifurcated into anoxic and aeration tanks, the latter of which included an HPJD (**Fig. 1a**). This study was therefore undertaken to broaden the applicability of an HPJD to an MLE process for organic carbon and nitrogen removals. An HPJD was installed into a return activated sludge line in a pilot-scale MLE process continuously receiving municipal wastewater. By also operating an MLE process without an HPJD in parallel, the reduction of excess sludge discharge, the organic carbon, nitrogen, and phosphorus concentrations were compared. The microbial community compositions and spatial distribution of specific bacteria in activated sludge flocs were investigated for the MLE processes with and without an HPJD using a combination of microscopic observation for eukaryotes, fluorescence *in situ* hybridization (FISH), and 16S rRNA gene amplicon sequencing. In particular, this research investigated the retention of ammonia-oxidizing bacteria (AOB) in an MLE process with an HPJD, and its effect on nitrogen removal performances.

MATERIALS AND METHODS

Overview of the pilot-scale system

Two pilot-scale MLE processes were assembled and operated for 111 days (from May 2017 to August 2017) at a municipal WWTP (Saitama, Japan). Each MLE process comprised an anoxic tank (0.5 m³), an aeration tank (1 m³), a sedimentation tank (0.8 m³), and a concentrated activated sludge return line connecting both tanks, as shown in **Fig. 1**. The difference between the two MLE processes lies in the presence or absence of an HPJD. In the MLE process with the HPJD (hereafter termed MLE-HPJD), the concentrated activated sludge return line was divided to join both anoxic and aeration tanks and connected to an HPJD before the aeration tank. Because an HPJD aspirates air when applied in sludge reduction [5] and eventually increases the dissolved oxygen (DO) concentration of the mixed liquor [6], the treated activated sludge cannot be simply recycled back to an anoxic tank. The volumetric ratio of returned activated sludge

Fig. 1 Schematic diagram of the pilot-scale MLE processes.

divided into both tanks was 1:1. The dimensions of the HPJD were 1 m in length, with 13 mm and 28 mm pipe diameters in the middle and at both edges, respectively. Activated sludge was transferred by a high-pressure pump (HPJ-160, Tsurumi, Osaka, Japan) via a nozzle (Φ 1 mm) set at one edge of the pipe or supplied from the top port by a magnetic pump. The volumetric ratio of the activated sludge supply from the top and lateral ports was consistently 1. Because the minimum supply of the high-pressure pump (8.4 L/min) is much larger than the required amount of concentrated activated sludge recycle (0.80 L/min), the pump was intermittently operated, turning on and off for 1 min and 20 min, respectively. The intermittent operation ensured sludge treatment of 570 L/day. The energy output of the pump was adjusted to provide an applied pressure of 5–6 MPa at the entrance of the HPJD.

Reactor operation

The wastewater in this study was drawn from a primary settling tank in the WWTP. The flow rate of the wastewater supplied to each process was consistently 4 m³/day. Hydraulic retention times (HRTs) were three hours for the anoxic tank and six hours for the aerobic tank in both sys-

tems. Excess activated sludge was withdrawn to ensure solid retention times (SRTs) of 22 days in the MLE-HPJD and 9.6 days in the MLE. These SRTs were set to attain comparable mixed liquor suspended solids (MLSS) concentration in each aeration tank. The average DO concentrations in the aeration tanks were 1.79 ± 0.54 mg/L and 1.71 ± 0.37 mg/L in MLE and MLE-HPJD ($p < 0.05$), respectively (As for the details, please see **Fig. S2**).

Chemical analyses

The influent and effluent samples were taken three times a week. The samples were filtered using a 0.45- μ m syringe filter prior to analysis. Dissolved organic carbon (DOC) and dissolved total nitrogen (DTN) were measured using a total organic carbon (TOC) analyzer (TOC5000A, Shimadzu, Kyoto, Japan). Nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) ions were measured via ion chromatography (ICS-90, Thermo Scientific, Waltham, US) equipped with columns for anion (Dionex IonPac AS12A, Thermo Scientific) and cation (Dionex IonPac CS10, Thermo Scientific) measurements. DO concentration and pH were measured using a multi-parameter water quality meter (D55,

Table 1 Oligonucleotide probes and the corresponding formamide concentrations in this study.

