Enhanced biological phosphorus removal deterioration without significant change in microbial population under trace element shortage

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1 Introduction

One of wastewater treatment method based on microorganisms remove organic matters, nitrogen (N) and phosphorus (P) from wastewater is called “activated sludge”. Activated sludge can be modified for object of remove such as Enhanced Biological Phosphorus Removal (EBPR) process, which removes P from wastewater by phosphorus-accumulating organisms (PAOs). EBPR basic structure is an anaerobic phase followed by aerobic, where in anaerobic phase PAOs proliferates then in aerobic phase they accumulate P from wastewater into their cells. In a result, wastewater P pollutants shifted to biomass, which readily remove by settling.
EBPR process can be affected by temperature, pH, wastewater composition ratio, micronutrients, bacterial community change and operational parameters, which triggers unstable performance. Impact of trace elements shortage to EBPR performance, which investigated in previous study by Yuki Sato (Sato, 2012) who concluded Fe is the most responsible element than other seven elements (Co, Cu, Mo, I, Bo, Mn, Zn) by his lab-scaled sequencing batch reactor experiments. During his experiments, EBPR performance deteriorated significantly in the experiment reactor that compares to control reactor, which had been performed good.
Microbial community analysis is an essential tool in wastewater treatment technology, because of treatment methods based on microorganism character. Investigating relationship between microbial community and deterioration impact is a key point to improve ways to control EBPR process.
DNA sequencing technology is rapid developing techniques, which enables comprehensive analytical ways in microbiology, which was limited by culture-dependent methods few decades ago.

2 Research frameworks

This research has two objectives,

1. To confirm reproducibility of the trace elements shortage effect to the EBPR process.
2. To clarify relationship between microbial population change and EBPR deterioration caused by trace elements.

For achieve these goals, following studies has done in scope of this research,

a. Operated two Runs on the lab-scaled 2 EBPR reactors in parallel and observe the performance between normal and trace element shortage, based on chemical analyses.
Run_A was aimed to confirm trace element shortage effect and Run_B was dedicated for investigate impact of the Fe shortage.

b. Made microbial community analyze based on pyrosequencing of 16S rRNA and 16S rDNA gene amplification on the samples of Yuki Sato experiment Run8 and Run10 (Yuki Sato, 2012), which EBPR performance affected by trace elements.

3 Material and Methods

3.1 Reactor operation

Control and experiment 2 sequencing batch EBPR reactors operated in parallel in 2 runs, which Run_A intended to confirm effect of the omission of trace element, Run_B focused on Fe effect to the EBPR performance.
Reactors operated under same condition which total volume of 10L, hydraulic retention time (HRT) was 8 hours, sludge retention time (SRT) was 7days. One cycle continued 4 hours in total, consisting of: 1 hour anaerobic, 2 hours aerobic, 1 hour settling and discharge. 5L synthetic wastewater was supplying per cycle. The composition of the synthetic wastewater is, sodium acetate 113mg/L, sodium propionate 53.6mg/L, peptone 100mg/L, yeast extract

20mg/L, potassium chloride 42mg/L, calcium chloride dihydrate 13.2mg/L, heptahydrate magnesium sulfate 110mg/L, 2 dipotassium phosphate 72mg/L. About trace element concentration in influent is Fe 56.9μg/L*、 Mo 4.4μg/L、 Cu 1.4μg/L, Co 8.2μg/L、 B 4.8μg/L、 I 25.3μg/L、 Mn 6.5μg/L、 Zn 5.0μg/L. Influent trace element content referred to each Runs and experiment described in Table 1.

