Bacterial communities in different locations, seasons and segments of a dairy wastewater treatment system consisting of six segments

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ABSTRACT

A dairy wastewater treatment system composed of the 1st segment (no aeration) equipped with a facility for the destruction of milk fat particles, four successive aerobic treatment segments with activated sludge and a final sludge settlement segment was developed. The activated sludge is circulated through the six segments by settling sediments (activated sludge) in the 6th segment and sending the sediments beck to the 1st and 2nd segments. Microbiota was examined using samples from the non-aerated 1st and aerated 2nd segments obtained from two farms using the same system in summer or winter. Principal component analysis showed that the change in microbiota from the 1st to 2nd segments concomitant with effective wastewater treatment is affected by the concentrations of activated sludge and organic matter (biological oxygen demand [BOD]), and dissolved oxygen (DO) content. Microbiota from five segments (1st and four successive aerobic segments) in one location was also examined. Although the activated sludge is circulating throughout all the segments, microbiota fluctuation was observed. The observed successive changes in microbiota reflected the changes in the concentrations of organic matter and other physicochemical conditions (such as DO), suggesting that the microbiota is flexibly changeable depending on the environmental condition in the segments. The genera *Dechloromonas*, *Zoogloea* and *Leptothrix* are frequently observed in this wastewater treatment system throughout the analyses of microbiota in this study.

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Introduction

Dairy wastewater is highly polluted in terms of its scale as well as concentration of organic matter. Actually, the amounts of dairy wastewater are 35,000 and 67,000 m^3/day in Hokkaido, Japan and in all of Japan, respectively. It contains high amounts of proteins, fatty substances and lactose (Tocchi et al., 2012). Dairy wastewater exhibits a biochemical oxygen demand (BOD)

of approximately 2000–5000 mg/L. Considering that the BOD of ordinary sewage is approximately 200 mg/L, which of dairy wastewater leads to a high-load operation of a wastewater treatment system owing to the concentrations of organic matter. Therefore, if dairy wastewater were dumped into a river or lake, it would be a heavy burden to the natural environment. Because of the amount of dairy wastewater and its high density of organic matter, it should be properly treated by appropriate methods.
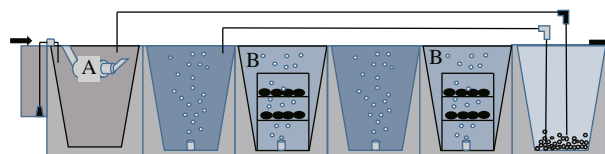
Among the organic matter contained in dairy wastewater, milk fat is difficult to degrade owing to its water insolubility, and fatty substances are fundamentally difficult to degrade by microorganisms in a short time. Furthermore, milk fat particles are too large to be easily degraded by microorganisms. In a wastewater treatment system, owing to the milk fat on the surface of the wastewater, vigorous aeration is difficult for the degradation of organic compounds by aerobic microorganisms. Vigorous aeration produces large amounts of foam and scum, which cover the surface of the wastewater. Moreover, dairy wastewater contains various chemicals for cleaning (Kosseva et al., 2003) such as detergent and hormones (Cai et al., 2012, 2013). In addition, it sometimes contains milk that cannot be shipped owing to the remaining high concentration of antibiotics. For the reasons described above, it is difficult to degrade organic compounds using an ordinary wastewater treatment system. Therefore, the establishment of a new and effective treatment system for dairy wastewater is desired.

