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生物学的りん除去廃水処理プロセスにおいて生成したグラニュール汚泥の処理性能と微生物群集構造の経時変化

Temporal sludge performance and microbial community change of aerobic granular sludge from an EBPR wastewater treatment bioreactor

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LIST OF ACRONYMS

AGS	aerobic granular sludge
bp	base pair
DGGE	Denaturing gradient gel electrophoresis
DO	Dissolved oxygen
DOC	Dissolved organic carbon
EPS	Extracellular polymer substances
MLSS	Mixed Liquor Suspended Solids
OTU	Operational Taxonomic Unit
OTUMAMI	Operational Taxonomic Unit Management And Mining
PCR	Polymerase Chain Reaction
QIIME	Quantitative Insight Into Microbial Ecology
RDP	Ribosomal Database Project
SBR	Sequential batch reactor
SVI	Sludge volume index
T-RFLP	Terminal resistriction fragment length polymorphism
TRFs	Terminal resistriction fragments
TOC	Total organic carbon
WCDS	Water content of dewatered sludge
WWTPs	Wastewater treatment plants

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CHAPTER ONE

1.1 Introduction

1.2 Objectives and Scope

1.3 Research Framework Structure

1.4 Composition of the dissertation

1.1 Introduction

1.1.1 Domestic wastewater treatment

Domestic wastewater contains organic matters, minerals, and microorganisms 0which may cause human health and environmental problems if discharged into water body without proper treatment. Different kinds of domestic wastewater treatment processes-physical, chemical, biological- are thus used to remove these matters which are related with human health and environment. As a result of urbanization, the goal of domestic wastewater treatment has been the removal of these pollutants. But nowadays, under global problems such as global warming and the limitation of energy supply, the objectives of domestic wastewater treatment now also includes energy saving and material recycle to reach sustainable society.

1.1.2 Aerobic granular sludge process

Aerobic granular sludge process is one kind of activated sludge treatment processes, which makes the use of microorganisms that remove organic matters in wastewater. It utilizes microorganisms that self-immobilize to form granules, which are denser and bigger than normal activated sludge flocs. Granules can settle very quickly and be easily separated in the secondary settler.

Granulation is an attractive technology due to its two main advantages. First, granular sludge guarantees high biomass retention inside the reaction tank, which improves the efficiency of the wastewater treatment system in general. Secondly, granular sludge demonstrates high settling velocities facilitating efficient solid-liquid separation in the clarifier, which reduces the costs related to waste sludge dewatering and disposal. Thus, aerobic granular sludge has represented technological breakthroughs for

environmental protection.

In this research, the granular sludge process was combined with the enhanced biological phosphorus removal (EBPR) process, rather than conventional wastewater treatment process. Aimed to promote removal of phosphorus (P) from wastewater without the need for chemical precipitant, EBPR is an activated sludge process recirculating sludge through anaerobic and aerobic conditions. This process is widely used because P is a key nutrient that causes eutrophication in closed water bodies and should be removed following the regulation. Phosphorus is accumulated inside the cell of microorganism, and then be removed from the system as excess sludge. The combination of the granular sludge process and the EBPR can be a breakthrough technology for easier P removal with easier solid-liquid separation of P containing biomass, which finally leads to an effective P recovery technology.

1.1.3 Temporal microbial diversity changes

Aerobic granulation is a complex process, which is dependent on the concerted activity of multiple microbial populations interacting in a trophic web. However, often, microorganisms in activated sludge are regarded as "black-box" entities, with little consideration for the structure and/or function of the microbial communities underpinning the biotechnology. There is, therefore, much scope for the application of molecular techniques to describe the community structure of wastewater treatment biomass and, more importantly, to monitor the temporal fate of practically relevant groups or species in bioreactor systems. With these molecular techniques, it is possible to investigate the key species in granule formation.

Different molecular methods are available now. In order to observe the dynamics of microbial population, denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) are often employed, both in combination with polymerase chain reaction (PCR). In order to grasp whole bacterial population, PCR is performed targeting at a partial 16S rRNA gene. By both DGGE and T-RFLP, bacterial population is profiled as electropherogram in which each species is thought to give a band at a unique position, and the change of band intensity is interpreted as the dynamics of the corresponding species. But in these techniques, it can happen that two or more species yield bands at the identical position. On the other hand, new and powerful sequencing technologies are being rapidly developed, which are often using the principle of pyrosequencing. In pyrosequencing, sequences of as many as or more than 100,000 DNA fragments can be determined in one experiment. The results can give not only information of the identity of bacterial species but also their quantity in the samples. The pyrosequencing approach is thought to be a very promising tool.

1.2 Objectives and Scope

Under the circumstances described above, the present study was focused to grasp the temporal sludge performance and microbial community change of aerobic granular sludge from an EBPR bioreactor.

The key challenge is to achieve granulation while keeping EBPR activity. Thus specific objectives of the study are:

1. To form granulated sludge from conventional activated sludge using anaerobic-aerobic laboratory-scale SBR reactor by controlling settling time, evaluating its treatment performance in EBPR process and capacity of solid-liquid separation.

2. To reveal the microbial community structure and dynamics of aerobic granules cultivated by molecular community analyses.

3. To clarify the relationship between granular sludge treatment performance and bacterial community.

1.3 Research Framework Structure

To achieve the above objectives, a number of experiments were conducted under several main parts as follows;

1. Operation of Lab-scaled reactors and taking samples routinely, to monitor treatment behavior and sludge characteristics.

2. Analysis of the bacterial community of collected sludge samples using T-RFLP and Unidirectional Pyrosequencing methods

3. Exploration on the relationship between granular sludge treatment activity and bacterial populations

4. Further data analysis and interpretation with the sequencing result

1.4 Outline of Dissertation

The dissertation includes 6 chapters and the content of each chapter is briefly described below.

Chapter 1 is the introductory chapter of the present study. In this chapter, the background, objectives, framework of the present study, and the structure of the dissertation are briefly described.

The literature review is given in Chapter 2 and it describes: 1) the fundamentals of aerobic granular sludge processes, the advantage and disadvantage, microbiology and related problems; 2) EBPR process and reclaimed phosphorus reuse; and 3) different methods of bacterial community analysis in brief.

Chapter 3 discusses the details of the experimental designs for the methodology development on reactor operating, sample preparation and arrangement of data.

The results of experiments conducted in this study are presented in Chapters 4 and 5. Chapter 4 discusses the operation of laboratory activated sludge reactors and the behavior of the reactors. Chapter 5 is focused on the analysis of bacterial communities in collected samples. T-RFLP and pyrosequencing data were further analyzed to find relationship between sludge treatment behavior and sludge community.

In Chapter 6, conclusions of the outcomes of present study are summarized, and recommendations for future researches are proposed.

CHAPTER TWO

REVIEW OF LITERATURE

This chapter includes a brief review on aerobic granular sludge (AGS) processes, enhanced biological phosphorus removal (EBPR) and different methods of bacterial community analysis in brief. The sub-sections on AGS illustrate its derivation, its merit and disadvantage, and its microbiology and related problems. The sub-sections of EBPR contain some important part of the process and the possibility of domestic wastewater reclaimed phosphorus reuse. Two latest applicable microbial techniques are introduced as their mechanisms and applications.

2.1 The Aerobic granular sludge (AGS) processes

2.1.1 Activated sludge processes for wastewater treatment

For human health and the environment protection concern, domestic sewage and industrial liquid waste often have to be treated before discharge into natural water bodies. Numerous wastewater treatment technologies have been developed till now. Among these wastewater treatment technologies, biological wastewater treatment is becoming one of the largest bio-industry in the world.

As a method for biological wastewater treatment, activated sludge processes are widely employed in many countries. The process was so named because the treatment performance depends largely on the formation and growth of activated biomass. By mineralization and assimilation process, this activated sludge can remove dissolved and colloidal carbonaceous organic matters in wastewater, and transform them into carbon dioxide gas, water, new cells, and other end products. For successful biodegradation of the organic pollutants, sufficient oxygen has to be provided to satisfy the oxygen demands of aerobically respiring organisms(mainly bacteria and protozoa) and fully mixing of the liquid with biomass has to be achieved (Tchobanoglous et al., 2004).

Activated sludge flocs, which are aggregates formed by diverse group of microorganisms, mainly bacteria, some inorganic particles, and extracellular polymers, are the most usually found activated biomass forms (Snidaro et al., 1997; Seviour and Blackall, 1999; Wilén et al., 2004). For successful treatment, these

bioflocs generated should also have good settling properties to allow them to separate efficiently from the supernatant in the clarifiers (Olofsson, 1998; Liao et al., 2002;Sheng et al., 2006).

2.1.2 Development of aerobic granular sludge process

During the last 20 years, biofilms have demonstrated to be often more efficient for water purification than conventional suspended activated sludge by intensive research in the field of biological wastewater treatment. Considered to be a special case of biofilm, granular sludge were densely packed microbial aggregates whose densities were much higher than that of conventional activated sludge. Granular sludge formation was first described in strictly anaerobic systems in 1980 (Lettinga et al., 1980). And not until the late 1990s had the formation and application of aerobic granules been reported (Morgenroth et al., 1997, Beun et al., 1999 and Dangcong et al., 1999).

Condition favoring the formation of aerobic granules has been extensively studied. To date, the previous studies have demonstrated that sequencing batch reactor (SBR) is the most suitable reactor configuration for aerobic granulation. (Morgenroth et al., 1997, Beun et al., 1999, Tay et al., 2001a, Yang et al., 2003, Liu and Tay, 2004 and Adav et al., 2007a). Granulation process was also thought to be affected by a number of other operational parameters, such as seed sludge (Wilen et al., 2008), substrate composition (Liu and Tay, 2004), organic loading rate (Moy et al., 2002), feeding strategy (Liu et al., 2003), reactor design (Liu and Tay, 2002), settling time (Liu and Tay, 2002), exchange ratio (Wang et al., 2006), and aeration intensity (hydrodynamic shear force) (Adav et al., 2007b).

