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Study of Suspended Impurities Origin and Composition in the Treatment Process of Johkasou System

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Johkasou system is widely used in Japan for household wastewater treatment and have played important roles in maintaining the water environment. However, out of the total units used in Gifu Prefecture, about 10% cannot meet the quality requirement for the effluent transparency above 30 degrees. Previous studies have shown clearly that the reason is mainly attributed to suspended impurities that remain in the water after treatment, with more than 96% being consisted of fine particles with sizes of 0.5-1.0 μm. A detailed investigation for identifying the origin and composition of the fine particles is necessary in order to find the approaches to improve the treatment function of the related Johkasou units. The analysis by Flow Cytometry suggested that the organic fine particles could be grouped into bacterial fraction, existed in all tanks of the Johkasou, and non-bacterial fraction that was confirmed mainly existed in anaerobic tanks, thus suggesting the lower transparency of Johkasou's effluent was greatly contributed by the bacterial fraction. PCR-DGGE further identified that some of the bacteria contained in the effluent were originated from the anaerobic tank.

Keywords : fine particles, Flow Cytometry, johkasou system, PCR-DGGE, water quality, wastewater treatment

INTRODUCTION

Up until World War II, Japan adopted vault toilet system with the night soil collected were use as agricultural fertilizers and soil conditioners. However, the wet flushing toilets were spread rapidly in 1970s, when the demand for it heightened strongly with the modernization of the citizen's life. This leads to the development of mass production technologies of decentralized on-site night soil treatment tank, namely Johkasou. Since then, sewers and Johkasou have developed side by side. It was reported that by the end of 2012 Johkasou installed throughout Japan

reached 7.76 million units (Setiyawan, 2015).

Johkasou is the Japanese word for on-site wastewater treatment. It is a combination of the words *jouka*, which means purification and *sou*, which means a tank or tub. Johkasou are mainly used in two situations: 1) when there is no access to sewer; and 2) in densely populated areas for on-site wastewater treatment including water reclamation and reuse (Gaulke, 2006). Johkasou system is a sophisticated technology because, in addition to an anaerobic process like that used normally in a septic tank, a contacting aerobic biological process is also included and followed before the effluent is discharged to the local water bodies after disinfection. As small-scale Johkasou can be installed on a household level and treat and discharge wastewater locally, these facilities have remarkable advantages compared to sewerage systems from the perspective of protecting the water environment and economical values.

In the on-site management and maintain of Johkasou, transparency is used as the main evaluation index for water quality for its efficient measure method. But, due to structural and/or maintenance problems by residents, about 10% of *gappei-shori* Johkasou of the total number of 70,053 installed in Gifu Prefecture, cannot meet the quality requirement for effluent transparency above 30 degrees. The reason is mainly attributed to suspended impurities that remain in the water after treatment. Suspended impurities are microscopic materials which are not completely soluble in water and contribute to water transparency level. These substances can be organic and inorganic particles and also microorganisms with different sizes. A previous investigation showed that Johkasou's treated water contained a large number of suspended particles, with more than 96% being consisted of fine particles with sizes of 0.5-1.0 μm. However, concerning the origin and composition of the fine particles little is known.

A detailed investigation for identifying the origin and composition of the fine particles, as the main objective of this study, is necessary in order to find the approaches that can improve the treatment function of the related Johkasou units. In order to achieve this purpose, the investigation was conducted on selected Johkasou with effluent transparency below 30 degrees to study its performance by analyzing its physicochemical properties, fine particles distribution inside each tanks using a flow cytometer, and microbial community structure with the aid of a PCR-DGGE-based bacterial structure.

EXPERIMENTS

Sampling collection

Two selected Johkasou (Johkasou A and B) of the same structure were selected with having treated water transparency level below 30 degrees. The water samples were collected from the 1st anaerobic tank (①), the inlet of the 2nd anaerobic tank (②), the outlet of the 2nd anaerobic tank(③), the aerobic tank (④), and the sedimentation tank (⑤) as shown in Figure 1. The sludge samples were collected from the 1st anaerobic tank (❶),

2nd anaerobic tank (❷), sedimentation tank (❸), and 2nd anaerobic filter bed (❹). Biofilm (**B**) on the filter media of the aerobic contact tank was also collected.

