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Changes in bacterial composition during *in vitro* oil degradation experiments using activated sludge from different sources

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Abstract

We monitored bacterial compositional changes during *in vitro* food oil degradation experiments, wherein activated sludge (AS) from different sources was employed as seed. Two AS samples were collected from different aeration processes at the same plant and one sample was collected from a different plant; these were used for the degradation of synthetic wastewater containing commercial food oil as the sole carbon source. A 16S rRNA gene-based next generation sequencing technique was applied to study the bacterial community in the seed AS samples and resultant water after *in vitro* degradation for two days. Out of the total assigned genera of 319, 153 were present in all three AS seed samples, while the remaining 166 genera, observed in one or two AS seed samples, accounted for only 5% of the total operational taxonomic units. We found that different bacterial groups of each AS capable of oil degradation were present in the samples after two days' incubation. This suggests that the oil degradation process differently affected the key microbial groups from different sources. Therefore, monitoring of the AS seed and optimizing the degradation condition may be required to achieve efficient degradation performance.

Key words: high throughput sequencing, membrane panel, oil-degrading bacteria, proteobacteria

INTRODUCTION

Serious water pollution concerns due to contaminated oil released by food manufacturers and/or restaurants have been raised in Southeast and East Asia (Vijayaraghavan *et al.* 2007). Biological treatment techniques have been widely applied for the removal of waste food oil in wastewater treatment plants because of their high treatment efficiencies and low cost (Takeno *et al.* 2005). Oil interceptor tanks trap fats, oil, and grease from organic wastewater from small business establishments (kitchens/restaurants and food-processing factories) before they enter a wastewater collection system. Nevertheless, municipal wastewater contains large amounts of organic matter and food oil, which enter wastewater treatment plants, where they are treated via conventional activated sludge (AS) processes.

A wide variety of bacteria that can utilize and degrade different organic matter exist in AS. However, treatment efficiency largely depends on the ability of microbial communities to metabolize organic and inorganic pollutants. Efficient oil degradation may be difficult to achieve; for instance, seasonal variations of such diverse microbial communities may affect process efficiency (Ju *et al.* 2014). Effective and sustainable removal systems for waste food oil can be developed if the bacterial group in AS responsible for the degradation of oil can be identified. However, information on which bacterial groups can degrade oil in sewage is limited. High-throughput sequencing (HTS) technologies have been applied to investigate the diversity and abundance of microorganisms in wastewater treatment systems (Ye *et al.* 2011; Hu *et al.* 2012; Zhang *et al.* 2012). We used an HTS technology

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to study the proximal changes in microbial community structures during the degradation of synthetic oil-contaminated water with AS from three different sources.

Various AS-based wastewater treatment systems have been developed and applied practically. For example, conventional AS processes take 6–8 hours for hydraulic retention time (HRT), whereas it expends a longer time in extended aeration processes (16–24 hours HRT) and oxidation ditch processes (24–36 hours HRT) (Japan Sewage Works Association 2013). Membrane panel (MP) type aerators are one such system and have the advantage of higher aeration efficiency (i.e. oxygen diffusion) as they give rise to smaller air particles than conventional mechanical type aerators (Fatone *et al.* 2011). We have, therefore, hypothesized that AS from an MP type system would have improved capacity for microbial aerobic oxidation of organic matter and would hence be more efficient for oil degradation. The microbial community structures and oil degradation efficiencies of the AS samples collected from the two types of treatment processes (MP and mechanical) operating in parallel in the same plant were compared.