Several researchers hypothesized that the formation of granules are result from bacteria self-immobilization and found the aerobic granules they got were densely packed with a dark core, indicating the presence of an anaerobic zone within the aerobic granule. (Kim et al., 2004, McSwain et al., 2004, Qin et al., 2004a, Qin et al., 2004b, Wang et al., 2004, Hu et al., 2005 and Liu et al., 2005).

2.1.3 Merits and disadvantages of AGS

Aerobic granules showed many advantages for the applications in wastewater treatment, compared with the conventional activated sludge flocs. Firstly, they have excellent settleability, which shorten the time and space needed for settling tank. Secondly, the dense and strong microbial structure and EPS (extracellular polymeric substances) matrix protect the cells, which thus lead to resilience to shock loadings and tolerance to toxins. Thirdly, long biomass residence time results in high and stable rates of metabolism. Moreover, the biomass immobilization inside the aggregates gives the possibility for bioaugmentation, which can be regarded as an effective tool in the removal of xenobiotics from wastewater (Wuertz et al., 2004;

Bathe et al., 2004). All of these merits have resulted in the fast development of aerobic granular technology.

Despite of the obvious merits aerobic granular sludge systems may have, and the increasing number of publications on lab-scale operation, few reports concerning the engineering application of aerobic granular sludge systems can be found in literatures. This may because the formation and maintenance of aerobic granules in SBR may need relatively high cost associated with aeration and relatively sophisticate operation manners to handle different operational parameters. These could act as the main defect and limit for the scaling up of aerobic granular sludge reactors.

2.1.4 Microbiology of aerobic granule sludge

As one of the most complex activated sludge systems, granular sludge presents a microbial ecosystem consisting of various kinds of microorganisms including bacteria, protozoa, metazoan, viruses, fungi, and algae (Snidaro et al., 1997;Seviour and Blackall, 1999; Liu et al., 2007; Wilén et al., 2008). The core of them is thought to be bacteria. In general, the diverse group of bacteria could divide into two groups depended on the main roles they play: the bacteria which play key role of wastewater purification and the bacteria which produce EPS matrix to keep the structure of the granule.

These bacteria are the "heart" of the aerobic granule sludge system in treatment performance, and their population changes could result in treatment quality fluctuating and sometimes even cause serious problems such as bulking and foaming. When EPS-secreting bacteria or filamentous bacteria growth rate is too fast and accumulated in excessive amounts, they may grow in a dispersed and non-settleable form. This may make sludge settling and compaction slower, and create foam or "scum" which floats on the surfaces of aeration basin and final clarifiers. These settling problems can also lead to washout of the biomass which not only worsen effluent quality but also cause loss of the plant's treatment capacity and even failure of the process.

2.2 The enhanced biological phosphorus removal (EBPR)

Wastewater is increasingly recognized as a viable source of resource and energy. Many wastewater treatment processes are expected not only to produce clean water but also value-added chemicals such as bioplastics and fuels. As one of the most advanced and complicated wastewater treatment processes applied, Enhanced biological phosphorus removal (EBPR) can not only remove contaminating carbon, nitrogen, and phosphate (P) from wastewater but also can recover the P in a more amendable form for reusing comparing with traditional chemical P precipitation. As a nonrenewable resource, the prices of phosphate is in the long trend expected to

rise due to increasing demand and limited global reserves, thus recycle P from EBPR process will become increasingly viable in the future.

2.2.1 Basic idea of EBPR

The process in the Fig 2.1 is the anaerobic-aerobic EBPR activated sludge process used to remove the organic pollutants and phosphorous in influent wastewater. In this process, polyphosphate accumulating organisms (PAOs) predominate in activated sludge and thought to accumulate polyphosphate under aerobic conditions and to use it for energy source to take up organic materials under anaerobic conditions. These organisms can accumulate P in excess of their growth requirement in the form of poly-P. Eventually, the phosphorus-rich waste sludge is removed from the system as excess sludge (Mino et al., 1998).

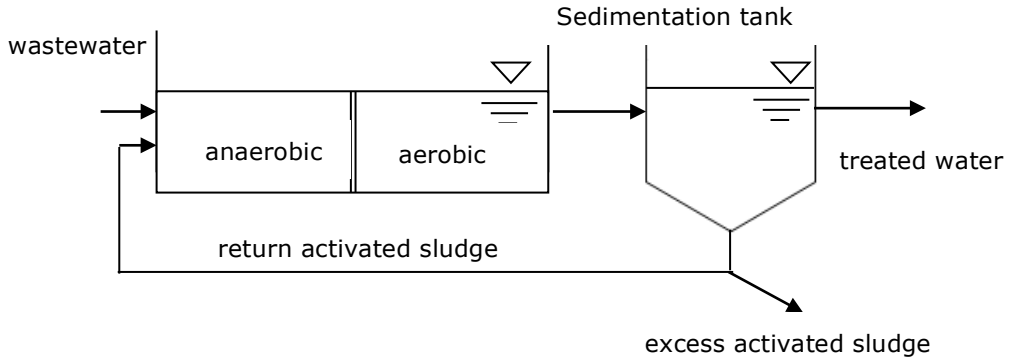


Fig 2. 1 Enhanced biological phosphorus removal (EBPR) processes.

2.2.2 Phosphorous recovery from EBPR excess sludge

Excess sludge from EBPR is phosphorus-rich, and is expected as a potential source of P for recovery and reuse. In excess sludge P is in a more amendable form for reuse comparing with traditional chemical P precipitation. If a sound recycling strategy is developed and applied, the waste sludge could be used as a viable source of raw material for the fertilizer industry.

Different researchers found that poly-P accumulated in excess sludge could be recovered by heating and adding precipitant with high recovery efficiency. Kuroda et al. (2002) reported that by heating sludge at 70°C for about 1 h, 75% poly-P of the total P can be recovered by the addition of $CaCl_2$ at room temperature without adjusting pH. Takiguchi et al. (2007) tested the influence of mineral elements on phosphorus (P) release from heated waste sludge, and found P was associated with Al, Ca, and Mg on the surface of waste sludge biomass. Kugimiya et al. (2008) further found an imprinted polymer showed high binding ability to and selectivity for phosphate in aqueous media, and nearly 70% of highly concentrated phosphate could be recovered. These discoveries can offer a distinct advantage in recovering P from the liquid phase as “biophosphorite”

However, an obvious disadvantage of these heating and precipitating techniques is the additional cost required for heating waste sludge (Kuroda et al. 2002). The more dense the sludge are, the less cost would be need. Thus to make the P recover process more efficient, we need to minimize the amount of the sludge and concentrate it to the best.

2.3 Combination of AGS with EBPR

As mentioned in 2.1.3, AGS has excellent settling ability and dense structure, which largely decrease the volume of waste sludge. Thus the combination of AGS with EBPR can be a promising chance for P reusing.

It is reported that AGS are able to simultaneously remove carbon, nitrogen, and phosphorus from wastewaters in laboratory SBR reactors (de Kreuk et al., 2005; Lemaire et al., 2008; Yilmaz et al., 2008). And two works are found specially for combing EBPR and AGS. Wu et al.(2010,2012) found aerobic granules were formed naturally without the increase of aeration intensity when EBPR was achieved and suggested that with the release of phosphorus during the anaerobic period, positively charged particles are formed, around which activated sludge flocs can be aggregated by electrical force. Dense and compact subgranules can be formed stimulated by these positive charged particles. Gonzalez-Gil et al.(2011) compared microbial populations dominated in propionate-and acetate-cultivated EBPR aerobic granules and reported that EBPR activity in the propionate granules was high and stable, whereas EBPR in the acetate granules was erratic throughout the study and suffered from a deterioration period.

Because aerobic granular sludge is a recent technology, still not much information is available in relation to the microorganisms in the granules, especially in EBPR granules. In contrast to suspended biomass, strong chemical gradients may develop within the granular environment, leading to the formation of distinct microbial niches and granule architecture where competitor such as GAOs populations may closely interact with PAOs. For full-scale applications, detailed knowledge about the dynamics of microbial community structure which guarantees granule robustness concerning efficient nutrient removal is needed.

2.4 Analytical method of bacterial communities

With the rapid development of molecular biological techniques, many microbial molecular ecological techniques have been applied to monitor the variance of the bacterial community in activated sludge (Forney et al., 2004).

2.4.1 T-RFLP method

Among the molecular methods, terminal restriction fragment length polymorphism (T-RFLP) seems to be a more practical and useful approach for bio-monitoring the bacterial community (Osborn et al., 2000). Based on the position of a restriction site closest to a labeled end, T-RFLP is designed for profiling of unknown microbial communities of restriction enzyme cut amplified gene. Because it gives reproducible results for many samples, the method is easy to compare the difference between different samples and experiments. Moreover, the T-RFLP data in digital numerical format allows further statistical analyzes.

Digesting fluorescently labeled PCR products at the 5' end using one or more restriction enzymes at selected region of bacterial genes, the method separate differently sized fluorescently labeled terminal restriction fragments by a capillary or gel electrophoresis and detects the fluorescence intensity. Later the result in form of terminal restriction fragment length and their fluorescence intensity could be used for further bacterial community comparison.

2.4.2 Unidirectional Pyrosequencing

Because of the decreasing cost, DNA sequencing methods have been applied extensively lately for the actual bacterial communities identification. In comparison to conventional Sanger sequencing method, pyrosequencing method is a rapid and cost effective sequencing method.

