Dynamics of dewaterability and bacterial populations in activated sludge
Ning Li, Hiroyasu Satoh and Takashi Mino

ABSTRACT
Relationships of bacterial populations and extracellular polymer substances (EPS) to dewaterability of activated sludge were studied on three laboratory-scale activated sludge reactors fed with synthetic wastewater. Dewaterability of activated sludge was evaluated by a novel method developed by the authors, in which small amount of sludge was centrifugally dewatered, and its water content was measured. Bacterial populations during the reactor operation were analyzed by the polymerase chain reaction/terminal-restriction fragment length polymorphism (PCR/T-RFLP) targeted at a partial 16S rRNA gene. Extracellular polymeric substances (EPS) were extracted using cation exchange resin (CER), and polysaccharides and total protein in EPS were determined. Some of the dominant terminal-restriction fragments (T-RFs) were observed to have significant relationships with dewaterability of sludge, and it was suggested that bacterial species corresponding to those peaks significantly affected dewaterability. On the other hand, significant relationships were not found between EPS concentration and dewaterability of sludge.

Key words | bacterial populations, dynamics of dewaterability, multiple regression, PCR/T-RFLP, water content

INTRODUCTION
Dewatering is a physical unit process to reduce the volume of sludge by removing water from wet sludge. Many factors, such as extracellular polymer substances (EPS) concentration, particle size distribution, specific surface area, density, particle charge, bound water content, pH and organic concentration, have been reported to affect dewaterability of sludge (Liu & Fang 2007). In the case of dewaterability of excess activated sludge, bacterial populations in the sludge would be one of the possible factors that affect dewaterability. Yet, so far, very limited studies have been done to clarify the effects of bacterial populations on dewaterability. Those limited studies often focused on the morphological characteristics of bacteria (Jin et al. 2004). However, bacterial characteristics other than morphology might also affect dewaterability. Thus, it should be worth studying the relationship between dewaterability and bacterial populations in activated sludge.

There are different molecular biological methods available to analyze whole bacterial populations in activated sludge, such as polymerase chain reaction/denaturing gradient gel electrophoresis (PCR/DGGE) and PCR/terminal-restriction fragment length polymorphism (T-RFLP). On the other hand, the authors developed a method to evaluate dewaterability of activated sludge with less than 50 mg-dry, and demonstrated that dewaterability of a laboratory-scale activated sludge reactor dynamically changed during two months of its operation (Li et al. 2010). By combining these methods, molecular methods and dewaterability evaluation method developed by Li et al. (2010), it is possible to examine the relationship between bacterial populations and dewaterability of activated sludge.

In the present study, the authors tried to examine the correlation between bacterial populations and EPS with dewaterability of activated sludge. For this purpose, three laboratory activated sludge reactors were operated for about two months. During the operation, dewaterability was monitored by the method developed by Li et al. (2010), bacterial populations were monitored by PCR/T-RFLP method targeted at a partial 16S rRNA gene. Meanwhile, protein and polysaccharides concentrations in EPS were monitored by the extraction of EPS using cation exchange
resin (CER) followed by the determination of total protein and total polysaccharides.