MATERIALS AND METHODS

Seed sludge was obtained from the aeration tanks of two sewage processing plants (two-stage nitrification/denitrification system) in Nagano prefecture, Japan. Seed sludges AS1 and AS2 were collected from different processes (MP and mechanical type aeration) of the same plant, while seed sludge AS3 was collected from a plant with an MP type system. The oil content of AS3 is 2.5 times higher than that of AS1 and AS2. The synthetic sewage used in this study is composed of 0.2 g/L of $(NH_4)_2HPO_4$ dissolved in distilled water and commercial food oil prepared from soybean (Nisshin Oil Co. Ltd., Japan, 100 mg/L) using a sonic disperser (GP-621D and SPN-620, BlueSpace Co. Ltd, 600 W 26 kHz). For the batch experiments, 300 ml synthetic wastewater was taken in 500 ml glass culture flasks. AS samples (100 ml) from different sources were used for each batch experimental run: AS1 for Run1, AS2 for Run2, and AS3 for Run3. Mixed liquor suspended solid concentration was 990 mg/L in Run1, 1,110 mg/L in Run2, and 2,380 mg/L in Run3, respectively. The batch experiments were carried out for 2 d at 20 °C using an agitating water bath (BW400: Yamato Scientific Co. Ltd., Tokyo, Japan) at a shaking width of 4 cm and a shaking speed of 100 time/min. The batch experiments were carried out for 2 d because preliminary experiments revealed that efficient oil degradation could be achieved with incubation for 2 d. The pH and dissolved oxygen contents during this incubation period were monitored daily using glass electrodes and luminescent dissolved oxygen probes (Hach, Loveland, CO), respectively. The pH and dissolved oxygen contents were not controlled, the dissolved oxygen contents were over 4.5 mg/L during the experiments and the initial pH were neutral in all the runs. Oil concentrations were analyzed by Oil Content Analyzer (solvent extraction, non-dispersive infrared spectroscopy, OCMA-505: Horiba, Kyoto, Japan) with oil extraction solvent (H-997: Horiba, Kyoto, Japan).

The total bacterial numbers for the six samples (the three AS samples and samples obtained after the three batch experiments carried out for two days), were determined using a real-time polymerase chain reaction (PCR) method. The genomic DNA of the microorganisms present in the samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and stored below –20 °C until analysis. The PCR conditions and primer sequences for total bacteria used in this study are according to those used in a previous study (Uyeno *et al.* 2017). Primer sets Eub338F (ACTCCTACGGGAGGCAG) and Eub522R (ACGTCRTCCMCNCCTTCCTC), a CFX96 Real-Time PCR detection system (Bio-Rad Inc., Hercules, CA), and an SYBR(R) Premix Ex Taq Kit (Takara Bio Inc., Otsu, Japan) were used. The PCR cycling conditions were initial denaturation at 95 °C for 10 s and 40 cycles at 95 °C for 5 s and 62 °C for 30 s; this was followed by melting curve analysis to confirm that the expected PCR products were obtained. Furthermore, the extracted

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bacterial genomic DNA was subjected to 16S rRNA gene amplicon pyrosequencing. Primer sets 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVHHHTWTCTAAT), a T-100 thermal cycler (Bio-Rad Inc., Hercules, CA), and an Ex Taq Kit (Takara Bio Inc., Otsu, Japan) were used to generate the amplicons. The PCR cycling conditions used for amplification were initial denaturation at 95 °C for 10 s and 25 cycles at 95 °C for 10 s, 57 °C for 30 s, and 62 °C for 30 s. A barcoded amplicon was subjected to paired-end sequencing on an IlluminaMiSeq (Illumina, San Diego, CA, USA). The sequencing results have been submitted to the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (Accession Number: DRX150611-DRX150616). Post analyses of the sequencing results were carried out after filtering the low quality reads and trimming the adapters, barcodes, and primers using QIIME. The reads from all the samples were clustered into operational taxonomic units (OTUs) at a 97% sequence similarity level. The data for species-level taxonomy were obtained by filtering the OTU tables containing taxonomic data generated using the Ribosomal Database Project (RDP) Classifier at the genus level. Representative sequences were then extracted, and species-level matches within the National Center for Biotechnology Information database were identified using the Basic Local Alignment Search Tool (BLAST). Further, alpha diversity (α-diversity) was measured using the Shannon index.

RESULTS AND DISCUSSION

The oil degradation efficiencies were 62%, 63%, and 73% (day 1) and 85%, 90%, and 90% (day 2) in Runs 1, 2, and 3, respectively. This suggests that the use of seed AS from different sources affected oil degradation efficiencies only slightly under the operating condition used in this study, while it seemed marginal difference of the degradation efficiency between seed AS type. Similarly, the total bacterial count varied only slightly ($1.6–2.7 \times 10^9$ copies/g sludge) among the experiments conducted with AS from different sources. The incubation period of 1–2 days is longer than HRT in conventional AS processes, probably due to (1) lower solids concentration in the mixed liquor, or (2) lower available nutrient concentration compared to practical conditions.