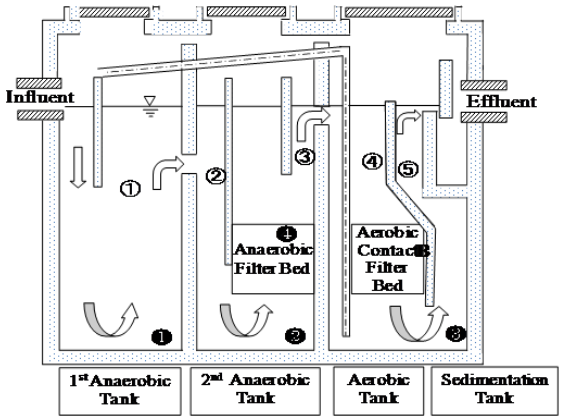

Fig. 1: Flowchart of the targeted Johkasou and sampling points for water (①-⑤), sludge (❶-❹), and biofilm (**B**)

Physicochemical properties

During water sampling process, transparency, dissolved oxygen (DO), pH, electrical conductivity (EC), and water temperature were measured. Water samples were transported to the laboratory at Gifu University and Gifu Prefectural Environmental Management and Technology Center, Japan, for measurements of other water quality measurement.

Flow cytometry

Staining and flow cytometry analysis was done as described previously (Hammes, 2008). Samples were analyzed with a CyFlow® Cube 6 (Partec, Germany) equipped with a 488 nm blue solid-state laser and the emitted green fluorescence was counted for 25 seconds at a flow rate of 0.5 µL/sec. Forward (FSC) and side scatter (SSC) signals were collected and processed using the FCS Express 5 software.

PCR-DGGE based bacterial structure

The microbial DNA was extracted from water, sludge and biofilm samples using The PowerSoil® DNA Isolation Kit. 1 µl of extracted DNA was added to 49 µl of the PCR mixture and then amplified by using the universal bacterial primers GC341F and 907R. The PCR cycling parameters were as follows: 95°C for 5 min (initial denaturation); 35 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 45 s; and 72°C for 10 min (final extension). Presence of PCR products was confirmed by electrophoresis on 1.2%-agarose gels stained with ethidium bromide.

Denaturing gradient gel electrophoresis (DGGE) was performed with the Dcode System (Bio-Rad Co., USA) with slight modification to a previously described method (Muyzer, 1993). PCR products were loaded onto 6% (w/v) polyacrylamide gels in 1X TAE using denaturing gradients ranging from 30 to 60%. The electrophoresis was carried out at 60 V for 16 hours and maintained at 60 °C. Gels were then stained with SYBR Green I (1 :10,000 dilution) and visualized on UV transilluminator and photographed (Image Lab Software). Bands were excised from the gel and DNA eluted in 80 µL ddH_2O at 4 °C overnight. The eluted DNA was re-amplified using the same primers and same PCR condition. PCR amplicons were tested on 1.2% agarose and the positive PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent and sent to Gifu University Molecular Genetics Research Center for sequencing. The

sequencing results was compared with the GenBank database to confirm the microorganisms origins (BLAST algorithm; NCBI, Bethesda, MB, USA).

RESULTS AND DISCUSSIONS

Physicochemical properties

The water indexes of Johkasou were measured over time, during this study, the aeration strength had been changed for two times. The aim is to promote the formation of biofilm by changing the amount of air blown. The transparency level of water samples from Johkasou A and B can be seen in Figure 2.

As shown in Figure 2, during the two times of aeration strength change, the transparency of Johkasou A's effluent had also changed. Increasing the aeration strength resulting in the increasing of transparency level. Moreover, Johkasou B had a high nitrification rate of 49.3% for its original condition so the aeration strength was decreased during the sampling period. The aeration strength and transparency level showed the opposite tendency, when the aeration strength decreased, the transparency level increased for a while and then decreased again.

Flow cytometry

The distribution of bacterial and non-bacterial particles in the water samples along the treatment processes of Johkasou A are shown in Figure 3. The