**MATERIAL AND METHODOLOGY**

**Laboratory activated sludge reactors**

Three laboratory-scale activated sludge reactors, Reactor I, II and III, were operated. They were operated with six cycles per day, each cycle consisting of 1 h anaerobic phase, 2 h aerobic phase, 55 min settling and 5 min effluent discharge. A volume of 5 L of synthetic wastewater was added during the first 9 min of the anaerobic phase. The hydraulic retention times (HRT) were 8 h, and the sludge retention times (SRT) were kept at 10 days by withdrawing mixed liquor at 1 L/d. When bulking happened, bio-retention times (SRT) were kept at 10 days by withdrawing shorter. The seed activated sludge for these reactors were obtained from full-scale wastewater treatment plants treating urban wastewater. The same seed sludge was used for Reactors II and III, and that for Reactor I was obtained from full-scale wastewater treatment plants treating urban wastewater. The same seed sludge was used for Reactors II and III, and that for Reactor I was obtained from another wastewater treatment plant. The components of synthetic wastewater are as follows: Reactors I and II—CH$_3$COONa·3H$_2$O (90 mg/L), CH$_3$CH$_2$COONa (43 mg/L), peptone (80 mg/L), yeast extract (16 mg/L), KCl (34 mg/L), CaCl$_2$·2H$_2$O (11 mg/L), MgSO$_4$·7H$_2$O (88 mg/L), KH$_2$PO$_4$ (29 mg/L); Reactor III—CH$_3$COONa·3H$_2$O (221 mg/L), CH$_3$CH$_2$COONa (78 mg/L), peptone (8.3 mg/L), yeast extract (16 mg/L), KCl (34 mg/L), MgSO$_4$·7H$_2$O (11 mg/L), KH$_2$PO$_4$ (42.5 mg/L), NH$_4$Cl (45 mg/L).

**Physical parameters**

During the operation of the activated sludge reactors, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), and sludge volume index (SVI) were monitored according to *Standard Methods for the Examination of Water and Wastewater* (APHA 2005).

**Dewaterability measurement**

Dewaterability of activated sludge was measured as follows. In each determination, 25 mL activated sludge mixed liquor was centrifuged at 3,500 rpm for 5 min, supernatant was decanted, and around 300 mg-wet of the settled sludge pellet was loaded on a piece of membrane filter (cellulose mixed ester, Millipore, 0.45 μm, 25 mm). The filter was placed in a Swinnex filter holder (25 mm, Millipore), and the filter holder was set in a bucket for 50 mL conical tubes (No.053-5010, Kubota). Then, centrifugal dewatering was applied by a Kubota 2800 centrifuge at 2,000 g (3,500 rpm) for 5 min. About 100 mg of the dewatered sludge was transferred onto a piece of aluminium foil (for which dry weight (A) had been predetermined), and the dewatered sludge weight including aluminium foil (B) was measured. In measuring the dewatered sludge weight, the foil was folded to cover the sludge to avoid drying during weight measurement. The sample was unwrapped, dried at 105 °C for 60 min, cooled to room temperature in a desiccator for 30 min, and the weight after drying (C) was measured. Water content of the dewatered sludge (WCDS) was calculated by $(B-C)/(B-A)$ as an indicator of dewaterability. Measurement of weight was done with an analytical balance XS105 (Mettler Toledo, USA) with a resolution of 0.01 mg. Analyses were done in quadruplicate (Li et al. 2010).

**Microbial analysis**

The PCR/T-RFLP analysis was done based on the method by Liu et al. (1997). From the activated sludge mixture, DNA was extracted by the sonication-dilution method that was developed by Satoh et al. (2008). In this method, strong sonication is applied to destroy bacteria cells in activated sludge, the sonicated sample is diluted, and used as template for polymerase chain reaction (PCR) without purification. A BRANSON Digital Sonifier-250DA with a 1/2 inch horn was used for sonication at amplitude of 100% for 20 sec to 30 mL of activated sludge mixed liquor, and the samples were diluted so that DNA concentration in PCR mixture is in a range between 1 and 10 pg/μL. Partial 16S rRNA was amplified using 27f forward primer (5’-AGAGTTTGGTCG (A/C)-TGGCTCAG-3’) labeled with the fluorophore FAM on the 5’ end and 519r reverse primer (5’-G(A/T)ATTACCGCGGCTG(T)GCTG-3’) (Lane 1991). AmpliTaq Gold (Applied Biosystems, USA) was used as the Taq polymerase. The thermal cycle was as follows: 95 °C for 10 min followed by 30 cycles of denaturing (94 °C for 30 sec), annealing (55.3 °C for 30 sec) and extension (72 °C for 30 sec), followed by a final extension at 72 °C for 10 min. The PCR products were purified using the QIA-Quick PCR Purification Kit (QIAGEN, USA), and digested with RsaI restriction enzyme (Biolab, USA). The digested PCR products were denatured, and analyzed by ABI 310 capillary sequencer (Applied Biosystems).
In T-RFLP analyses, bacterial population structures are profiled as peak patterns in the electropherograms. The same species are thought to give peaks at the same position because PCR products originated from them basically have restriction sites at the same positions. T-RF ± 1 bp was considered as one species in this experiment (Jin et al. 2010). Peak heights were standardized based on the sum of peak heights for the sample, and the relative peak heights were used as the indicator of the abundance of bacterial group corresponding to the peak. Multiple regression analyses between relative peak heights and WCDS values were done using statistical package R (R Development Core Team 2011).