Probe name	Probe sequence (5'-3')	Specificity	FA (%)	Reference
EUB338	GCTGCCTCCCGTAGGAGT	Most bacteria	0–40	[43]
EUB338 II	GCAGCCACCCGTAGGTGT	Planctomycetales	0–40	[43]
EUB338 III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	0–40	[43]
NSO1225	CGCCATTGTATTACGTGTGA	Ammonia-oxidizing betaproteobacteria	35	[44]
GNSB941	AAACCACACGCTCCGCT	Phylum Chloroflexi	35	[45]
CFX1223	CCATTGTAGCGTGTGTGTMG	Phylum Chloroflexi	35	[46]
XAN818	CAACATCCAGTTCGCATC	<i>Xanthomonas</i> branch	10	[47]
COM1242	ACCTACTTCTGGCGAGA	<i>Comamonas</i> spp.	25	[48]

HORIBA, Kyoto, Japan). Activated sludge was also sampled three times a week. Sludge settleability was evaluated considering sludge volume index (SVI). MLSS and mixed liquor volatile suspended solids (MLVSS) concentrations were quantified according to the standard method [17].

Prokaryotic community structure analysis

DNA was extracted from 50 mg of activated sludge using an ISOIL Beads Beading Kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The primer set targeting the V4 hypervariable region of 16S rRNA genes for all the bacteria and archaea, 515F (5'-GTGCCAGCMGCCGCGG-3') and 806R (5'-CCGTCAATTCMTTTRAGTTT-3'), was used. The reverse primer was labeled by the 12-bp barcode [18]. The PCR condition was as follows [19]: initial denaturation at 98°C for 20 sec, 28 cycles of amplification consisting of 98°C for 10 sec, 53°C for 30 sec, 72°C for 120 sec and final extension at 72°C for 7 min. PCR amplicons were purified by an AMPure XP Kit (Beckman Coulter, Brea, US) and a Wizard SV Gel and PCR Clean-up System (Promega Corporation, Madison, US). DNA concentration and purity of amplicons were measured by Qubit (Thermo Fisher Scientific). A 300-cycle MiSeq Reagent kit (Illumina, San Diego, US) and a MiSeq sequencer (Illumina) were employed for the adjustment and sequencing of samples and initial control (bacteriophage phiX; Illumina). Subsequently low-quality ($Q < 30$) and chimeric sequences were removed, and paired-end sequences were performed [20]. Retrieved sequences were assigned to operational taxonomic units (OTU) with a 97% identity using QIIME version 1.7.0 [21]. Following this, 7,424 sequences per sample were used as a basis for the calculation of alpha-diversity (Chao1 index) by QIIME. Ten-times subsampling was performed for the calculation of alpha-diversity indices (Chao1 and Shannon indices).

Statistical analysis

One-way analysis of variance (one-way ANOVA) was used to detect a significant difference ($p < 0.05$) in extracted DNA and alpha-diversity indices (Chao1 and Shannon indices) using Microsoft Excel.

Fluorescence *in situ* hybridization (FISH)

Activated sludge samples taken from each aeration tank were fixed for three hours at 4°C by 4% (v/v) paraformaldehyde (PFA) and then washed three times with $1 \times$ phosphate-buffered saline (PBS). After ethanol dehydration in series at 50%, 80% and 98% (v/v), hybridization with oligonucleotide probes listed in **Table 1** was performed. Formamide (FA) concentrations were chosen by ProbeBase (<http://www.probebase.net> [22]). A universal probe EUBmix (EUB338, EUB338II, and EUB338III) was labeled at the 5'-end with FITC, and targeted probes were labeled at the 5'-end with Cy3. In parallel, non EUBmix was applied to examine the presence of autofluorescence and unspecific probe adhesion for all samples. The hybridized samples were covered with SlowFade Diamond Antifade Mountant (Invitrogen, CA, USA) and then observed with a fluorescence microscope (Biozero BZ-8100, Keyence, Osaka, Japan). The relative abundances of target bacteria in the total population were determined from the ratio of the area stained in yellow (red + green) to the area for all bacteria (green) hybridized with EUBmix. More than 10 images of each sludge sample were attained to determine an average relative abundance using the digital image software, daime [23].

Floc particle size distributions and Areal Cell Density of AOB

The fixed activated sludge image used for FISH analysis was also subjected to the quantification of the Feret diameter by an image software, Image J (<https://imagej.nih.gov/ij/>) [24,25]. More than 10 sludge samples were used for quan-

tification. Areal cell densities of AOB (detected by FISH probe NSO1225), as an indicator of the HPJD effect on AOB abundance and destruction, were evaluated by dividing cell numbers by each microcolony surface area [26].