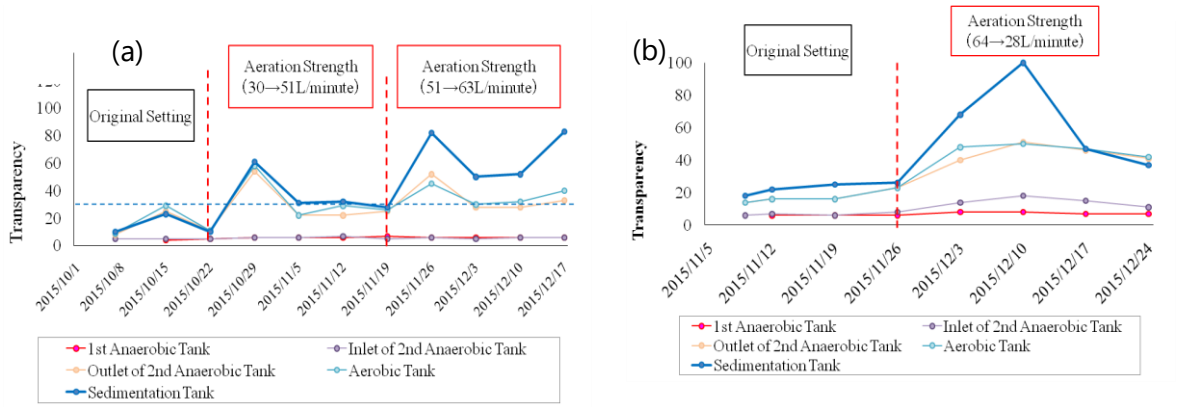

Fig. 2: Transparency level of water samples during sampling period. (a) Johkasou A ; (b) Johkasou B

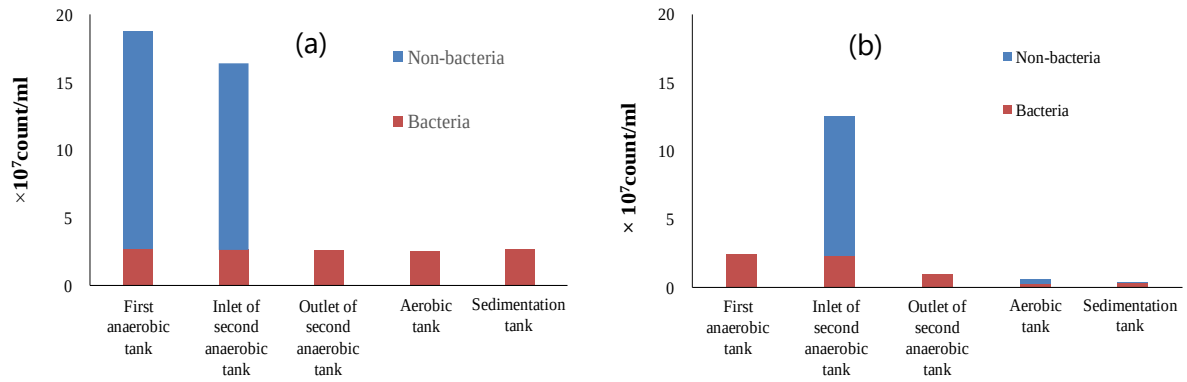

Fig. 3: The distribution of fine particles along the treatment process of Johkasou A. (a) Effluent transparency level of 10 degrees; (b) Effluent transparency level of 83 degrees

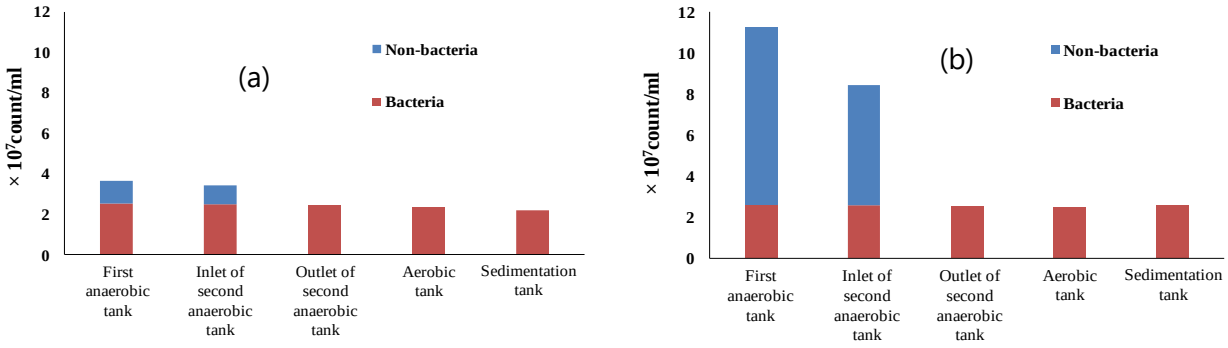

Fig. 4: The distribution of fine particles along the treatment process of Johkasou B. (a) Effluent transparency level of 26 degrees; (b) Effluent transparency level of 37 degrees

effluent water transparency level was 10 degrees and 83 degrees, respectively.

For the non-bacterial particles, a significant decrease was noticed between the inlet and the outlet of the second anaerobic tank, indicating that non-bacterial particles were mainly eliminated from water in this tank. For the bacterial particles, a trend of gradual decreases in their number along the treatment processes seemed to be existent; however, the trend was less obvious. By comparing Figure 3 (a) and (b), it can be seen that there were differences of non-bacterial particles number between effluent with low transparency and high transparency level. Non-bacterial particles were mainly present on water of anaerobic tank and contributed to effluent's low transparency level. Bacterial particles were also higher on effluent with low transparency level. This indicates that the effluent transparency level was affected by fine particles.