A dairy wastewater treatment system composed of six separate activated sludge water treatment segments equipped with a facility for the physical destruction of milk fat particles was developed. The main segments of the dairy wastewater treatment system are one segment equipped with a facility for the destruction of milk fat particles, four activated sludge treatment segments, and the last segment for activated sludge settlement. This system has already run in several dairy farms in Hokkaido, Japan. It can reduce the BOD of the original wastewater from 4600 to 4.4 mg/L. Concentrations of nitrogen and phosphate are decreased from 340 and 80 mg/L to 9.8 and 22 mg/L, respectively. Although the system has proven its efficiency for dairy wastewater in different locations and seasons, there is no information available on whether the microbiota is changed depending on the location and season. In addition, although BOD is sequentially decreased from the 1st to the 5th segments, the difference in microbiota in the six separate segments is unknown. Microbiota was examined to ascertain the efficiency of the dairy wastewater treatment system placed at different locations and in different seasons and segments of the total system by a culture-independent method.

1. Materials and methods

1.1. Configuration and operation of the wastewater treatment system

The total scheme of this wastewater treatment system is shown in Fig. 1. This system consists of six separate segments of 10 m³ volume each. These segments are connected in line in the order of treatment step (from the 1st to the 6th). The total

Fig. 1 – Total scheme of wastewater treatment system consisting of 6 segments of 10 m³ volume each. Each segment is connected following the sequence of treatment steps from the 1st to the 6th segments. The particle size of the milk fat that accumulated on the surface of the 1st segment of the wastewater treatment system was reduced by aspiration in the facility and by beating against a ceramic balls with a surface having a sharp corrugation (at the 1st segment, indicated as "A"). Wastewater is treated with activated sludge by vigorous aeration from the 2nd to the 5th segments. Among them, the 2nd and 4th segments are for ordinary aeration with the activated sludge. Activated coal, the surfaces of which possesses pores for carrying bacteria, is equipped at the 3rd and 5th segments (indicated as "B"). The activated sludge is allowed to settle in the 6th segment. The sludge is occasionally fed back to the 1st and 2nd segments for recycling.

volume is 60 m³, consisting of 50 m³ for the wastewater treatment segment and the last 10 m³ for the sludge settlement. The flow volume is 8–9 m³/day. Therefore, the retention period of wastewater in the system is approximately 7 days. The sediment in the 6th segment is sent back to the 1st and 2nd segments. Wastes from cattle shed include wasted milk, water used for washing the floor and detergents. A facility for the reduction of the size of milk fat particles is attached to the 1st segment of the system. The milk fat that accumulated on the surface of the 1st segment of the wastewater treatment system was aspirated using a pump into a tube and the particle size was physically broken down by strongly stroking on the sharp corrugation on the surface of the ceramic balls that filled the tube. By this treatment, the size of the milk fat particles is reduced from 1.90 to 0.82 μm on average. In the 1st segment, no aeration is performed. Wastewater is treated with activated sludge by vigorous aeration from the 2nd to the 5th segments. A carrier consisting of activated coal, the surface of which possesses pores for carrying bacteria, is equipped at the 3rd and 5th segments. The activated coal has bacterial cells in the pores on the surface. The activated sludge is allowed to settle in the 6th segment and the supernatant is made to flow outside as treated water. The settled sludge is sent back to the 1st and 2nd segments for recycling. One of the typical stepwise BOD reductions in this system is as follows: 2800, 1200, 660, 80 and 60 mg/L in the 1st, 2nd, 3rd, 4th and 6th segments, respectively. On the other hand, the stepwise nitrogen removal is as follows: 170, 160, 130, 23 and 23 mg/L in the 1st, 2nd, 3rd, 4th and 6th segments and the phosphate degradation is as follows: 49, 51, 46, 22 and 22 mg/L in the 1st, 2nd, 3rd, 4th and 6th segments, respectively. These values are changeable depending on the wastewater characteristics such as the concentration of organic matter, the amount of activated sludge and temperature.