Microscopic observation for identification of the eukaryotic community

A phase-contrast microscope (ECLIPSE 80i, Nikon, Japan) was used to identify Eukaryotes in activated sludge. The quantification of eukaryotic cell counts was done according to the method applied by Canals *et al.* [27]. Twenty-five microliters of an activated sludge sample were homogenized by reciprocal pipetting, followed by mounting onto each well of a glass slide and sealing each well with a 20 × 20 mm cover glass. Eukaryotes in the sludge suspension were counted under the microscope. For > 10 µm flagellates, 1 × 1 mm gridded glass slides were used to count flagellates in each grid. The observation was performed in triplicate for each sample. Abundance was estimated as individuals per milliliter (ind./mL). Eukaryotic identification was performed using morphological features according to a previous study [6].

Accession numbers

The 16S rRNA gene sequence data acquired in this study were deposited in the GenBank/EMBL/DDBJ accession number DRA008788.

RESULTS

Wastewater treatment performance

The time courses of the influent and effluent concentrations of DOC, DTN, SS, and $\text{PO}_4^{3-}\text{-P}$ are shown in **Fig. 2**. The average removal efficiencies of DOC were $64.4 \pm 17.8\%$ and $68.1 \pm 8.1\%$ in MLE-HPJD and MLE, respectively, with no significant difference ($p < 0.05$). The average removal efficiencies of DTN were $45.8 \pm 11.0\%$ and $48.6 \pm 11.1\%$ in MLE-HPJD and MLE, respectively ($p < 0.05$). A major nitrogen constituent in the effluent was nitrate (NO_3^-): effluent NO_3^- concentrations in MLE-HPJD and MLE were 9.10 ± 2.43 and 9.27 ± 2.67 mg-N/L respectively, concomitant with the comparable NH_4^+ removal efficiencies of $83.1 \pm 8.1\%$ and $80.1 \pm 8.1\%$ in MLE-HPJD and MLE, respectively ($p < 0.05$). The high NH_4^+ removal efficiencies underscore that nitrification successfully occurred in both systems. The average suspended solids (SS) concentrations in the treated water were 18.6 ± 7.1 and 22.0 ± 8.3 mg/L in MLE-HPJD and MLE, respectively, displaying a statistically insignificant difference between the two processes. The average phosphate (PO_4^{3-})

concentrations in the effluent were 0.54 ± 0.30 and 0.67 ± 0.27 mg-P/L in MLE-HPJD and MLE, respectively. There was also no significant difference observed between both processes ($p < 0.05$). Given the PO_4^{3-} balance in the influent and effluent water, neither biological PO_4^{3-} removal nor deterioration of PO_4^{3-} removal was observed. These results show that removal performances of organic carbon, nitrogen and phosphorus were comparable between the MLE-HPJD and MLE processes throughout the operation period ($p < 0.05$).

Sludge reduction performance

The change in MLSS concentration is shown in **Fig. 3a**. Despite the different SRTs, average MLSS concentrations in each aeration tank throughout the entire operation period were statistically identical ($p < 0.05$), *i.e.*, $1,943 \pm 247$ mg/L in MLE-HPJD and $1,931 \pm 318$ mg/L in MLE. MLSS concentration in the anoxic tank was 17.3% lower ($1,606 \pm 189$ mg/L) than that in the aeration tank in MLE-HPJD, whereas it was comparable in both anoxic ($1,942 \pm 320$ mg/L) and aeration tanks in MLE. The SVI of each activated sludge is shown in **Fig. 3b**. Average SVIs were comparable, 113.5 ± 15.9 and 104.6 ± 19.2 mL/g in MLE-HPJD and MLE, respectively. The time course of the cumulative amount of excess activated sludge is shown in **Fig. 4**. The difference in the cumulative amount of excess sludge between MLE-HPJD and MLE was evident immediately after the operation was commenced. After 111 days, the cumulative amount of wasted excess sludge discharged from the MLE-HPJD process was reduced by 73.7%, compared to that from the MLE process. Taking the cumulative amount of SS discharged from the treated water into account, the total amounts of waste sludge were 28,947 and 66,675 g in MLE-HPJD and MLE, respectively, showing a reduction in excess sludge of 56.6% as a result of the introduction of an HPJD (**Fig. S1**).