Analysis of EPS

To extract EPS, CER AG 50W-X8 (BIO-RAD, USA) was used. First, 2 mL activated sludge mixed liquor was centrifuged for 10 min at 12,000 g. Then, supernatant was decanted, sludge pellet was resuspended in 2 mL PBS buffer, CER was added at a ratio of 60 g of CER to 1 g of MLVSS, and shaken for 3 h in an incubator at 250 rpm, 5°C (Park et al. 2008). Protein concentrations in the extracts were determined using DC Protein Assay (BIO-RAD), and polysaccharides concentrations were determined by the anthrone method (Koehler 1952).

RESULTS AND DISCUSSION

Performance of the activated sludge reactors

The performances of the three reactors monitored are shown in Figure 1. For Reactors I and II, after about one month of operation, filamentous microorganisms proliferated and caused bulking, and SVI increased when MLSS and MLVSS reduced. Reactor III was operated without bulking.

Dynamics of dewaterability

As an indicator of dewaterability, WCDS was monitored. The results are as shown in Figure 2. The higher the WCDS value, the worse the dewaterability. The WCDS values ranged from 83.5% (Reactor III) to 94.0% (Reactor I), and the values fluctuated dynamically during the operation of each reactor (Figure 2). Li et al. (2010) reported that WCDS measurement...
has standard deviation (SD) of about 0.5% in quadruplicate. In the present study, to more clearly grasp the behavior of dewaterability, WCDS values were analyzed in quadruplicate.

Jin et al. (2004) reported that sludge containing higher amounts of bound water showed higher SVI, reflecting poorer sludge compressibility. However, in this experiment, the monitored fluctuation of WCDS was not negatively related with SVI shown in Figure 1.

Microbial analyses

The results of the microbial population analyses by the PCR/T-RFLP method are shown in Figure 3. In each reactor, dominant species changed during the operation. The authors selected major T-RFs by the following criteria. For each sample, cumulative relative peak heights were calculated until the value exceeded 50% of the total relative peak height in a descending order of relative peak heights. Those T-RFs that were used at least once in this calculation for the reactor were regarded as major T-RFs. Minor T-RFs were combined into ‘others’ in this study.

Selected major T-RFs by the above criteria were: 91, 101, 124, 414, 451, 468, 513 and 518 bp in Reactor I (Figure 3(a)), 78, 116, 465, 468, 479 and 513 bp in Reactor II (Figure 3(b)), and 116, 119, 468 and 481 bp in Reactor III (Figure 3(c)).

**Figure 3** | Relative height analysis of T-RFLP profiling.
The cause of the change of microbial population is not yet known. There have been reports on the change of microbial populations in activated sludge reactors operated under the same conditions (Okunuki et al. 2004; Satoh et al. 2007; Zengin et al. 2010). Bacteriophages may be one of the causes of bacterial population changes (Otawa et al. 2010; Barr et al. 2010).