Pyrosequencing of the 16S rRNA genes was conducted to examine the bacterial diversity in these seed AS samples and the synthetic wastewater used for the oil degradation experiments. After filtering the low quality reads, 142,569–266,401 effective reads were obtained. To conduct downstream analyses of the different samples at the same sequencing depth, the library size of each sample was normalized to 47,522 sequences, which is the smallest library size among the six samples, by denoising, filtering out chimeras, and removing the archaeal sequences. For all the samples, the median sequence length after preprocessing and before clipping was 283. Comparative analyses revealed a core microbiota across all the seed AS samples. As shown in Figure 1, among the 319 assigned

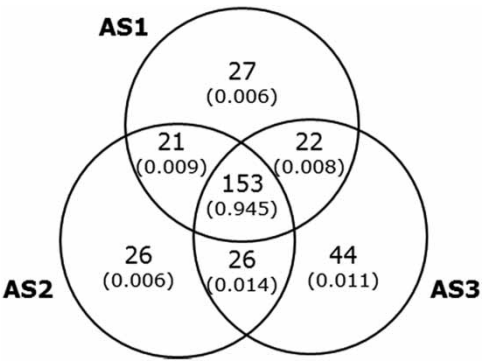

Figure 1 | Venn diagram depicting the number of common (or uncommon) bacterial genera among the AS seed samples. A total of 319 genera were identified by sequencing. Numbers in parentheses indicate the proportion of cumulative operational taxonomic units within genera classified into respective regions.

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genera, 153 were present in all samples, and accounted for 94.5% of the total OTUs. The remaining 166 genera appeared only in one or two samples, thereby accounting for only 5% of the total OTUs, which is a minor fraction of the bacterial community in the seed AS samples. Shannon indices ranged between 4.29 and 4.63 for all the six samples.

Proteobacteria was the most abundant phylum in all the samples, accounting for 36–65% of the total assigned bacterial sequences (Figure 2 and Table S1). This is similar to the analytical results for bacterial communities in AS obtained by other studies employing HTS technologies (Hu *et al.* 2012; Yu & Zhang 2012; Zhang *et al.* 2012) and other methodologies such as microarray technology and gene cloning (Xia *et al.* 2010; Wan *et al.* 2011). Other dominant phyla were Bacteroidetes, Firmicutes and Actinobacteria. Some other major phyla in AS1 and AS2 were Verrucomicrobia, Chloroflexi, and Acidobacteria, while Cyanobactereia was marginally more dominant in AS3. Certain amount of retrieved sequences either did not belong to classified organisms or displayed relatively low levels of similarity with known 16S rRNA gene sequences. Overall, minor differences in the microbial community structures of the seed AS samples were observed, suggesting that the treatment process and source locations affected these structures but it was rather subtle compared to our expectation.