Microbial community structure analysis by MiSeq

As many as 1,700–2,000 OTUs were identified from 37,000–59,000 sequences of high-throughput sequencing of 16S rRNA gene amplicons. Alpha-diversity indices, *i.e.*, Chao1 and Shannon, of activated sludge in MLE-HPJD and MLE were not significantly different before and after the operation (**Table S1**). The installation of the HPJD did not affect the diversity indices.

As shown in **Fig. 5a**, the microbial community composition at the phylum level demonstrated that there was no remarkable difference between the microbial communities in the MLE-HPJD and MLE processes at the beginning of the operation. The phyla Proteobacteria (MLE-HPJD: 50.1%;

Fig. 2 Time courses of influent and effluent concentrations in the MLE-HPJD and MLE processes: (a) DOC; (b) DTN; (c) SS; (d) Phosphate; (e) Ammonium; and (f) Nitrate.

Fig. 3 Time variations of (a) MLSS concentration and (b) SVI in an aeration tank of MLE-HPJD and MLE.

Fig. 4 Cumulative amount of wasted excess activated sludge from the MLE-HPJD and MLE processes.

MLE: 44.8%) and Bacteroidetes (MLE-HPJD: 15.8%; MLE: 17.7%) were dominant in both processes. In the MLE process, the dominant phyla did not alter even at the end of the operation. On the other hand, in the MLE-HPJD process, the increases in the relative abundances of Firmicutes (4.8%) and Synergistetes (1.3%) were observed at the end of the operation. By contrast, these phyla showed low relative abundances (0.6% for Firmicutes and 0.0% for Synergistetes)

in the MLE process. As shown in **Fig. 5b**, the community structures at the class level were distinct between the two processes. The relative abundances of Saprospirae, Chloroflexi, and Chloracidobacterium on day 111 were significantly reduced to 3.0%, 0%, and 0% in the MLE-HPJD, whereas they were 13.8%, 3.2% and 2.3% in the MLE, respectively. On the contrary, Clostridia and Bacteroidia significantly increased in relative abundance from 0.3% to 3.6% and from

Fig. 5 Bacterial community compositions at the (a) phylum and (b) class levels in the MLE and MLE-HPJD processes.

Fig. 6 Top 12 OTUs detected after the 111 days-operation in the MLE and MLE-HPJD processes.

0.2% to 3.8% in the MLE-HPJD process, respectively. Meanwhile these abundances in the MLE on day 111 were 0.5% and 0.14%, respectively.

The top 12 OTUs in descending order of relative abundance and their phylogenetic classification in each process on day 111 are listed in **Fig. 6** and **Table S2**, respectively.

OTUs 1, 4, and 7 were commonly present with relative abundances higher than 1%. Irrespective of the HPJD installation, OTU 1, affiliated with the genus *Comamonas*, had highest abundance. OTUs 3, 5, 9, and 12 were found dominant only in the MLE process and OTUs 3, 7, and 10 were found only in the MLE-HPJD process.

Detection of specific bacteria by FISH

The application of nonEUB probes indicates neither auto-fluorescence nor unspecific bindings of applied probes. The installation of an HPJD did not cause dispersion and disaggregation of activated sludge flocs. Noticeable difference in floc size was not found between the MLE ($56.08 \pm 92.59 \mu\text{m}$) and MLE-HPJD ($76.02 \pm 93.31 \mu\text{m}$) processes, nor in the activated sludge morphology. FISH was performed to detect *Comamonas* spp., Xanthomonadaceae, beta-proteobacterial AOB, and Chloroflexi, which from the amplicon sequencing result were deemed abundant and important in both processes (Fig. 6). FISH micrographs and the quantification results are shown in Fig. 7 and Table 2, respectively. *Comamonas* spp. were detected with clusters (Fig. 7a and 7b), accounting for $7.02 \pm 4.29\%$ and $5.70 \pm 1.96\%$ in the MLE and MLE-HPJD processes, respectively. The oligonucleotide probe XAN 818 targeting the genus *Xanthomonas* did not confirm any specific fluorescence despite detection by the amplicon sequencing (Fig. 6). The application of GNSB941 + CFX1223 for Chloroflexi detected substantial portions of filamentous bacteria affiliated with the phylum Chloroflexi (Fig. 7c), which was predominant in the MLE with a relative abundance of $31.9 \pm 12.3\%$. The abundance was much higher than that in the MLE-HPJD process ($4.22 \pm 5.16\%$). Although the relative abundance of beta-proteobacterial AOB was as close to the detection limit by the amplicon sequencing (Fig. 6), the application of NSO1225 explicitly detected fluorescence from AOB cell clusters even in the MLE-HPJD process. The relative abundances were $7.34 \pm 2.89\%$ in the MLE and $11.8 \pm 9.78\%$ in the MLE-HPJD processes, respectively ($p < 0.05$). The detection of AOB in the MLE-HPJD process indicated that an HPJD did not destroy the AOB cells. Aerial cell densities in each AOB cluster in the MLE and MLE-HPJD processes were $0.52 \text{ cells}/\mu\text{m}^2$ (R^2 value of the correlation between cluster size and cell numbers: 0.91) and $0.53 \text{ cells}/\mu\text{m}^2$ (R^2 value: 0.86), respectively. Average AOB cluster sizes were 49.9 ± 46.2 and $39.4 \pm 48.3 \mu\text{m}$ in diameter, respectively ($p < 0.05$). Together, no significant differences in AOB density or cluster size were observed.