Furthermore, the distribution of bacteria and non-bacterial particles along the treatment process of Johkasou B is shown in Figure 4.

From Figure 4 (a) it can be seen that non-bacterial particles were mainly distributed on the anaerobic tank and the number was decreased on the outlet of second anaerobic tank, indicating that the particles were mainly removed in this tank. However, the bacterial particles were seen distributed in all tanks of Johkasou and the trend of gradual decrease were seen despite not so significant. On the other hand, from Figure 4 (b) it can be seen that a significant decrease was noticed between the inlet and the outlet of the second anaerobic tank, indicating that the non-bacterial particles were mainly eliminated from the 2nd anaerobic tank. For the bacterial particles, the trend was less obvious.

PCR-DGGE based bacterial structure

Bacterial community structures in water, sludge and biofilm samples from each sampling point of Johkasou A were investigated by PCR-DGGE-based method, and the results are shown in Figure 5 below.

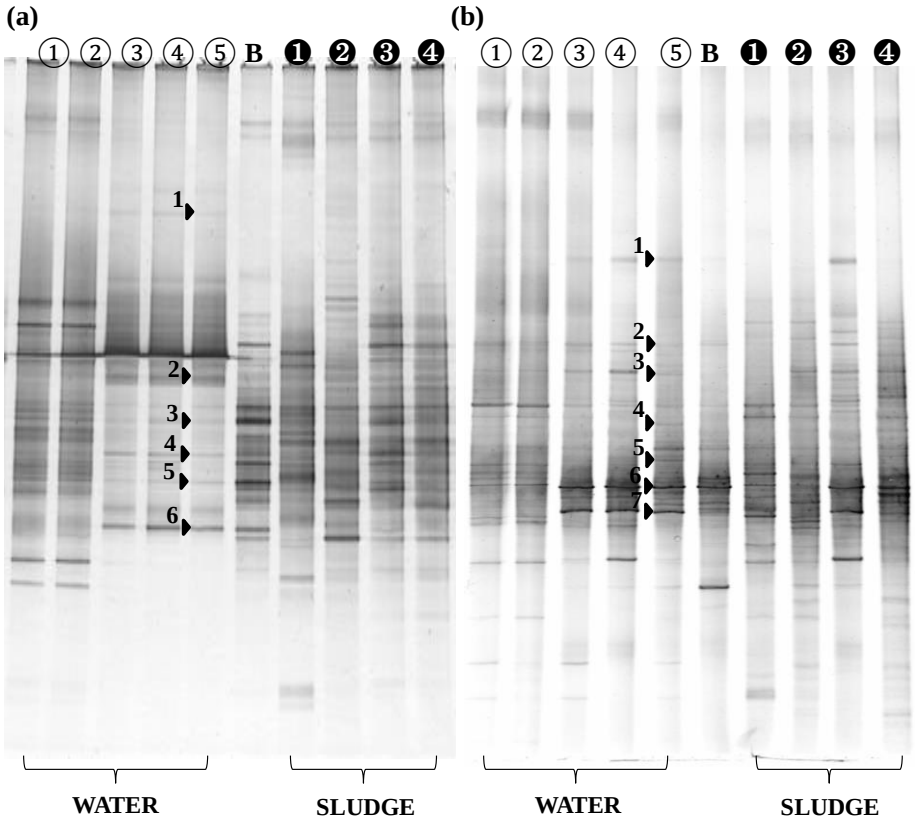

Fig. 5: Band images of PCR-DGGE for bacteria contained in water, sludge and biofilm of Johkasou A. (a) Effluent transparency level of 10 degrees ; (b) Effluent transparency level of 83 degrees.

From Figure 5(a) it can be seen that there were similarities in some bacteria community structure among the treated water, sludge and biofilm samples, indicated by arrows. Some of the bacteria contained in the sedimentation tank water have similarities with those on the 2nd anaerobic tank, suggesting that the bacteria probably were washed out together with the flow as shown by arrow **1** and **2**. These two arrows have no significant similarities with bacteria contained in the sludge and biofilm samples. In Figure 5(a) there were also few bands that possessed similarities with biofilm, suggesting a detachment of biofilms and resuspended to the water phase, as shown by arrows **3**, **4**, **5**, and **6.**

To further confirm variability contain in the each sampling points, 27 dominant DGGE bands with high intensities in the DGGE gel were selected for sequencing. Sequence analysis by the BLAST algorithm revealed that the entire bacteria present in this study belonged to the phylum of *Proteobacteria* (Brigmon, 2003; McGarvey, 2004; Oren, 2014; Panday, 2010).