Unidirectional Pyrosequencing or One-way reads Amplicon sequencing is a special design for pyrosequencing, where increasing the number and length of unidirectional reads is more informative than obtaining a mixture of forward and reverse reads on an Amplicon and the added accuracy that can be derived from bidirectional sequencing is not needed.

In case like this experiment, the author want only metagenomics data that help identifying bacterial species in a complex sample by the sequence of their 16sRNA, thus obtaining more long reads from a common primer starting point will allow to interrogate more species, whereas reverse reads would provide little benefit. The basic design is similar with normal pyrosequencing method but uses fusion primers made with the 'Lib-L' Primer A and Primer B sequences instead. The fusion primers are designed such that directionality of the reads is known and libraries prepared and amplified using the Lib-L chemistry can be sequenced only from the Primer A end. So this design provides only unidirectional sequencing

The data obtained by pyrosequencing could be analyzed by comparing with global DNA data bases such as ribosomal database project (RDP) and DNA data bank of Japan (DDBJ) to know the actual bacterial species in the samples and their phylogenetic relationships.

CHAPTER THREE

MATERIALS AND METHODS

In this chapter, the detailed methodology of the whole experiment is described. The study was accomplished by operating three runs of a granular activated reactor to monitor temporal dynamics of its performance and bacterial population. The seed sludge was from three wastewater treatment plants (Run1 Run2 from municipal wastewater treatment plant, Run3 from a human excreta wastewater treatment plant).

3.1 Reactors Operation

The three granular activated sludge reactors were operated using the same sequencing batch reactor (SBR) which had a working volume of 10L installed in an air conditioned room at 20±2˚C.

Each cycle was controlled to be 4hr in total, consisting of an inflow and anaerobic phase (70min), an aerobic phase (changeable from130 to151 min), a settling phase (changeable from 30 to 3min) and effluent discharge (10min). Nominal sludge retention time (SRT) was controlled at around 7days by discharge of mixed liquor every cycle. Hydraulic retention time (HRT) was 8hr by supplying five liters of synthetic wastewater to the reactor during the influent phase. The composition of the synthetic wastewater was CH3COONa·3H2O 136mg/L, CH3CH2COONa 64mg/L, PEPTONE 120 mg/L, East extract 24 mg/L, KCl 50 mg/L, CaCl2·2H2O 15.8 mg/L, MgSO4·7H2O 132 mg/L, K2HPO4 43 mg/L and trace element solution containing B, Fe, Mo, Co and Cu. The influent carbon concentration was around 120mgC/L and P was around 30mgPO4/L.

Dissolved oxygen (DO) concentration was monitored with a DO electrode (DO-21P, DKK-TOA, Japan), and DO during the aerobic phase was controlled between 2.3 mg/L to 2.5 mg/L. The pH was monitored with a pH electrode (WM-22EP, DKK-TOA, Japan) and pH was not controlled during the operation..

As mentioned in Character 2, granulation process was thought to be affected by a number of operational parameters, such as seed sludge, substrate composition, organic loading rate, feeding strategy, reactor design, settling time, exchange ratio, and aeration intensity (hydrodynamic shear force). Here the granulation was achieved simply by reducing the settling time and the shortened settling time was added to aerobic phase to extended starvation period, which stimulate the granulation as some

researcher argued.

In general, during the first 10 days, the settling time was gradually reduced from 35 to 10 min; then gradually it was decreased to the minimum (2-3) min; and finally, it was increased a little and maintained at 5 min. The concentration of mixed liquor suspended solids (MLSS) was controlled in between 1000 mg/L and 2000 mg/L by adjusting settling time.

3.2 Sampling

To monitor the reactor performance, liquid samples were taken and analyzed for dissolved organic carbon (DOC), PO4–P and other anion and cation. During the experimental period, samples for the measurement of dissolved organic carbon (DOC) and anions including phosphate were collected at the end of the anaerobic and aerobic phases of selected cycles. Samples for the measurement of mixed liquor suspended solids (MLSS), sludge volume index (SVI), dewaterability, and microbial population analyses were collected at the end of the aerobic phases.

3.3 Treatment performance analysis

The samples for the measurement of DOC and anion analyses were filtered through a piece of 25mm glass fiber filter (GF/C , Whatman, England), and then stored at 4˚C until analyses.

Dissolved organic carbon (DOC) concentration in supernatant was measured with a TOC analyzer (TOC-Vcsn, Shimadzu, Japan).

Anions were analyzed 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.

The concentrations of MLSS were measured according to the Standard Methods (APHA, AWWA,2005).

Settling ability which represented with sludge volume index (SVI) was determined by following the methods described in APHA (2005).

Dewaterability was monitored by the WCDS testing method which was developed by Dr Ning Li in our lab. The protocol is as follows:

As shown in Fig 3.1, 25 ml mixed liquor was filtered by 45mm filter (15 min) for

thickening, and then around 300mg-wet thickened sludge were loaded on piece membrane filters which were placed in a Swinnex filter holder (25mm, Millipore, Japan) for dewatering. The filter holder was set in a bucket for 50 ml conical tubes (No. 053-5010, Kubota, Japan) and centrifuged at 2000g for 5 min to exert dewatering process. At last, the dewatered sludge cake (about 100 mg) was moved onto a piece of prepared aluminum foil (dry weight c of which had been predetermined), and the dewatered sludge weight including aluminum foil a was measured, with the foil folded to cover the sludge to avoid drying during weight measurement. After unwrapped and dried at 105°C for 24 hour and cooled to room temperature in a desiccators for 30 min, the dry weight of the sample b was measured. Water content of dewatered sludge (WCDS) was calculated as (a-b)/ (a-c). Measurement of weight was done with an analytical balance XS105 Du (Mettler Toledo, Switzerland) with a resolution of 0.01mg. Analyses were done in duplicate.

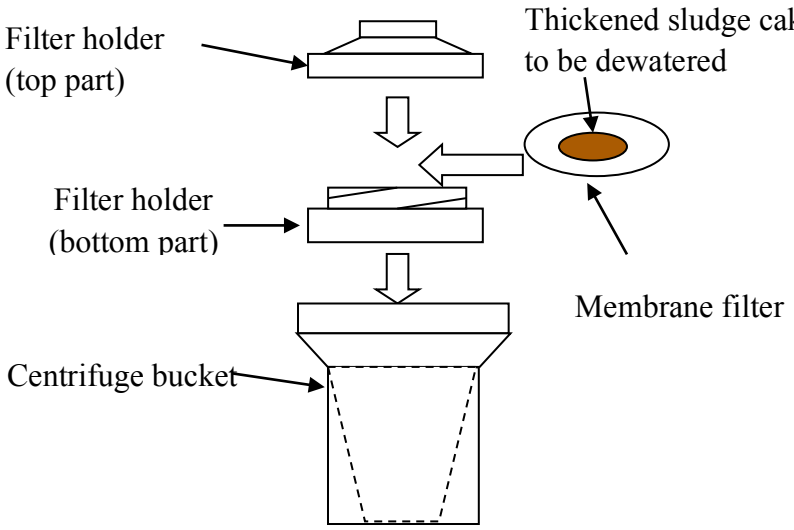


Fig 3. 1 WCDS dewaterability testing method and applied apparatuses

Granule size distribution analysis was done under microscope The particle size distribution for a full-scale granular sludge reactor has been characterized experimentally by Vlaeminck et al. (2010), resulting in 6 granule size classes with relative abundance, which was used in our study to group the granules.

3.4 Bacterial community analysis

3.4.1 PCR compatible DNA template Preparation

Activated sludge samples for bacterial community analyses were taken from the reactors during the aerobic phase and immediately 1:1 mixed with pure ethanol (0.75mL mixed liquor with 0.75mL pure ethanol) and stored at -80 °C after sonicated at amplitude of 30%(around 7W) for 15s by 250DA advanced digital sonifier (Branson).

The above stored samples were thawed in room temperature, remixed, and their DNA concentrations were checked with a Pico-Green dsDNA Quantification kit (Invitrogen) according to the manufacturer's instructions. Then the samples were diluted to reach final DNA concentrations to about10 to 30 pg/μL and used as template for PCR(which will further10 times diluted when mixed with PCR mixture).

3.4.2 PCR and T-RFLP

PCR reaction was performed using universal primer 27f forward primer (5'-AGAGTTTGATC(A/C)-TGGCTCAG-3') labeled with 6-carboxyfluorescein (FAM) on the 5'end and 519r reverse primer (5'-G(A/T)ATTACCGC -GGC(G/T)GCTG-3'), which is targeted at a partial 16S rDNA gene. ExTaq Hot Start Version (Takara, Japan) was employed as the enzyme for PCR, and the composition of the PCR mixture was as follows when the reaction was done at a 20 μL scale: 2μL 10× buffer, 1.6μL 2.5mM dNTP mixture, 0.4μL forward primer, 0.4μL reverse primer, 0.1μL ExTaq, 13.5μL autoclaved ultrapure water, and 2μL template solution. And thermal cycles were programmed with 95°C for 600s followed by 30 cycles of (94°C for 30 s, 55.3°C for 30s and 72°C for 30s) followed by 72°C, 600s using Thermal Cycler Dice (Takara, Japan). PCR product concentration was quantified using Pico-Green dsDNA Quantification kit (Invitrogen) according to the manufacturer's instructions.

PCR products were purified by a QIAQuick PCR Purification kit (Qiagen, Valencia, USA) and digested with *Rsa*I (which recognize a sequence of 5'-GTAC-3', and cut between T and A in the restriction site) (New England Biolabs, Ipswich, Massachusetts) at a 10μL size including 5μL of purified PCR product (around 50-75ng of DNA), 1μL of 10×restriction buffer, 1.6 U of restriction enzyme, and sterilized Milli-Q water, at 37°C for 4 hours followed by heating at 65°C for 15 minutes.