Trace elements	Run_A		
	Preparatory period (Days 1-4)	Comparison period (Days 5-7)	
	Both Control and Experimental Reactors (μg/L)	Control Reactor (μg/L)	Experi-mental Reactor (μg/L)
Fe	56.9	56.9	
B	4.8	4.8	
Cu	1.4	1.4	
I	25.3	25.3	
Mn	6.5	6.5	
Mo	4.4	4.4	
Zn	5.0	5.0	
Co	8.2	8.2	

Table 1. Description of the Run_A and trace element concentration

Trace elements	Run_B		
	Preparatory period (Days 1-4)	Comparison period (Days 5-7)	
	Both Control and Experimental Reactors (μg/L)	Control Reactor (μg/L)	Experi-mental Reactor (μg/L)
Fe	56.9	56.9	
B	4.8	4.8	4.8
Cu	1.4	1.4	1.4
I	25.3	25.3	25.3
Mn	6.5	6.5	6.5
Mo	4.4	4.4	4.4
Zn	5.0	5.0	5.0
Co	8.2	8.2	8.2

Table 2. Description of the Run_B and trace element concentration

During observation period of Run_B, experiment reactor fed by trace element solution, which omitted Fe only, in order to generate Fe shortage condition.
In Run_A, control reactor fed by trace element, experiment reactor stopped feeding by trace element after 6 days preparatory period and observed both reactor performances during observation period. In Run_B, control reactor fed as normal, experiment reactor fed by trace elements, which omitted Fe.

3.2 Analytical method

MLSS and SVI measurement were done according to Standard method (APHA, 2005). Concentration of dissolved organic carbon (DOC) in the supernatant was measured by a TOC analyzer (TOC-VCSN, Shimadzu, Japan). PO4-P concentration was determined by an ICS-3000 ion chromatograph with an AS12A column and an ASRS suppressor (Dionex). Carbonate buffer solution (containing 2.7mM sodium bicarbonate and 0.3mM sodium carbonate) was used as the eluent at a flow rate of 1.5mL/min. Anion standard mixture solution from Kishida Chemicals Inc., Japan, was used as the standard.

3.3 Microbial community analysis

Total 28 samples for microbial community analyses were obtained from the four reactors (a control reactor and an experimental reactor in both Run8 and Run10) by Yuki Sato (Sato, 2012) for 7 successive days and were stored with 1:1 ethanol at -80^{0}C freezer taken from reactors immediately.
In 2013, in scope of this research, samples thawed in room temperature and sonicated by Advanced Digital Sonifier 250AD cell disrupter (Branson) at amplitude of 30% (7W) for 10 seconds to extract DNA and ribosomes from cells.
PCR and RT-PCR were performed targeting 16S rRNA gene or 16S rRNA using 27f and 519r primers (Lane, 1991). For PCR, ExTaq HotStart Version (Takara, Japan), and for RT-PCR, PrimeScript One Step PCR Kit Version 2 (Takara) were used. Then second step PCR with 8 base barcoded 27f and 519r universal primers made with 5 cycles.
After each process, DNA product concentrations were checked by PicoGreen dsDNA Quantification Kit (Invitrogen, USA). To confirm the sizes of the products, products were run on 1% agarose gel by gel electrophoresis in TAE buffer, stained with

GelRed (Wako, Japan), and band patterns were visualized in a GelDoc system (BioRad, USA). Mixed samples run on 1% low melting agarose gel, which contained 0.5µL GelRed/20mL and manually cut 600bp sized DNA products under UV light, in order to remove 130bp sized products, which unacceptable small products for pyrosequencing. Gel slice that containing targeted DNA products, purified by QUAQuick Gel Extraction Purification Kit and concentration and contamination checked by Bioanalyzer 2100.

Pyrosequencing has done in the Omics and Bioinformatics center, Graduate School of Frontier Science, University of Tokyo.

Pyrosequencing result or sequence reads were processed by a pipeline of QIIME (Caporaso et al., 2010), and developed by OTUMAMi (Satoh et al., 2012). Low quality reads (shorter than 300bp or average quality of reading smaller than 25) were omitted. Operational taxonomic units (OTUs) were formulated by uclust algorithm (Edgar et al., 2010) at 97% similarity. Taxonomic assignments were done by RDP classifier Version 2.2 (Wang et al., 2007) using GreenGenes 12_10 (97% similarity OTUs) database (McDonald et al., 2012, Werner et al., 2012) as the reference.