Furthermore, the bacterial community structure in water, sludge and biofilm samples from each sampling point of Johkasou B is shown in Figure 6.

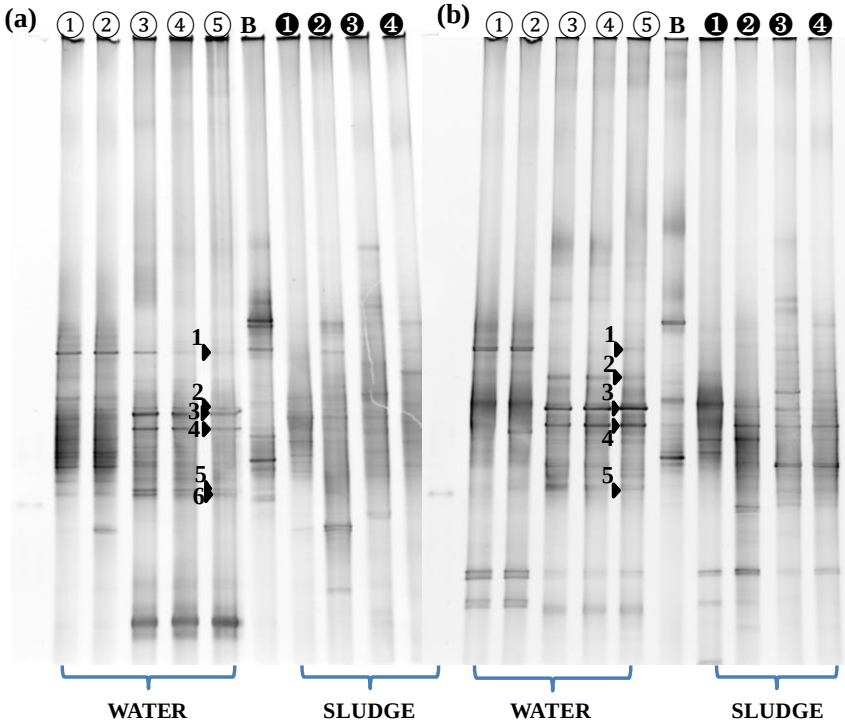

Fig. 6: Band images of PCR-DGGE for bacteria contained in water, sludge and biofilm of Johkasou B. (a) Effluent transparency level of 26 degrees ; (b) Effluent transparency level of 37 degrees.

From Figure 6(a) it can be seen that some of the bacteria contained in the effluent water were coming from the 2nd anaerobic tank that probably were washed out together with the flow as shown by arrow **3**, **4** and **5**. On the other hand, some bacteria contained in the sedimentation tank water also displayed similarities with those on each tank of water sample as indicated by arrow **1**, **2** and **6**.

After changing the setting condition of Johkasou B, there are changes in bacterial community structure as shown in Figure 6(b). From Figure 6(b) it can be seen that most of the bacteria in the sedimentation tank displayed similarities with water samples of the outlet 2nd of anaerobic tank, aerobic tank, and could be clustered into one group as shown by arrow **2**, **3**, **4** and **5**. However, water samples of the 1st anaerobic tank and the inlet of the 2nd anaerobic tank displayed the same bacterial diversity, which differs with that in the following tanks. It's suggested that the bacteria contained in effluent of Johkasou came from 2nd anaerobic tank water that were flown out together with the effluent. Furthermore, some bacteria contained in sedimentation tank water also possessed similarities with those on the 1st anaerobic tank water and sedimentation tank sludge as shown by arrow **1**. However, the band intensity is not so significant, suggesting that this species is not dominant.

CONCLUSION

Flow cytometry analysis suggested that the water samples of johkasou have

proven to contain bacteria and non-bacterial particles. The bacteria are detected occupying each tank, both anaerobic and aerobic tank, including the sedimentation tank and flown out together with effluent. Particles with larger size also had proven affecting the effluent water quality and needs to be removed.

The existence of bacteria as suspended impurities inside the johkasou's effluent was investigated using PCR-DGGE-based bacterial structure. The results suggest that some of the bacteria contained inside the effluent were originated from the 2nd anaerobic tank that were probably washed out together with the flow, and also from the detachment of biofilms and resuspended to water phase. Further sequencing was required to determine the microbial composition and species.

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