Relationships between bacterial populations and dewaterability

The relationship between the bacterial populations and dewaterability was studied by multiple regression analysis between the selected major T-RFs and WCDS values for each reactor. As a result, following results were obtained:

Reactor I:

\[
WCDS_{\text{est}} = 0.074RH_{101}^* - 0.080RH_{414}^* - 0.135RH_{451}^* - 0.032RH_{518}^* + 0.916
\]

Reactor II:

\[
WCDS_{\text{est}} = 0.069RH_{116}^* - 0.047RH_{465}^* - 0.059RH_{468}^* - 0.148RH_{479}^* + 0.025RH_{513}^* + 0.862
\]

Reactor III:

\[
WCDS_{\text{est}} = 0.164RH_{116}^* - 0.135RH_{119}^* - 0.091RH_{481}^* + 0.868
\]

where \( WCDS_{\text{est}} \) is for estimated WCDS value based on the multiple regression, and \( RH_X^* \) is for relative peak height of the T-RF with a size of \( X \) bp. The superscript P for RH indicates the level of significance for the correlation (\(*\) for 90%, ** for 95%, *** for 99%, and **** for 99.9%).

The observed and estimated WCDS values had coefficient of determination (\( R^2 \)) values of 0.77, 0.71, and 0.71, respectively. The relative heights of T-RFs had strong correlations with WCDS values. This also means that bacterial populations corresponding to these T-RFs had positive or negative correlations to dewaterability.

Those T-RFs that have positive coefficients in Equations (1)–(3) are thought to be related to bacterial populations that were more when dewaterability became poor. Such T-RFs are: 101 bp for Reactor I, 116, 468 and 513 bp for Reactor II, and 116 bp for Reactor III. On the other hand, those which have negative coefficients in Equations (1)–(3) are thought to be related to bacterial populations that were more when dewaterability was better. Such T-RFs are: 414, 451, and 479 bp for Reactor I, 465 and 479 bp for Reactor II, and 119 and 481 bp for Reactor III.

Except for T-RF sized with 116 bp, no common T-RFs were found to be shared in Equations (1)–(3). This most probably means that different kinds of microorganisms can affect dewaterability differently. Indeed, seed sludge for Reactor I was different from that for Reactors II and III, and the feed compositions were different for Reactors II and III. It is not surprising even if bacterial populations that affected dewaterability in these reactors were different. In order to more precisely interpret the outcomes, bacterial species corresponding to these T-RFs will have to be identified.

Correlation between EPS and dewaterability

As shown in Figure 5, protein, polysaccharide, and their sum were determined during the operation of the three reactors, and their correlations to WCDS values were examined. As can be seen, none of protein, polysaccharide, and their sum had significant correlations with WCDS (\( R^2 \leq 0.1 \)). One of the ways to interpret the result is that EPS amount does not affect dewaterability.

On the other hand, not a small number of researchers are suggesting that EPS affect dewaterability (Eriksson &
Hardin 1984, Pere et al. 1993, Liu & Fang 2005), and further, Yu et al. (2008) reported that protein amounts in EPS had significant correlation with CST values. It is also possible that the present results with low correlation were obtained even though EPS affects dewaterability. The effect of EPS on dewaterability may have been hidden by the relative standard deviation (RSD) of EPS, which were about 5% for protein and 15% for polysaccharide. Yet, in comparison to the effect of microbial population, the effect of EPS on dewaterability was smaller in the activated sludge examined here.

CONCLUSION

At the current state of knowledge, authors can only speculate that bacterial species related to the suggested T-RFs might govern dewaterability. Bacterial species that are related to T-RFs 101 (Reactor I), 116, 468, 513 (Reactor II), and 116 bp (Reactor III) were suggested to negatively affect dewaterability, and those related to T-RFs 414, 451, 518 (Reactor I), 465, 479 (Reactor II), 119 and 481 bp (Reactor III) may be relevant to the improvement of dewaterability. Meanwhile, extracted EPS (neither protein nor polysaccharide) did not show their correlation with dewaterability. These results of relationships to dewaterability might provide the basis for a new viewpoint on the specific role of bacterial species to dewaterability.

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