Transition of eukaryote community structure

There was no significant difference in the number and type of eukaryotes at the start in both processes and they both substantially increased the number of eukaryotes from day 0 to 29. Surprisingly, the MLE-HPJD process resulted in a much higher abundance of eukaryotes by a factor of 4.6 on day 111 compared to the MLE process. The dominant order was Testacida with the amount of 7.00×10^3 and 8.70×10^3

ind./mL in the MLE and MLE-HPJD processes, respectively. The order Protomastigida considerably increased in abundance in the MLE-HPJD process (3.50×10^4 ind./mL) from day 0 to 29 and was subsequently kept constant till the end of the operation. However, the trend was not identical in the MLE process: Protomastigida became dominant once but then decreased, and Euglenida and Hypotrichida became dominant in its place.

DISCUSSION

Sludge reduction by HPJD and the effect on wastewater treatment performance

This study is the first pilot-scale demonstration that a high-pressure jet device (HPJD) is a promising lysis-cryptic method to reduce excess sludge production without deteriorating organic carbon and nitrogen removals from municipal wastewater in an MLE process. To avoid a decrease in nitrogen removal performance by the oxygen intrusion to an anoxic tank, we advocated the addition of an HPJD and eventually an aeration tank to the return activated sludge line. This connection avoids oxygenation of an anoxic tank, which would potentially impair denitrification. The installation of an HPJD, with the modification of the return activated sludge line as drawn in Fig. 1, into a pilot-scale MLE process, achieved a sludge reduction of 73.7% compared to the control MLE process but keeping the comparable organic carbon and nitrogen removal performances. Excess sludge reduction is equivalent to previous research [6], where 65% sludge reduction was achieved by introducing an HPJD into a pilot-scale CAS. Importantly, an increase in the effluent SS concentration was not observed in the MLE-HPJD process, whereas there was a severe increase where an HPJD was previously installed in a CAS (CAS-HPJD) [6]. The lack of increased SS concentration indicates that the HPJD in the MLE did not undermine sludge disintegration nor a decrease in the cumulative sludge reduction percentage down to 23% [6]. Maintaining a high sludge reduction potential in the MLE-HPJD process was corroborated by the comparable effluent SS concentrations with the MLE (Fig. 2c), resulting in a reduction of 56.6% in total sludge reduction by an HPJD. What deters sludge disintegration and the resultant increase in SS concentration in the treated water is the frequency of HPJD treatment within the set SRT. The activated sludge present in the MLE-HPJD process theoretically experiences 14 times the HPJD treatment within the assigned SRT (22 days), whereas the CAS-HPJD process does a 29 times re-iteration within the SRT (10 days) [6] (Details can be found

Fig. 7 FISH micrographs of targeted bacteria over the total microbial community (green) in activated sludge in MLE (Panels a, c, and e) and MLE-HPJD (Panels b, d, and f): (a) and (b) are activated sludge samples stained with Cy3-labeled COM1424 (red) and FITC-labeled EUBmix; (c) and (d) are those stained with Cy3-labeled NSO1225 (red) and FITC-labeled EUBmix. (e) and (f) are those stained with Cy3-labeled CFX1223+GNSB941 (red) and FITC-labeled EUBmix. Each scale bar represents 10 μm .

in the Supplementary files). The higher frequency of batch HPJD treatments in the previous study likely dispersed floc

structures in the activated sludge [5], suggesting a necessity to control the sludge ratio treated by an HPJD over the total

Table 2 Relative abundances of target bacteria by specific oligonucleotide probe (N.D.: not detected).