The digested samples were added with DNA marker (GeneScan 500 ROX Size Standard, Applied Biosystems) and HiDi Formamide (Applied Biosystems), and heated shortly and cooled quickly to denature the double-stranded DNA molecules. And then samples were analyzed by an ABI300 GeneticAanalyzer (Applied Biosystems, Japan) to obtain the electropherograms (T-RFLP profiles). POP-4 (Performance Optimized Polymer 4, Applied Biosystems, USA) was used as the gel and 45cm capillary was used.

3.4.3 PCR- Prosequencing

For pyrosequencing, PCR was conducted by the same method as described in the previous section except for the primers used. The primerse 27f and 519r were modified according to the instructions provided by Roche Inc. For each sample, a unique forward primer was used. That is, each of forward primer was appended with a unique barcode sequence and an adapter sequence (adapter A). On the other hand, the

519r reverse primer was appended only with an adapter region (adapter B), and was commonly used for all samples. In total, 64 samples were analyzed by pyrosequencing. The primers and their assignments to each sample are listed in Appendix 1 and Appendix 2.

PCR product concentration was quantified using Pico-Green dsDNA Quantification kit (Invitrogen) according to the manufacturer's instructions.

PCR products were purified by High Pure PCR clean Up Micro Kit (Roche, USA) and then QIAQuick PCR Purification kit (Qiagen, Valencia, USA) according to the manufacturer's instructions.

The purified samples, at the same DNA amount, were all mixed together, and a 100µl solution containing 1 µg of DNA was prepared. To check the quality of purified samples, Agilent 2100 Bioanalyzer was used to obtain the electropherogram.

The pyrosequencing was done by Center for Omics and Bioinformatics/Department of Computational Biology, Graduate School of Frontier Sciences, The University of Tokyo.

Read names were assigned automatically by QIIME as follows:

(f/r).samplename_readnumber

Where

(f/r): if the read was sequenced from the forward primer side, "f" is assigned, and if from the reverse primer side, "r" is assigned.

samplename: the name of the sample,

readnumber: read number in the same sample.

The reads named as above were further analyzed by QIIME. The combined reads were grouped into operational taxonomic units (OTUs) at 97% similarity, and the taxonomic identities were assigned by the Ribosomal Database Project classifier.

Then at last all the outcomes from QIIME analyses were imported to OTUMAMi by the command pick_otus_through_otu_table.py. And the QIIME pretreated pyrosequencing data were analyzed by OTUMAMi developed by Satoh et al. (2012) with the help of QIIME 1.5.0 (Caporaso et al., 2010).

CHAPTER FOUR

RESULT 1 Treatment Performance

4.1 Run 1:

4.2 Run 2

4.3 Run 3

4.4 Comparison between Results of the three Run

The author operated 3 different runs (from 2011 January to 2012 April) with seed sludge from three wastewater treatment plants (Run1 Run2 from municipal wastewater treatment plant, Run3 from a human excreta wastewater treatment plant). Run1 was successfully operated for nearly 3 months which confirmed the possibility of getting EBPR AGS by shortening settling time. Unfortunately, Run 1 had to be ended because of the great earthquake on March 11, 2011. Then Run2, Run3 was operated for a long time till deterioration happened to compare bacteria community. Granule size distribution and dewaterability have been monitored accurately in Run 3, hoping to clarify the relationship of microbial community to the fluctuation in sludge performance.

4.1 The First Run

The first Run is operated to establish the operational manner and check the efficiency of get granular sludge by shortening settling time. The performance of Run 1 is shown in Fig 4.1~4.3.

4.1.1 Granulation and settling ability

Monitoring was started when granules started to appear, which was referred to as day 0. Granules grew gradually on subsequent days and thus settling time was cut off dramatically. Mature granules were formed and stabilized in the reactor quickly within 24 days. And from day 25 to day 40, most biomass was in granular form (approximately 90% of granules had diameters of >0.5 mm). The average granule sizes of granules were around 1.5 ± 0.5 mm.

As shown in Figure 4.1, MLSS was initially at 2000 mg/L, and kept above 1200 mg/L during stable period. From day 4th to day 8th, MLSS declined dramatically with the rapid shortening of settling time. And to stop the significant biomass loss, shortening of settling time was slowed down after day 8. Because of the granules' good settling properties, settling time was further shortened from 5to 2 minutes after day 24.

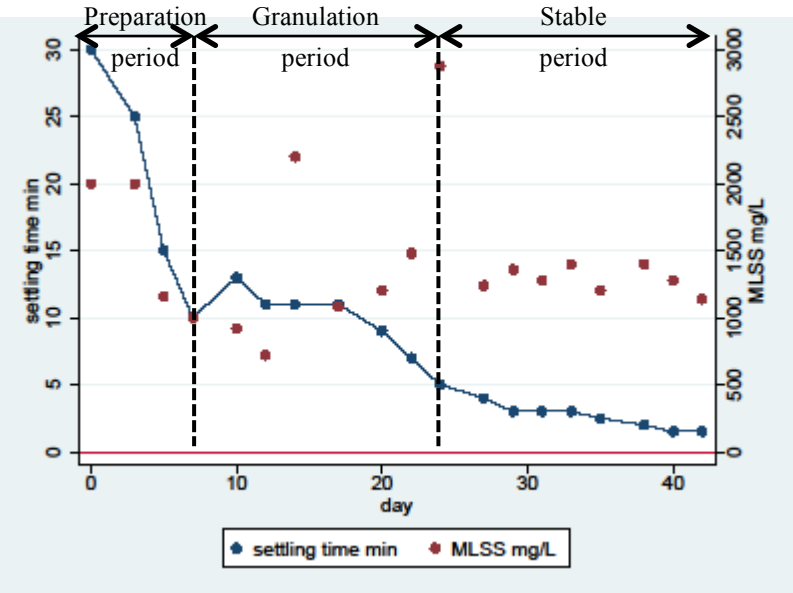


Fig 4. 1 Granulation process of Run1

4.1.2 Water quality

Fig 4.2 shows DOC both at the end of anaerobic (An-DOC) and aerobic phase (Ox-DOC). And Fig 4.3 shows PO_4-P both at the end of anaerobic (An-PO_4-P) and aerobic phase (Ox-PO_4-P). Both carbon removal and P removal kept stable during the operation. The TOC removal efficiencies remained, in general, higher than 98%, while almost 100% of the influent P was removed. The P-release to C-uptake ratio is a good indicative of the EBPR activity (Pijuan et al., 2009). The experimental values were 0.317 mmol P/ mmol C. These values are normal in comparison with other EBPR systems, where this ratio was expected between 0.250 and 0.320 mmol P mmol/C, depending on the carbon source. (Pijuan et al., 2009)