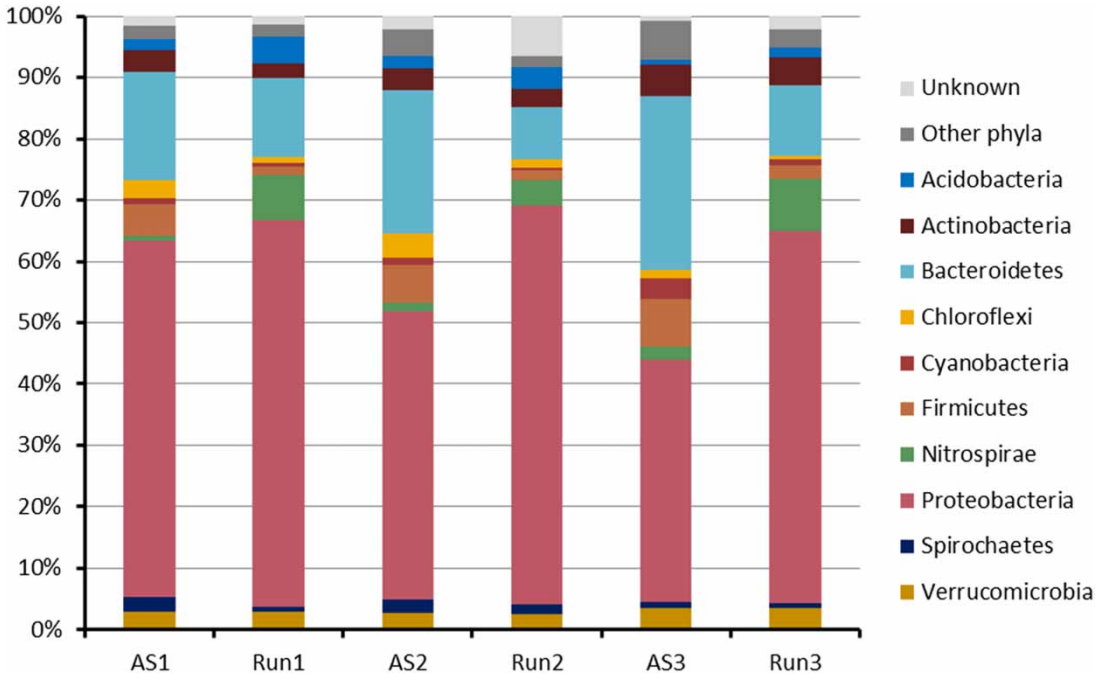

Figure 2 | Proportional distribution of respective bacterial phylum in AS seed samples and *in vitro* experiments.

During the experiments, the β-subdivision and γ-subdivision of Proteobacteria, particularly genera *Acinetobacter* and *Pseudomonas* belonging to the γ -subdivision, were the most dominant in Run1 and Run2 (Table S1). *Acinetobacter* has been identified in biological oil decomposition processes (Lal & Khanna 1996; Rahman *et al.* 2002). *Pseudomonas aeruginosa* produces biosurfactant, which enables efficient degradation of crude oil, and has been reported to be a potent oil degrader (Thavasi *et al.* 2011). Genera belonging to Burkholderiales (*Azohydromonas*, *Caldimonas*, and *Pelomonas*), and Sphingobacteriales (*Sphingopyxis*) of Bacteroidetes were found in a certain proportions in Run3, whereas they were not observed in the AS and the post-run bacterial communities. Furthermore, a well-defined nitrifying bacterium of Nitrosomonadales comprised a certain fraction of the β-proteobacterial community. An increased proportion of the genus *Nitrospira* during the experiments indicates that aerobic nitrification from ammonia to nitrate via nitrite likely

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occurred. This is further supported by the decrease in pH during the experiments (to pH 4.9–5.7). Although Bacteroidetes and Firmicutes were dominant in all the AS samples, their population marginally decreased during the experiments (Figure 2). As the reactor influent did not contain any organic matter other than the tested oil, it is possible that heterotrophic bacteria incapable of degrading oil could not thrive.

In practical applications, the composition of influent organic matter and environmental conditions will have a large impact on the physiological state, structure, and population dynamics of the bacterial community. Therefore, a better understanding of the microbial communities in wastewater processing will provide useful information for the stable operation of wastewater treatment systems, and enrich our knowledge of microbial ecology so that the overall kinetics of the microbial community can be reasonably optimized to environmental changes.

CONCLUSIONS

We made two important observations during our study. Approximately 90% food oil degradation could be achieved in synthetic wastewater within two days, thereby reducing the oil content to lower levels than those achievable with current practical treatment methods. Degradation efficiency was similar for AS samples regardless of different sources that possessed similar bacterial composition. However, the impact of the oil degradation process on key microbial groups in AS from different sources is likely to be different, as significant variations in the 16S rRNA gene profiles of the microorganisms capable of oil degradation were observed. Therefore, monitoring of the AS seed and optimizing the degradation condition may be required to efficiently degrade food oil contaminated sewage.

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SUPPLEMENTARY DATA

The Supplementary Data for this paper is available online at http://dx.doi.org/10.2166/wpt.2019.076.

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