1.2. Water quality measurement

The pH and DO in the wastewater treatment system were determined using a pH and DO meter (Horiba D55 pH, DO, ORP meter). The transparency of the wastewater was determined using a transparency meter. It is expressed as the height of water from a mark on the bottom of the tube, which is barely visible when the wastewater is added into the tube. SV_{30}, which indicates the volume of sludge that settled in 30 min, was estimated by settling a 1000 mL sample of mixed liquor from the wastewater treatment system for 30 min in a 1000-mL graduated cylinder. It is expressed as SV_{30} = volume of sludge that settled in 30 min·sample volume^{-1}. The amount of suspended solids (SS) was measured by weighing the residues obtained by filtering the mixed liquor with a 1-μm-pore-size glass fiber filter. The concentration of suspended solids in a 1000 mL sample of mixed liquor is denoted as mixed liquor suspended solids (MLSS). BOD after 28 days was determined from the amount of oxygen consumed. The oxygen consumption was measured using a Coulometer OM3100 (Okhura Electronic Co., Japan), which is a BOD meter in a closed system.

1.3. microbial composition analysis

Total DNA was extracted from a sludge sample, which was obtained from a water sample centrifuged at 15,000 g for 10 min, using ISOIL (Nippon Gene) following the manufacturer's instruction. To construct a 16S rRNA gene library from DNA extracted from water from a dairy wastewater treatment system, PCR was performed with the universal primer sets 9F (5′-GAGTTTGATCCTGGATCAG-3′) and 1514R (5′-AAGGAGGTGATCCAGCC-3′). PCR was performed in a 100 μL solution containing 10 μL of 10× sequence buffer, 8 μL of 2.5 mM dNTP mix, 100 ng of isolated DNA, 5 U of Ex Taq DNA polymerase (Takara) and 20 pmol of each primer. The reaction mixture was subjected to PCR under the following conditions: 94°C for 1 min followed by 30 cycles at 94°C for 30 sec, 56°C for 30 sec and 72°C for 1.5 min.

The PCR products were purified with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instruction. Thus, purified PCR products of 16S rRNA genes were cloned in *Escherichia coli* DH5α with the pT7Blue-2 Vector system (Novagen) according to the manufacturer's instruction. Approximately 20–25 randomly selected clones were checked for the correct insert size by vector-targeted PCR and gel electrophoresis for the PCR product. The approximately 500–600 bp partial sequences from the amplified full sequence of approximately 1500 bp were determined as described below.

The DNA sequence was determined by the dideoxy chain termination method with a BigDye terminator cycle sequence kit (Applied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). The assignment of determined sequences was performed by searching for matching sequences using BLAST. The clone sequences that exhibited higher than 95% and 90% similarities to that in the database were identified at the genus and family levels, respectively. The clone sequences that exhibited lower than 90% similarity were identified at the phylum level.

1.4. Phylogenetic analysis

Phylogenetic analysis was performed using the determined 16S rRNA gene sequence and corresponding phylogenetic neighbor gene sequences in the gene database. The sequences were aligned and the consensus sequence was determined using the program CLUSTAL W (Thompson et al., 1994). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) in MEGA 5 (Tamura et al., 2011). For the neighbor-joining method, the distance between sequences (K_{unc} value) was calculated using Kimura's two-parameter model (Kimura, 1980). The similarity between sequences was calculated using the GENETYX computer program (Genetyx, Tokyo, Japan).

1.5. OTU and rarefaction curve processing

Operational taxonomic units (OTUs) and rarefaction curves were defined at an average intra-OTU sequence identity of 97% on the Mothur platform (Schloss and Westcott, 2011). The SILVA bacterial database provided by Mothur (available at http://www.mothur.org/wiki/Silva_reference_files) was used for the template of aligning the clone sequences.

1.6. Comparison of clone libraries

The relationship between microbiota determined using dairy wastewater based on the bacterial 16S rRNA gene libraries was assessed using the phylogenetic-based metrics, UniFrac PCA (principal component analysis) (Lozupone and Knight, 2005). A phylogenetic tree file subjected to the UniFrac program was constructed by the neighbor-joining method using MOTHUR (Schloss and Westcott, 2011).