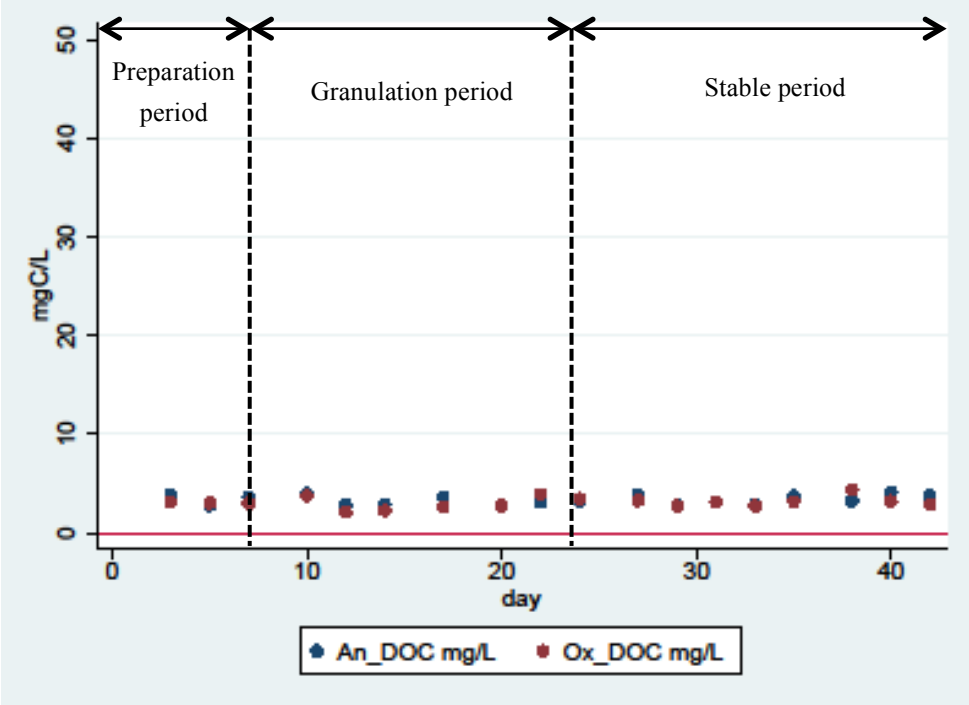


Fig 4. 2 Profiles of An-DOC and Ox-DOC of Run 1 during operation

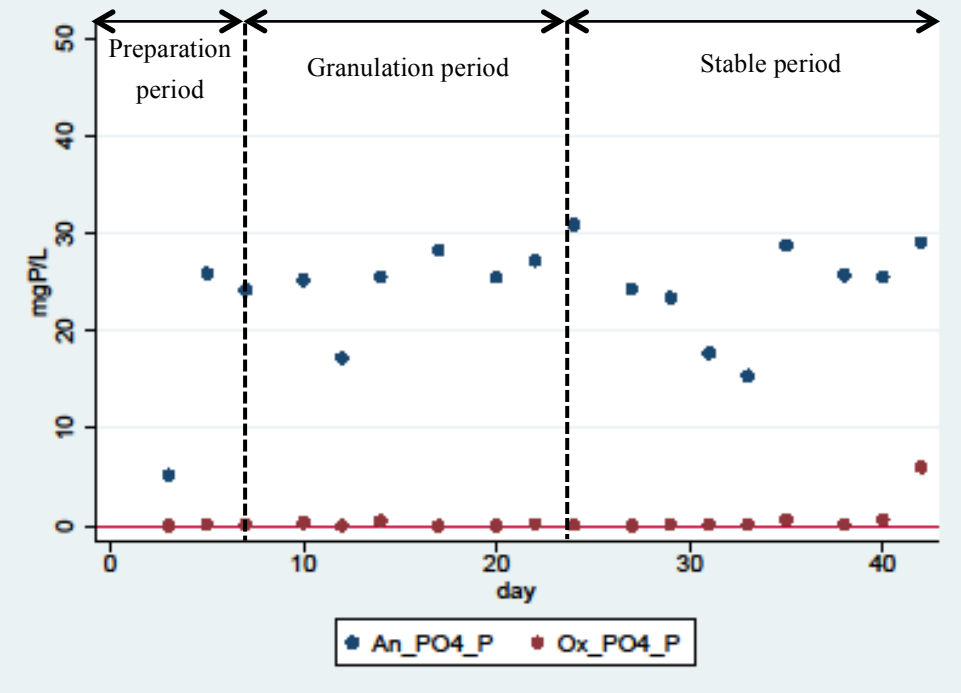


Fig 4. 3 Profiles of An-PO4-P and Ox- PO4-P of Run1 during operation

4.2 The Second Run

Figures 4.4~4.6 show the performance of Run 2.

4.2.1 Granulation and settling ability

As shown in Fig 4.4, monitoring of Run2 was started when granules started to appear, which was referred to as day 0. Big granules started to appear on the 11th day and then grew gradually on subsequent days. Mature granules get prevailed in the reactor within 28 days operation. Stable granule treatment was observed from day 31 to day 69 when most biomass was in granular form (approximately 90% of granules had diameters of >0.5 mm). The average granule sizes of granules were around 1.5 ± 0.5 mm.

As shown in Figure 4.4, MLSS was initially at 2500 mg/L, and kept around 1200 mg/L during stable period. However after day 69th, with settling ability getting worse, MLSS dropped to less than 1000 mg/L. In general the granules had worse settling properties than Run 1, and settling time was shortened to 8 minutes at minimum. To prevent significant biomass loss, settling time was increased back to 18min after day 110th to recover the biomass.

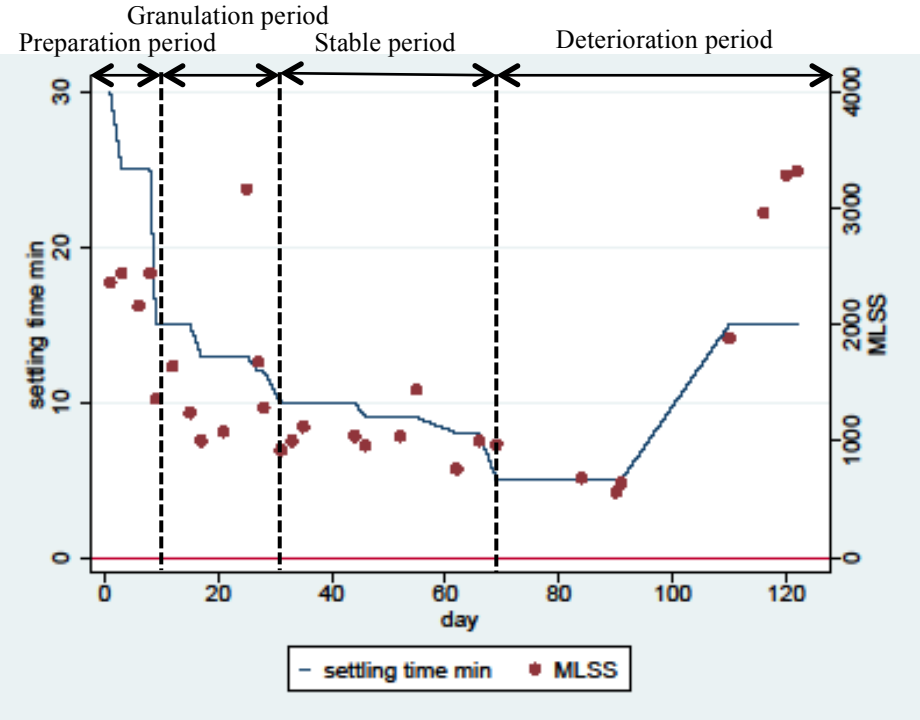


Fig 4. 4granulation process of Run2

4.2.2 Water quality

Fig 4.5 shows DOC both at the end of anaerobic (An-DOC) and aerobic phase (Ox-DOC). And Fig 4.6 shows PO_4-P both at the end of anaerobic (An-PO_4-P) and aerobic phase (Ox-PO_4-P).

EBPR treatment behavior changed in a pattern that independent with granulation period. During good EBPR period before day 53th, both carbon removal and P removal was relatively stable, though from time to time phosphate concentration in water after the aerobic phase was high. The TOC removal efficiencies remained higher than 98%, while, in general, almost 90% of the influent P was removed. The

P-release to C-uptake ratio is a good indicator of the EBPR activity (Pijuan et al., 2009).

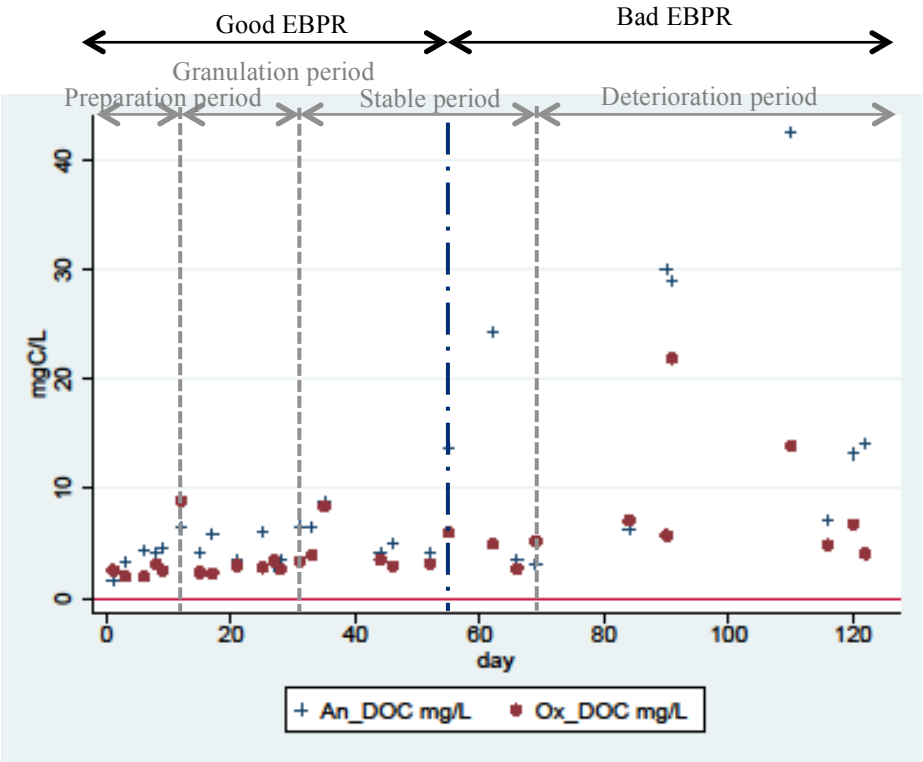


Fig 4. 5 Profiles of An-DOC and Ox-DOC of Run 1 during operation

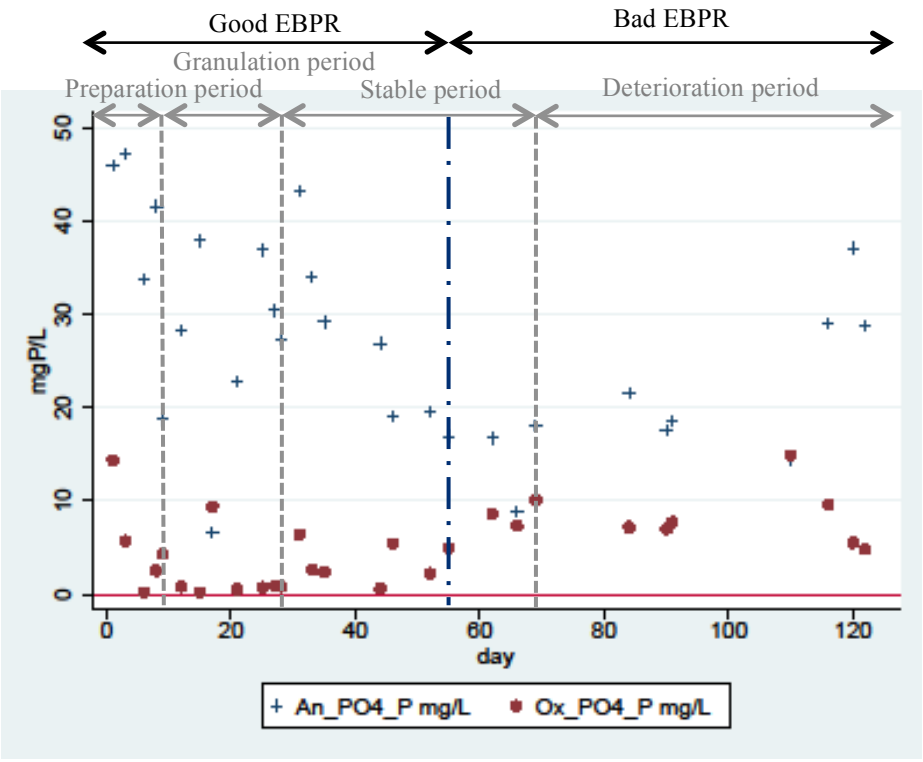


Fig 4. 6 Profiles of An-PO4-P and Ox- PO4-P of Run1 during operation

However, EBPR treatment behavior was poor after the day 53th. At this bad EBPR

stage, the microbial community consumed all carbon during the aerobic phase, and there was only 25% or even no phosphorus removal. At day 63, the P-release to C-uptake ratio during the anaerobic phase became extremely low which means a loss of EBPR property. During this period, overgrowth of finger-type and filamentous microorganisms over the granules was observed under microscope and granules lost their compactness.