Probe name	Abundance (%)	
	MLE	MLE-HPJD
XAN818	N. D.	N. D.
CFX1223+GNSB941	31.9 ± 12.3	4.22 ± 5.16
NSO1225	7.34 ± 2.89	11.8 ± 9.78
COM1424	7.02 ± 4.29	5.70 ± 1.96

return in activated sludge. Although the optimization of the HPJD treatment frequency is a future challenge, this study underscores the significance of adjusting the reiteration number, which potentially determines the degree of excess sludge reduction, sludge disintegration, and eventual effluent water qualities.

No significant differences in the organic carbon and nitrogen removal efficiencies were observed between the MLE-HPJD and MLE processes, revealing that the HPJD installation did not undermine performances of activated sludge. Sludge solubilization by a lysis-cryptic method results in an increase in DOC and nitrogen concentrations [4]; nevertheless, these constituents were sufficiently removed in the MLE-HPJD process. Although phosphate ions were not removed because an MLE is not designed for enhanced biological phosphorus removal (EBPR), no increase in the concentration in the treated water was observed. It has been reported that conventional lysis-cryptic methods using ozone and ultrasonic waves reduce phosphorus removal performances [28,29]. Such deterioration may also happen to EBPR with an added HPJD. The fate of phosphorus compounds is, therefore, a future challenge.

Effect of HPJD on bacterial community structures

The operation did not result in distinct Alpha-diversities of the microbial community in the activated sludge of the MLE and MLE-HPJD processes. The comparable Alpha-diversities indicate a marginal impact of the HPJD installation on the microbial diversity. On the other hand, the microbial community compositions significantly differed. The mechanism to disintegrate bacterial cells is different between Gram-negative and positive bacteria [30], the phylum of Gram-positive bacteria increasing their abundance in the MLE-HPJD system being only Firmicutes, alluding that HPJD process does not select bacterial taxa for cell decay based on cell membrane and wall structures. On the other hand, distinct microbial communities caused by the HPJD installation suggest selective microbial disruption, likely depending on cell morphologies or the form of cell structures

as previously indicated [31]. Further research on the selectivity is necessary.

Interestingly, an HPJD appears to selectively disintegrate some bacteria include filamentous bacteria. The microbial community compositions at the class level were distinct between the MLE and MLE-HPJD processes (Fig. 5b). The relative abundances of the filamentous bacteria Saprospirae [32] and Chloroflexi [33] in activated sludge decreased only in the MLE-HPJD process (Fig. 5b). Detailed taxonomic identification showed the presence of species (OTU 3) affiliated with the phylum Chloroflexi only in the MLE process (Fig. 6) and this more ubiquitous presence of the phylum Chloroflexi in the MLE process was confirmed by FISH analysis (Fig. 7e and Table 2). These molecular analyses confirm that an HPJD selectively destroys filamentous bacteria that reportedly cause sludge bulking [33,34]; therefore, this study additionally demonstrates that an HPJD is capable of suppressing filamentous bacterial growth and, eventually, avoiding sludge bulking. This newly found trait is exclusive among lysis-cryptic methods, potentially indicating the significance of an HPJD for not only excess sludge reduction but also as a selector of microbial community compositions likely based on cell morphologies.

The combination of FISH quantification and 16S rRNA gene amplicon sequencing displayed similar or different results depending on the genera involved. The abundances of the genus *Comamonas* (Figs. 6 and 7), reportedly responsible for phenol degradation and heterotrophic ammonia removal [35], were similar between the two methods. In contrast, these methods showed different abundances of the Xanthomonadaceae family: The OTU 2 (Fig. 6) belonging to the genus *Thermomonas* was found by sequencing to be dominant only in the MLE-HPJD process, whereas no positive signal was attained by FISH. This inconsistency was likely caused by the low activity of the genus or incomplete coverage of the probe by *Thermomonas*. It has been reported that *Thermomonas* consumes biomass-associated products, e.g., extracellular polymeric substances (EPS) [36]. However, the activity may be low so that rRNA contents were not

Fig. 8 Numbers of eukaryote detected in activated sludge in the MLE-HPJD and MLE processes.

likely detectable by FISH. Alternatively the applied probe (XAN818) *in silico* may not cover the uncultured *Thermomonas* detected in this study despite a broader coverage for the genus *Thermomonas* by XAN818 [646/710; RDP (<http://rdp.cme.msu.edu>)].