The sequence reported in this study was deposited in the DDBJ database under DDBJ/EMBL/GenBank accession numbers AB936317–AB936430 for different locations (two different locations), seasons (summer and winter) and segments (1st and 2nd segments) and AB936648–AB936744 for five segments (1st anaerobic and four successive aerobic segments) in one location.

2. Results and discussion

2.1. Analysis of microbiota in different locations in summer

Analysis of microbiota was performed in the samples obtained from two different locations, O and S Farms, using the same dairy wastewater treatment system composed of one segment equipped with the facility for the destruction of milk fat particles and four activated sludge treatment segments. Physicochemical wastewater characteristics are listed in Table 1. The water temperatures in the two farms were within 26.7–28.7°C (Table 1). The examined microbiota in S Farm in summer and winter and O Farms in summer are shown in Fig. 2. The list of the bacterial species that exhibit the highest similarity with the obtained clone from wastewater in the database of EMBL is shown in Supplementary Table S1.

The major group in the 1st segment in S Farm was *Coriobacteriia* (*Olsenella*-like bacteria) (Fig. 2 [SS1]; Table S1).

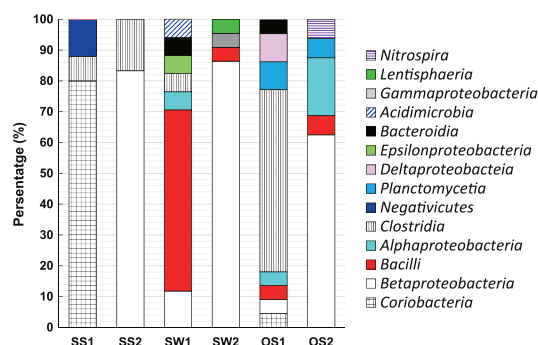| Table 1 – Physicochemical characteristics of segments in dairy wastewater treatment system at different sites and seasons.							
Location, season	Temperature (°C)	pH	BOD (mg/L)	SS (mg/L)	MLSS (mg/L)	DO (mg/L)	SV_{30} (%)
S farm, Summer-1							
1st segment	26.7	5.4	2,480	820	ND	0.06	ND
2nd segment	28.6	7.5	610	ND	3,060	0.92	50%
O farm, Summer							
1st segment	26.6	5.3	2,930	1430	ND	0.03	ND
2nd segment	28.8	7.4	1,610	ND	10,300	0.07	97%
S farm, Winter							
1st segment	14.3	6.4	410	330	ND	0.12	ND
2nd segment	14.3	7.5	15	ND	3,980	0.14	80%
[a]ND: no data.							

The genus *Olsenella* is known to be anaerobic bacteria (Dewhirst and Wade, 2012) (Fig. 2). This indicated that anaerobic microorganisms predominantly exist in the activated sludge in accordance with the physicochemical condition in the 1st segment. The major group in the 2nd segment was *Betaproteobacteria* (*Zoogloea* spp.) (Fig. 2 [SS2]; Table S1), which is found in organically polluted fresh water and wastewater at all stages of treatment. The measured BOD (2480 mg/L) in the 1st segment was effectively decreased in the 2nd segment (610 mg/L; Table 1). It was found that the concentration of the activated sludge was appropriate according to the values of MLSS (3060 mg/L) and SV30 (50%). These results indicated that the facility appropriately worked.
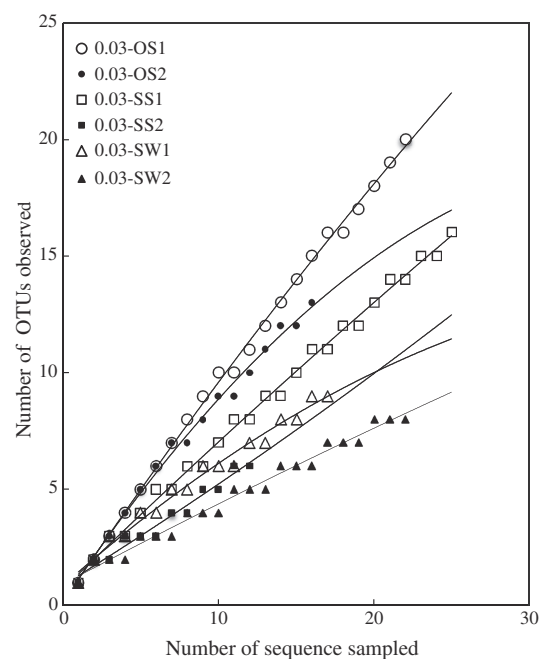
The major genus in the 1st segment in O Farm was the anaerobic *Clostridia* (Fig. 2 [OS1]). This also indicated that the condition of the 1st segment of the facility was anaerobic. On the other hand, the major order in the 2nd segment was *Betaproteobacteria* (Fig. 2 [OS2]), which mainly consisted of *Dechloromonas* spp. and *Leptothrix* spp. (Table S1). *Dechloromonas* spp. are reported as facultative anaerobic bacteria (Staley et al., 2005). On the other hand, *Leptothrix* spp. has been found in