This deterioration may be considered as a kind bulking. According to (Picioreanu et al.,2000) irregular biofilm structures tend to be formed when the values of the ratio between biomass growth rate to diffusive transport are high. In this reactor, the shear force of stirring may not enough to keep the granule size, the granule became bigger and this, on one side, caused DO concentrations lower than needed inside the granule, which means higher biomass growth rate to diffusive transport consequently. This rate thus favored the sharp-edged biofilms formation as Picioreanu (2000) mentioned.

Actually, Mosquera-Corral et al. (2005) also observed similar overgrowth of filamentous structures onto the granules surface in a SBR operating with similar influent concentrations when working at relatively low DO concentrations (3.5 mgO_2 /L) and the DO concentration here is less than that (2.3-2.5 mgO_2 /L). This may give an explanation of why granulation deterioration happened right after EBPR got worse.

Though Caravelli et al. (2004, 2007) suggested that dose of chlorine, ozone and other chemicals are effective to control bulking but in the present study, such countermeasures were not tried and Run 2 was stopped.

4.3 The third Run

Unlike Run1 and Run2, monitoring of run3 was started from the very beginning of inoculation. With the experience in Run1 and Run2, the author aimed to go further in Run3 to also observe every sludge solid-liquid separation capacity.

4.3.1 Granulation and process

As illustrated in Fig 4.7, monitoring was started when seed sludge was inoculated, which was referred to as day 0. A conservative settling time (30 min) was initially used for biomass acclimation to the influent. From day 14 to 35, the settling time was progressively reduced, from 25 min to 10 min, to increase the hydraulic pressure selection to select fast settling biomass, favoring granules development. In general the granules had worse settling properties than Run 1, and settling time was shortened to 8 minutes at minimum.

MLSS was generally stable with the shortening of settling time (decreasing right after the shortening and increasing slowly latter). However MLSS was not stable during stable period. After the day 50th, with settling ability getting worse, MLSS decreased

due to the flushing out. To prevent significant biomass loss, settling time was increased back to 12min after day 50th and to 18min after day 56th to recover the biomass.

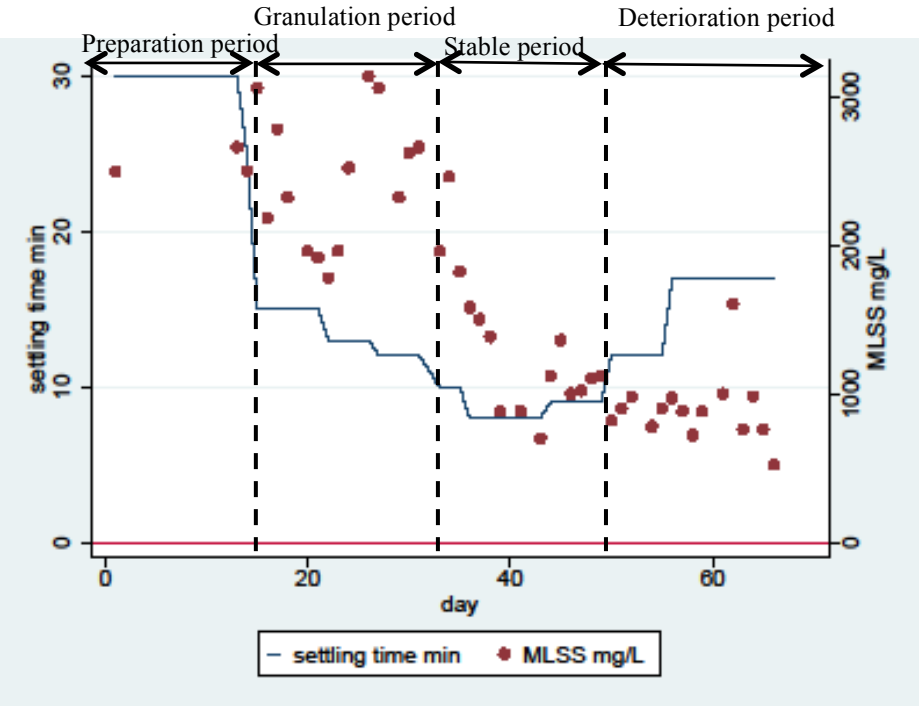


Fig 4. 7granulation process of Run3

During the reactor start-up period, granules started to appear on 14th day and then grew gradually on subsequent days. Complete granulation was observed after day 31. And from day 33 to day 49, most biomass was in granular form (approximately 90% of granules had diameters of >0.5 mm). The average granule sizes of granules were around 1.5 ± 0.5 mm. The average diameter distribution of granule before deterioration is showed in Fig.4.8.

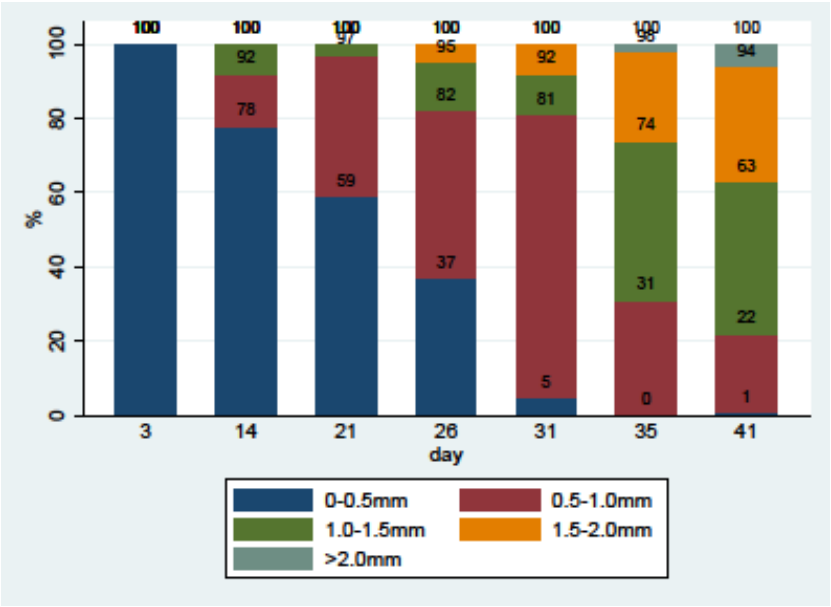


Fig 4. 8 average diameter distribution of granule of Run3

4.3.2 Sludge solid-liquid separation capacity

The SVI (sludge volume index) is used to monitor sludge settle ability and is usually measured after 30 min of sedimentation. The lower the SVI value, the better the sludge sediments. As a reference, the activated sludge used as the inoculum had an SVI30 value of 130 mL/g, which is common for this type of biomass.

Figure 4.9 shows the profile of SVI during the operation of the reactor. Compared with sludge EBPR performance fluctuating, solid-liquid separation showed more stable patterns. SVI30 of sludge stayed between 80 to 200 ml/mg before deterioration, which indicated good settling ability and sludge health condition. After day 34th, big flocs were the predominant biomass structures in the reactor with a higher SVI30 (150-200 mL/g). The SVI30 rapidly increased remarkably after day 50, which caused MLSS decreased due to the flushing out.

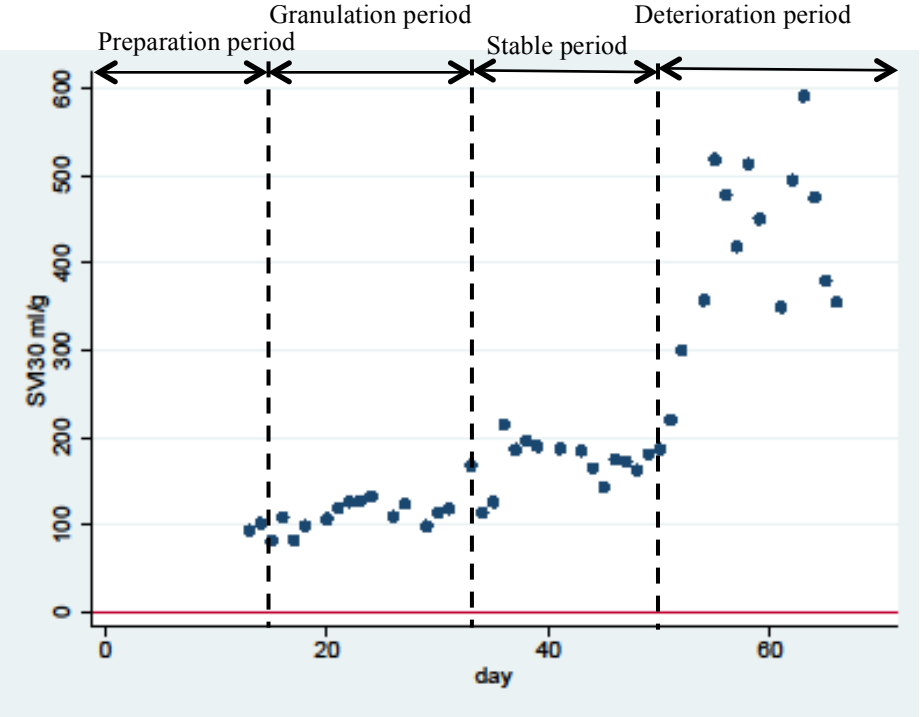


Fig 4. 9 SVI30 profile of Run 3 during operation

Water content percentage of centrifuging dewatered sludge (WCDS) was used to show the dewaterability of sludge, as shown in Fig.4.10. The higher the WCDS value, the worse the dewaterability is. The WCDS values ranged from 82.5% to 93.0%, and the values fluctuated dynamically during the operation of each reactor. It is interesting to point out that after day 56 in granule deterioration period, WCDs suddenly become lower, this may also indicate the broken of granule compact structure.