One paradox, *i.e.*, whether or not an HPJD destroys nitrifying bacteria, has remained. Our molecular analysis showed that the HPJD treatment under the tested conditions did not impair a nitrifying function. The short-term application of activated sludge to an HPJD underpinned marginal destruction of AOB by an HPJD [31]. Although the abundance of OTU 9 in the MLE-HPJD, belonging to the order Nitrosomonadales, was not as high as that in the MLE (Fig. 6), the FISH targeting AOB using probe NSO 1225 demonstrated the presence of AOB in both the MLE and MLE-HPJD processes (Fig. 7). It should be noted that the possibility of overestimation by autofluorescence was ruled out by applying a non-EUB probe. Given that quantitative analysis by FISH is deemed less susceptible to biases than PCR-based techniques [37], it is likely that AOB were present despite the installation of an HPJD. Moreover, the image analysis of the FISH micrograph confirmed the comparable AOB cluster size and cell density between MLE and MLE-HPJD ($p < 0.05$). Together, the ap-

plication of an HPJD to an MLE process did not affect the structure and abundance of the AOB population, resulting in the maintenance of the nitrification performance.

Effect of an HPJD on eukaryote community structures

The installation of an HPJD into an MLE and the continuous process operation allowed the formation of distinct eukaryote populations from those in a control MLE. Testacida was commonly present in both processes immediately after the startup, followed by a decrease in 29 days. It has been reported that the abundance of Testacida strongly depends on COD concentrations [38], thus the reduction in Testacida was due to the transitions of activated sludge from the wastewater treatment plant where the inoculum was originally present, to the MLE-HPJD and MLE processes. Surprisingly, Protomastigida, a small flagellate, significantly increased after 29 days only in the MLE-HPJD but not in the MLE. According to Madoni [39], small flagellates appear more in high organic content, while the metazoan population reportedly determines small flagellate abundances [38]. Furthermore, the increase in small flagellates suggests less presence of a metazoan community, which agrees with our observation (Fig. 8). In an engineering context, the emergence of

Protomastigida as an indicator of wastewater treatment is controversial: the presence represents a CAS performance [40] or does not show a clear correlation with treated water quality in a membrane bioreactor [41]. Our result supports the latter: no relation between the high abundance and water qualities was found. While treatment with an HPJD has been reported to disperse activated sludge [6], the decrease in activated sludge floc size and increase in effluent SS concentration were not confirmed. The present result indicates that sludge dispersion by HPJD treatment was likely offset by preferential predation of dispersed bacteria by eukaryotes as previously reported [42]. The high density of protozoa, e.g., small flagellates, found in the MLE-HPJD process could consume dispersed bacteria in activated sludge. Although the contribution to sludge reduction is likely not as high as an HPJD, protozoa may partially support sludge volume reduction and suppression of dispersed SS discharge in treated water. Although roles of small flagellates are still unknown, their physiologies and the interplay with treated water qualities warrant future investigation.

CONCLUSIONS

The introduction of an HPJD into an MLE process reduced the cumulative amount of excess sludge and SS in treated water by 73.7% and 56.6%, respectively. Furthermore, the continuous supply of municipal wastewater to both MLE-HPJD and MLE did not cause increases in the SS concentration, likely due to the adjustment of the HPJD treatment frequency. An appropriate frequency of HPJD treatment removed comparable amounts of organic carbon, nitrogen, and phosphate. The installation of an HPJD in an MLE process clearly changed the microbial community. Remarkably, the HPJD application allowed the suppression of filamentous bacteria including the phylum Chloroflexi. In addition, the appropriate HPJD operation maintained the structure and abundance of the AOB cluster in the activated sludge, displaying no adverse effect of an HPJD application on nitrification performance. The installation also changed the eukaryote community structure where a large number of small flagella appeared in the activated sludge. The emergence of small flagella may have synergistically contributed to sludge reduction and avoidance of SS discharge in effluent. The success of the proposed process leads to the implementation of a cost-effective and stable sludge reduction technology without impairing biological functions and activities in an MLE.

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SUPPLEMENTARY MATERIALS

Supplementary Materials file for this article is available at the link below. https://www.jstage.jst.go.jp/article/jwet/18/4/18_19-158/_supplement/_download/18_19-158_1.pdf

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