freshwater environments rich in soluble iron and manganese compounds (Spring and Kämpfer, 2005). The reduction rate of BOD in O Farm (45%) was not as high as that in the S Farm summer sample (75%) (Table 1). It is probably due to the inappropriate DO that caused the inappropriate amount of activated sludge.

Rarefaction analysis indicated that microbiota in O Farm samples were more diverse than those in S Farm samples (Fig. 3). This diversity may be attributed to the large concentration of activated sludge. PCA revealed that the bacterial communities of samples from different locations are distinct from one another (Fig. 4). The locational difference in the sewage treatment plant was reported before (Zang et al., 2012). It is considered that the difference is attributed to the operational difference. In the case of the two farms, there were differences in the efficacy of the treatment of dairy fat in
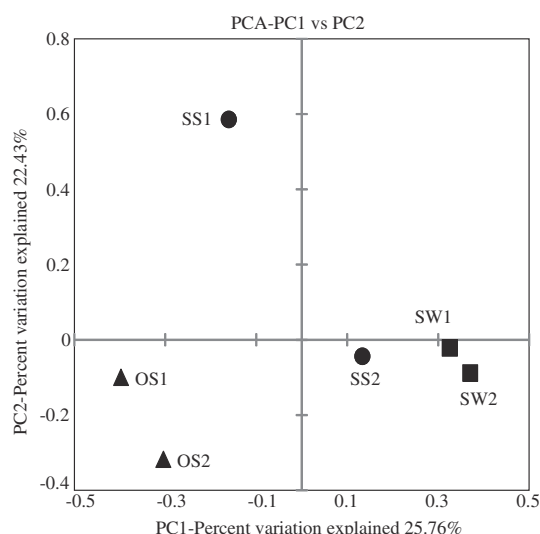

Fig. 2 – Comparison of taxonomic variation in the 1st and 2nd segments obtained from two different locations in summer and winter in the dairy wastewater treatment system. Relative abundances of orders observed in each sample are shown. SS1: S Farm 1st segment summer sample; SS2: S Farm 2nd segment summer sample; SW1: S Farm 1st segment winter sample; SW2: S Farm 2nd segment winter sample; OS1: O Farm 1st segment summer sample; OS2: O Farm 2nd segment summer sample.

Fig. 3 – Rarefaction curve of OTUs defined by 3% sequence variation in samples from 1st and 2nd segments obtained from two different locations in summer and winter in the dairy wastewater treatment system.