Jin et al. (2004) reported that sludge containing higher amounts of bound water showed higher SVI, reflecting poorer sludge dewaterability. However, in these experiments, the monitored fluctuation of WCDSs did not show clearly correlations with SVI.

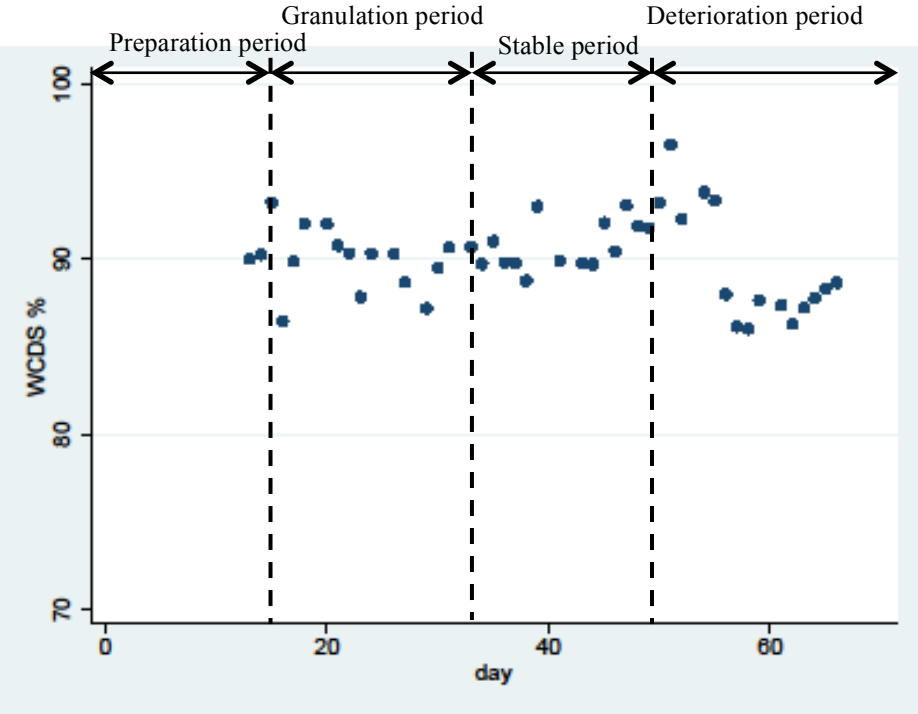


Fig 4. 10 dewaterability showed by water contend of centrifuging dewatered sludge of Run3

4.3.3 Water quality analysis

Results of the sludge treatment performance are shown as DOC both at the end of anaerobic (An-DOC) and aerobic phase (Ox-DOC) in Fig 4.11 and PO_4-P both at the end of anaerobic (An-PO_4-P) and aerobic phase (Ox-PO_4-P) in Fig 4.12.

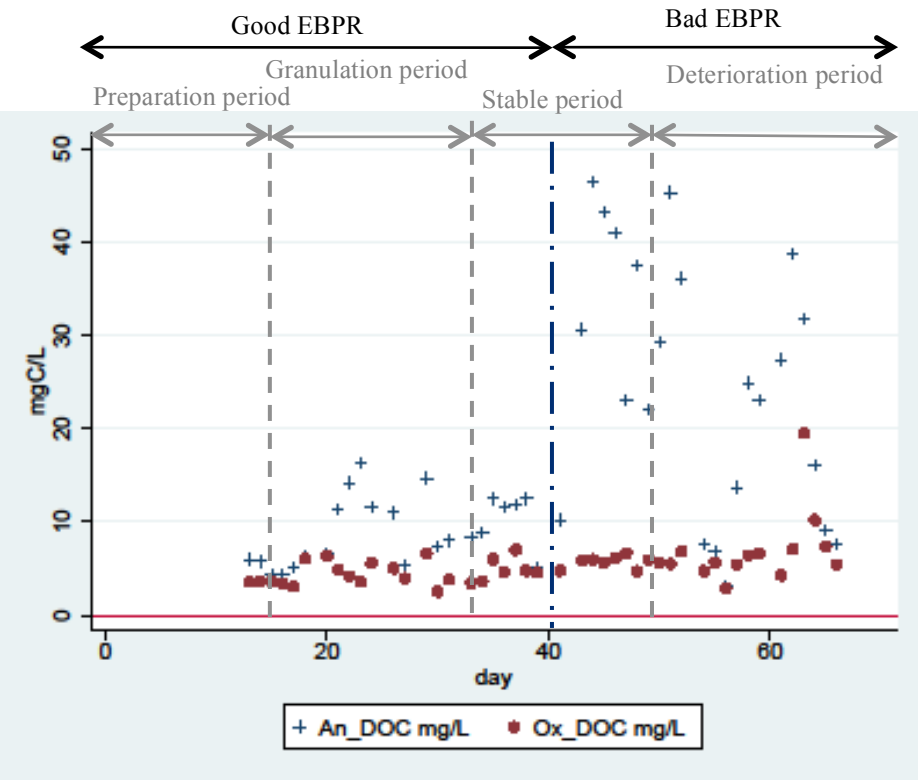


Fig 4. 11 Profiles of An-DOC and Ox-DOC of Run 3 during operation

EBPR treatment behavior changed in a pattern that independent with granulation period. During good EBPR period before day 41th, organic carbon concentrations before and after anaerobic and aerobic phase kept relatively stable and low, while P removal fluctuated depending on the settling time shortening and changes in granule diameter. The TOC removal efficiencies remained, in general, higher than 95%, while P removal fluctuated. .

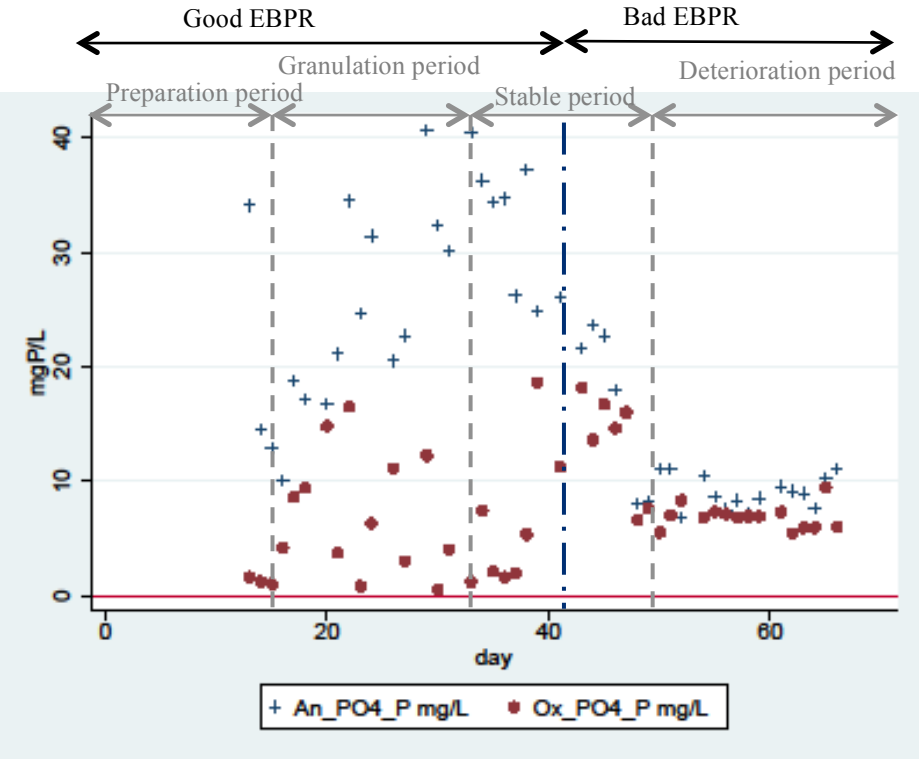


Fig 4. 12 Profiles of An-PO4-P and Ox- PO4-P of Run3 during operation

However, EBPR treatment behavior was poor after the day 41th. At this bad EBPR stage, the microbial community consumed all carbon only in the aerobic phase, and there was no phosphorus removal.

4.4 Comparison between Results of the Three Runs

In the present study, three different granulation EBPR runs were monitored. In all of these runs granular sludge treatment and solid-liquid separation behavior were to some extent satisfying, showing that granular EBPR process can be achieved by shortening settle time.

However, during granulation progress, different runs showed different performance patterns. In Run1, EBPR treatment was very stable and complete granulation was achieved with the settling time shortened to 2.5minutes. However in Run2 and Run3 EBPR performance fluctuated with time. In the next chapter, microbial population in Run 2 and Run 3 are analyzed.