Pyrosequencing made on 16S rRNA gene (DNA) and 16S rRNA (RNA) amplification, in order to discriminate total and active cells of same species.

4. Results and Discussion

4.1. Sequencing Batch Reactor experiments

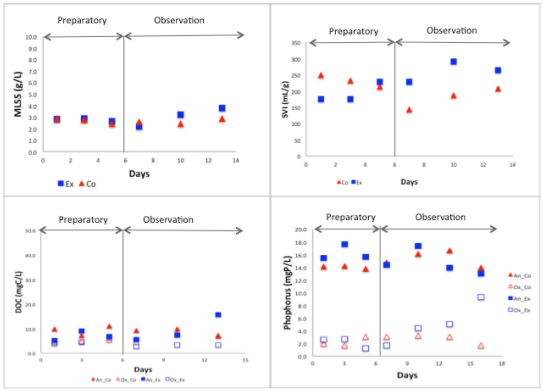

Figure 1. Performance of Run_A

In Fig.1, Settling Volume Index and P removal efficiency of the Run_A showed. After trace element supply stopped, experiment reactor P removal was deteriorated, which shown by Phosphorus concentration of the end of aerobic phase gradually increased (Ox_Ex). Settling ability deterioration observed in experiment reactor (Ex), which shown in SVI graphic. And control reactor performance was not deteriorated which fed by trace elements.

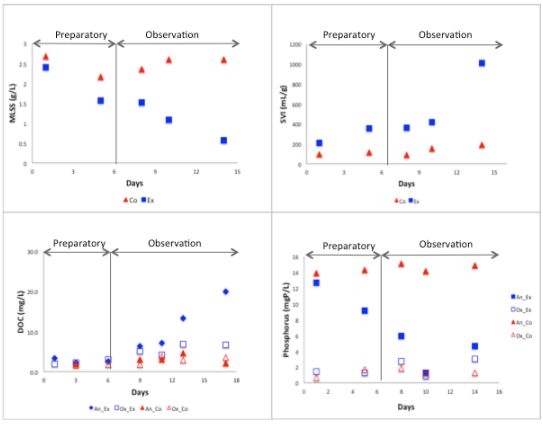

Figure 2. Performance of the Run_B

Run_B performance shown in Fig.2. Due to filamentous bacterial growth, settling ability deteriorated (Ex increased in SVI graphic) and biomass washed away by discharge. In result, poor biomass generated poor floc and affected to P removal efficiency.

4.2. Microbial population analysis

Total 67472 reads obtained from pyrosequencing and analyzed in the order of taxonomic hierarchical level from higher level to lower. Domain or kingdom level the highest, followed by phylum, class, order, family, genus, and OTU levels. As the OTUs were formulated at 97% similarity levels, OTU level here practically means species of the bacteria.

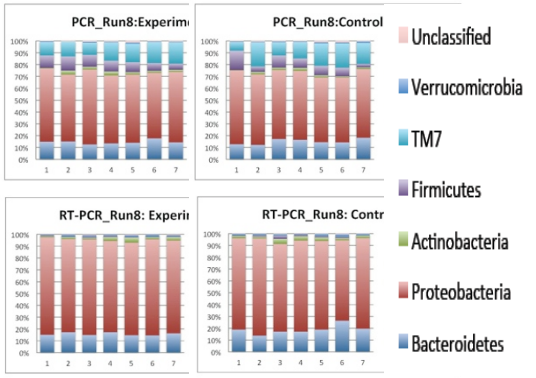

Figure 3. Taxonomic assignments of the Run8 showed in phylum level.

Fig.3 shows phylum level reads compositions of the PCR and RT-PCR products in Run8. Most dominant bacterial phylum was Proteobacteria, which showed by red in Fig.3. PCR and RT-PCR result based pyrosequencing showed different results on same species, which shown by different fluctuation on same day samples. Significant bacterial community change was not observed in last 3 days, which trace element supple changed.