Fig. 4 – Communities clustered using Unifrac PCA for bacterial community of dairy wastewater treatment system. Samples were obtained from the 1st and 2nd segments at two different locations in summer and winter. SS1: S Farm 1st segment summer sample; SS2: S Farm 2nd segment summer sample; OS1: O Farm 1st segment summer sample; OS2: O Farm 2nd segment summer sample; SW1: S Farm 1st segment winter sample; SW2: S Farm 2nd segment winter sample. PCA: principal component analysis.

the 1st segment (a large amount of dairy fat accumulated) and management for the adjustment of the amount of activated sludge. Microbiota between the 1st and 2nd segments in O Farm in summer and those between the 1st and 2nd segments in S Farm in winter exhibited similarity in PCA (Fig. 4). The similarity in OS1 and OS2 can be attributed to the low oxygen supply (low DO) owing to the excessive amount of activated sludge (MLSS) and organic matter (BOD) in the facility of O Farm. However, the 1st and 2nd segments at S Farm in summer were distinct from each other. This large difference in microbiota between the 1st and 2nd segments in S Farm in summer may reflect the appropriate DO owing to the appropriate concentrations of organic matter and activate sludge. This reflected the effective reduction of BOD between the 1st and 2nd segments.
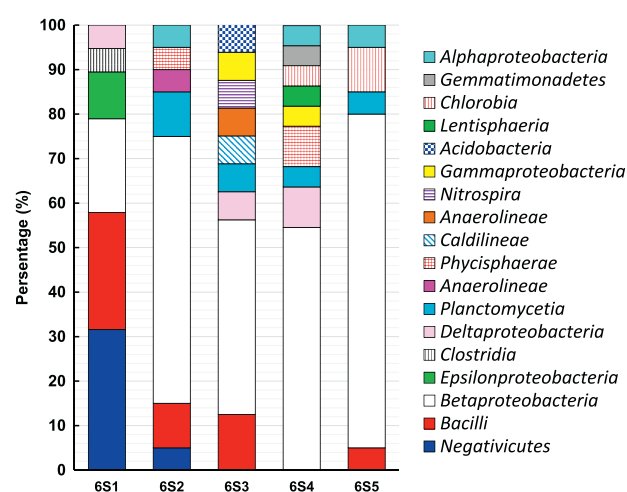
2.2. Microbiota of the wastewater treatment system in winter

It is considered that the microbiota of a wastewater treatment system changes depending on the changes in seasonal temperature. The microbiota of a wastewater treatment system in winter was examined in a sample obtained from S Farm (Fig. 2 [SW1, SW2]; Table 1). The water temperature was 14.3°C (Table 1). The most predominant group in the 1st segment was *Lactococcus*, which belongs to *Bacilli* (Fig. 2 [SW1]; Table 1). The most predominant group in the 2nd segment was *Betaproteobacteria* (*Zoogloea* spp.) (Fig. 2 [SW2]; Table 1). This result was similar to that obtained in summer at the same farm (Fig. 2 [SS2]). Phylogenetic analysis was performed on the *Zoogloea* species observed in summer and winter (Fig. S1). More diverse *Zoogloea* species were observed in

winter than in summer. The differences in *Zoogloea* species between summer and winter samples may be attributed to the difference in temperature between summer and winter. The measured BOD (410 mg/L) in the 1st segment was effectively decreased in the 2nd segment (15 mg/L; Table 1). Although we do not know the reasons, the BOD in the 1st segment was much lower than that in summer. Rarefaction analysis indicated that winter samples (SW1 and SW2) were less diverse than summer samples in S Farm (Fig. 3). There is a possibility that the temperature affects the microbial diversity of activated sludge. PCA revealed that although the bacterial communities of the 1st and 2nd segments are similar to each other, they are different from those of other samples obtained from O Farm and the summer samples at this farm (Fig. 4). The similarity in SW1 and SW2 can be attributed to the low concentration of organic matter (low BOD) and low DO in the 2nd segment, and the low microbial diversity of the sample due to the season (low temperature).

2.3. Analysis of microbiota in five separate segments

Although it is considered that the aerated 2nd to 5th segments have similar microbiota, there are some environmental differences, such as the concentrations of organic matter, nitrogen and phosphate. The microbiota of the total wastewater treatment system, anaerobic 1st segment and aerated 2nd to 5th segments was estimated (Fig. 5). The list of the bacterial species that exhibit the highest similarity with the obtained clone from wastewater in the database of EMBL is shown in Table S2. Although we did not estimate the BOD in each treatment step, it was considered that the system was in relatively good condition for the water treatment, as shown by the appropriate DO in the aerated segments (Table 2) and the transparency of the final treated water (30 cm). However, the amount of the activated sludge was relatively high considering
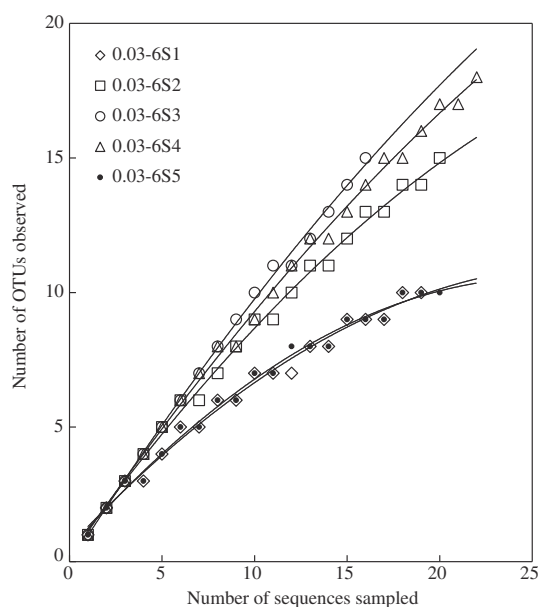

Fig. 5 – Comparison of taxonomic variations in the 5 segments (1st and 4 successive aerobic segments) at S Farm in summer. Relative abundances of orders observed in each sample are shown. 6S1: 1st segment; 6S2: 2nd segment; 6S3: 3rd segment; 6S4: 4th segment; 6S5: 5th segment.

Table 2 – Physicochemical characteristics of segments in dairy wastewater treatment system in S-Farm of summer sample at five different segments.

S farm, summer-2	Temperature (°C)	pH	DO (mg/)	SV_{30} (%)
1st segment	23.4	6.8		
2nd segment	23.9	7.4	2.07	93%
3th segment	23.8	7.6	2.52	94%
4th segment	23.9	7.5	ND^a	ND
5th segment	23.6	7.3	1.16	87%

[a] No data.

the SV_{30}. The major phylum in the microbiota of the 1st segment from S Farm was Gram-positive bacteria belonging to *Negativicutes* or *Bacilli*, whereas that of the 2nd segment was *Betaproteobacteria* (Fig. 5). In the 1st segment, *Veillonella* and *Lactococcus* were detected as the major genera, whereas in the downstream segments, *Dechloromonas*, *Leptothrix* and *Zoogloea* were predominantly detected (Table S2). Phylogenetic analysis was performed on the *Dechloromonas* species observed from the 1st to the 5th segments (Fig. S2). There were similar species observed from the 2nd to the 5th segments, while the same species were observed in specific segments. In addition, phylogenetic analysis of the *Leptothrix* species observed from the 2nd to the 5th segments was also performed (Fig. S3). In this genus also, there were similar species observed from the 2nd to the 5th segments. These results suggested that there is a similarity between the pre-maximally activated state (2nd segment) and the meta-maximally activated state (5th segment) depending on the efficiency of oxygen transfer and the concentration of organic matter. Rarefaction analysis indicated that a higher diversity of microbiota was observed from the 3rd and 4th segments (Fig. 6). It is considered that the difference can

be attributed to the organic matter (BOD) and DO. In other words, the highest activity was attained at the 3rd and 4th segments in the sludge in this facility at this sampling period. UniFrac PCA also revealed the independence of each segment (Fig. 7). It is considered that the microbiota is changeable depending on the physicochemical environment, such as the concentration of organic matter and DO and the period for the activation of the sludge. This characteristic may be effective for appropriate wastewater treatment under each corresponding condition in this wastewater treatment system.