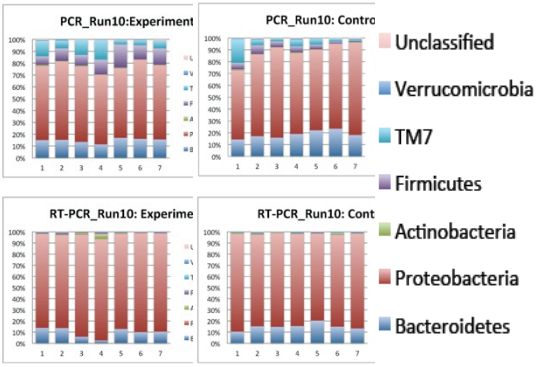

Figure 4. Taxonomic assignments of the Run10 showed in phylum level.

As shown in Fig.4, Run10 dominant bacterial phylum was Proteobacteria and TM7 and Firmicutes detected in PCR, but not detected in RT-PCR. After stopped trace element supply from 5th day, bacterial community did not change significantly in the experiment reactor.

Candidatus 'Accumulibacter Phosphatis' is responsible bacterial species for EBPR process and widely distributed in EBPR plants around the world. OTUs related to Accumulibacter extracted from the heatmap and further analyzed by Principal Component Analysis method, in order to investigate that affected by trace element shortage or not.

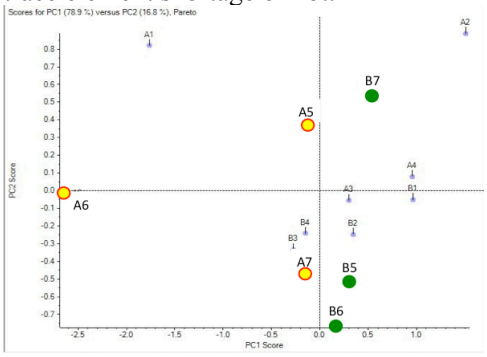

Figure 5. OTUs related to Accumulibacter PCA of the PCR result in Run8.

In Fig.5, experiment reactor condition was similar to control reactor, A6 plot changed to another position but then back to in A7 plots. A series plots located near to B series, which their condition was similar.

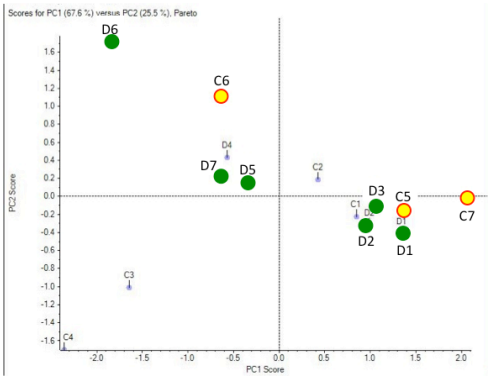

Figure 6. OTUs related to Accumulibacter PCA of the PCR result in Run10.

Plots, which after condition changed, C5, C7 were near with control reactor D plots. C6 showed some change, but C7 back to the initial place.

5. Conclusions

5.1 SBR operation experiment

EBPR deterioration observed in the experiment reactor after stopped trace element supply. At the same time, control reactor which fed by trace elements, performance was not deteriorated. Based on this result, author concluded trace element shortage affected to EBPR performance. In the Run_B, after Fe supply stopped, filamentous bacteria proliferated and affected to the settling of the sludge. Then biomass washed away by discharge and EBPR process was deteriorated.

5.2 Microbial community analysis

Significant microbial community change was not observed in phylum, class and OTUs level. Further analysis on Accumulibacter-related OTUs result showed EBPR deterioration was not related to PAOs.

And DNA sequencing and RNA sequencing gave different results on the same sample. It clearly observed on TM7, Clostridia related OTUs.