Overall analyses of microbiota suggested that the appropriate amount of activated sludge will lead to the appropriate DO in the aeration treatment. It has also been suggested that the marked change in microbiota between the segments is related to the good performance and effective oxygen transfer in the facility. It has been reported that DO is very important for wastewater treatment by activated sludge, not only for its performance (Melo et al., 2005) but also for the shift in microbial community (Yadav et al., 2014). It is also suggested that larger amounts of activated sludge will lead to a higher diversity of microbiota. However, the high diversity is not always related to the high performance of wastewater treatment.

In previous studies, *Pseudomonas*, *Clostridium*, *Turicibacter*, *Eubacterium* (McGarvey et al., 2005, 2007), *Roseobacter*, *Pirellula*, *Sedimentibacter* and *Trichococcus* (McGarvey et al., 2007) were observed in other dairy wastewater treatment systems. The results of this study indicated that *Dechloromonas*, *Zoogloea* and *Leptothrix* are frequently observed in this system regardless of the location, season and segment. These genera were observed in the activated sludge in wastewater in previous reports (Shao et al., 2009; Ebrahimi et al., 2010; Wang et al., 2012; Weissbrodt et al., 2012; Li et al., 2014). This suggests that the wastewater treatment system exhibits similarity to ordinary wastewater treatment systems.
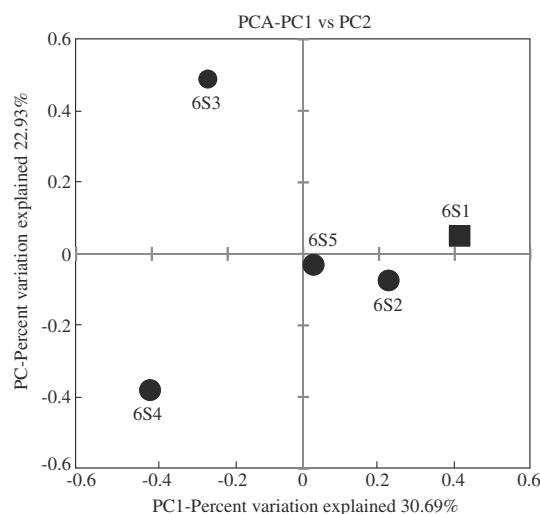

Fig. 6 – Rarefaction curve of OTUs defined by 3% sequence variation in samples from the 5 segments (1st and 4 successive aerobic segments) at S Farm in summer. OTUs: operational taxonomic units.

Fig 7 – Communities clustered using Unifrac PCA for bacterial community of dairy wastewater treatment system. Samples were obtained from the 1st to 5th segments at S Farm in summer. 6S1: 1st segment; 6S2: 2nd segment; 6S3: 3rd segment; 6S4: 4th segment; 6S5: 5th segment. PCA: principal component analysis.

3. Conclusions

The bacterial communities in different locations, seasons, and the 1st and 2nd segments of a dairy wastewater treatment system that consisted of six segments were examined. Microbiota between the 1st and 2nd segments in O Farm in summer and those between the 1st and 2nd segments in S Farm in winter exhibited similarity in PCA. This indicates that the changes in microbiota from the 1st to 2nd segments are related to the activation of the sludge related to DO and BOD.

The microbiota was also examined in the five segments (1st dairy fat degradation and four successive aerobic treatment segments) at one location. Although activated sludge circulates in this system, the microbiota of the five separated segments is distinct from one another. These results suggest that the difference in microbiota reflects the difference in physicochemical condition. This may mean that the microbiota is flexibly changeable depending on the water quality even though basically the same activated sludge is used.

The above analyses revealed that *Dechloromonas*, *Zoogloea* and *Leptothrix* are frequently observed in this wastewater system. These genera have been observed in wastewater treatment or in a freshwater system.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2015.09.025.

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