CHAPTER FIVE

Monitoring Of Microbial Population

In this chapter, the detailed microbial population changes in the run2 and run3 are described. To identify bacterial community, microbial population was first profiled by T-RFLP in section 5.1. With a list of relative higher intensity T-RFs, bacteria population dynamic was roughly graphed. Then in section 5.2, with the help of OTUMAMI software, pyrosequencing reads were digested. Major OUTs were listed out and their partial phylogenetic tree and heat map are given. OTU reads were further analyzed to different changing patterns and the expected T-RFs of these DNA reads were identified.

5.1 T-RFLP Results

Figure 5.1.1 shows an example of the obtained T-RFLP profiles, in which terminal restriction fragments (T-RFs) peaks are showed with the horizontal axis as the size and the vertical axis as the intensity.

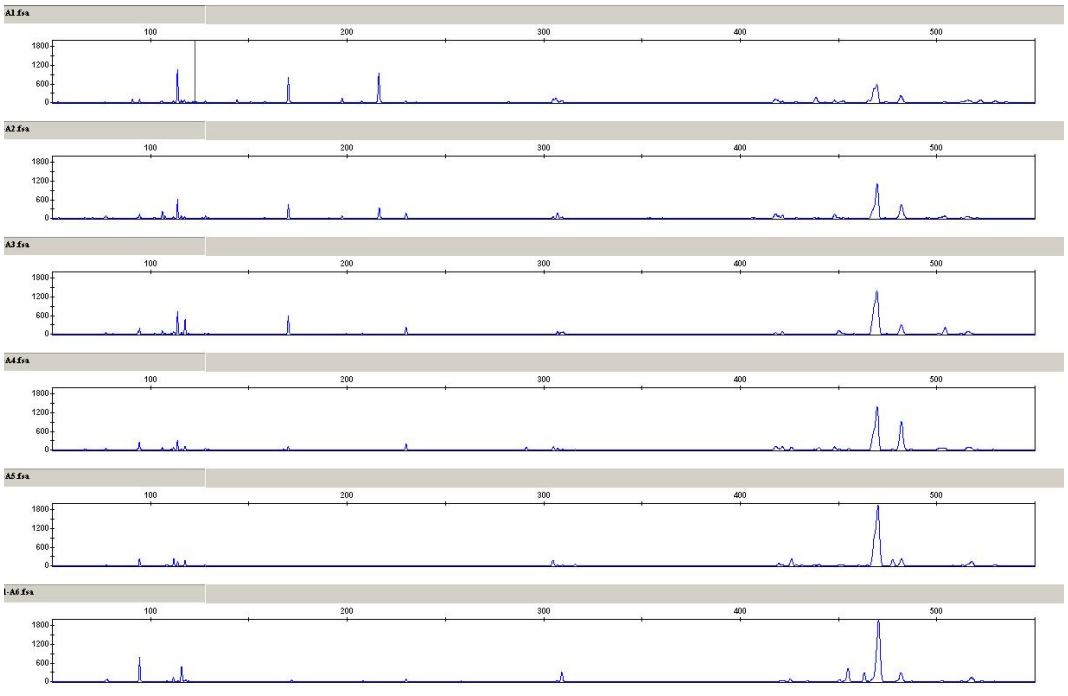


Fig 5. 1Examples of raw T-RFLP profile obtained by ABI 310

To compare bacterial populations in each sample, the T-RFLP profile should be further quantified. Since the assignment of T-RF size of the peaks is at a possible

error range of ±1 bp, the author first manually corrected the assignment of T-RF size by checked the T-RFLP pattern. Then intensity of each peak was standardized by dividing the peak heights by the sum of all the detected T-RFs heights for the sample. The standardized peak heights will be referred to as "relative intensity" here after.

Major T-RFs were selected by calculate cumulative relative intensity of peaks one by one in a descending order, until the value exceeded 50%. The T-RFs which were used at least one time in this calculation were selected as major T-RFs. Other T-RFs were combined into "others" as they only stand for minor of relative intensity.

The results of the microbial population of Run2 and Run3 analyses by the PCR/T-RFLP method are shown in Figure 5.2 and 5.3. In both reactors, dominant T-RFs changed during the operation. However, the dominant T-RFs were a little different with each other.

For Run 2, as shown in Fig 5.2, 10 T-RFs were selected as major T-RFs by the above criteri. During the operation, the most dominant T-RFs were 115bp, 117bp and 121bp. These three seems to show a competition relationship: when one increased, two others decreased. And the most dominant T-RFs were always changing inside these three T-RFs. In the initial 4 days or so, T-RF 115bp was the most dominant. Then from the day 9th, T-RF 115bp decreased, and T-RF 121bp became dominant. After day 99th, T-RFs 115bp were dominant once again. Other T-RFs, such as T-RFs 97bp, T-RFs 107bp, T-RFs 306bp and T-RFs 309bp also shifted during the observation.

For Run 3, as shown in Fig5.3, 12 T-RFs were selected as major T-RFs. During the operation, the most dominant T-RFs were 115bp, 119bp and 122bp. Unlike Run2, in Run3 T-RF 119bp was almost dominant during the whole operation. And T-RF 307bp in Run3 was much more than those in Run2 (compared with T-RFs 306bp in Run2 for possible error range of ±1 bp). This may because the initial seed sludge was different: Run2 from a domestic WWTP and Run3 from a night soil treatment plant. An interesting thing here is that before day 45th T-RFs 82bp and T-RFs 97bp existed with average relative intensity larger than 5%, however, after day 49th, T-RFs 82bp and T-RFs 97bp disappeared with average relative intensity larger than 2%. At the same time before the day 45th T-RFs 93bp was almost less than 1%, however after the day 49th T-RFs 93bp suddenly began to increase, and on the day 66th it occupied more than 15% relative intensity. As described in Chapter 4, in Run3 the treatment performance and settling ability began to deteriorate after the day 45th. These T-RFs may thus give a clue of the cause of deterioration.

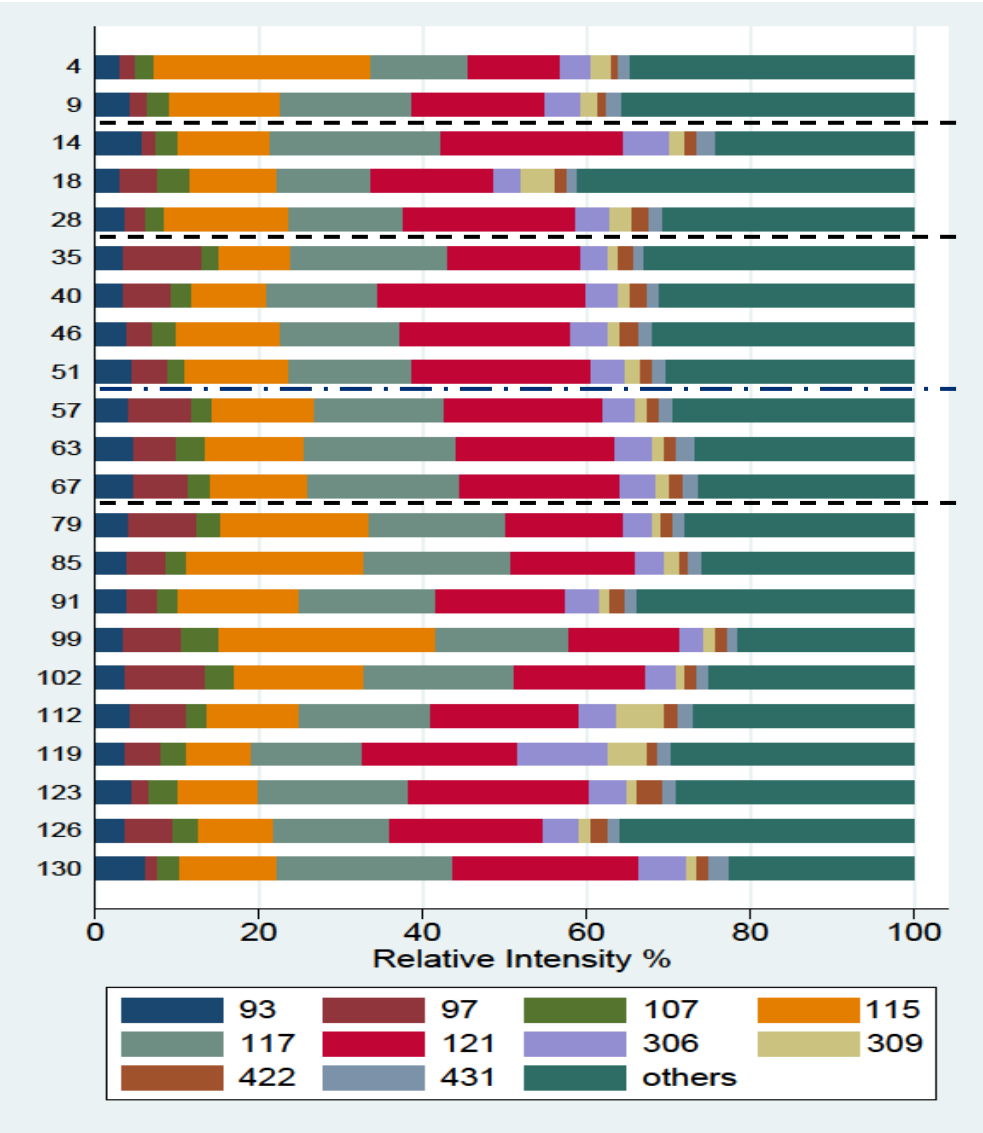


Fig 5. 2 Relative intensity analysis of T-RFLP profiling of Run 2

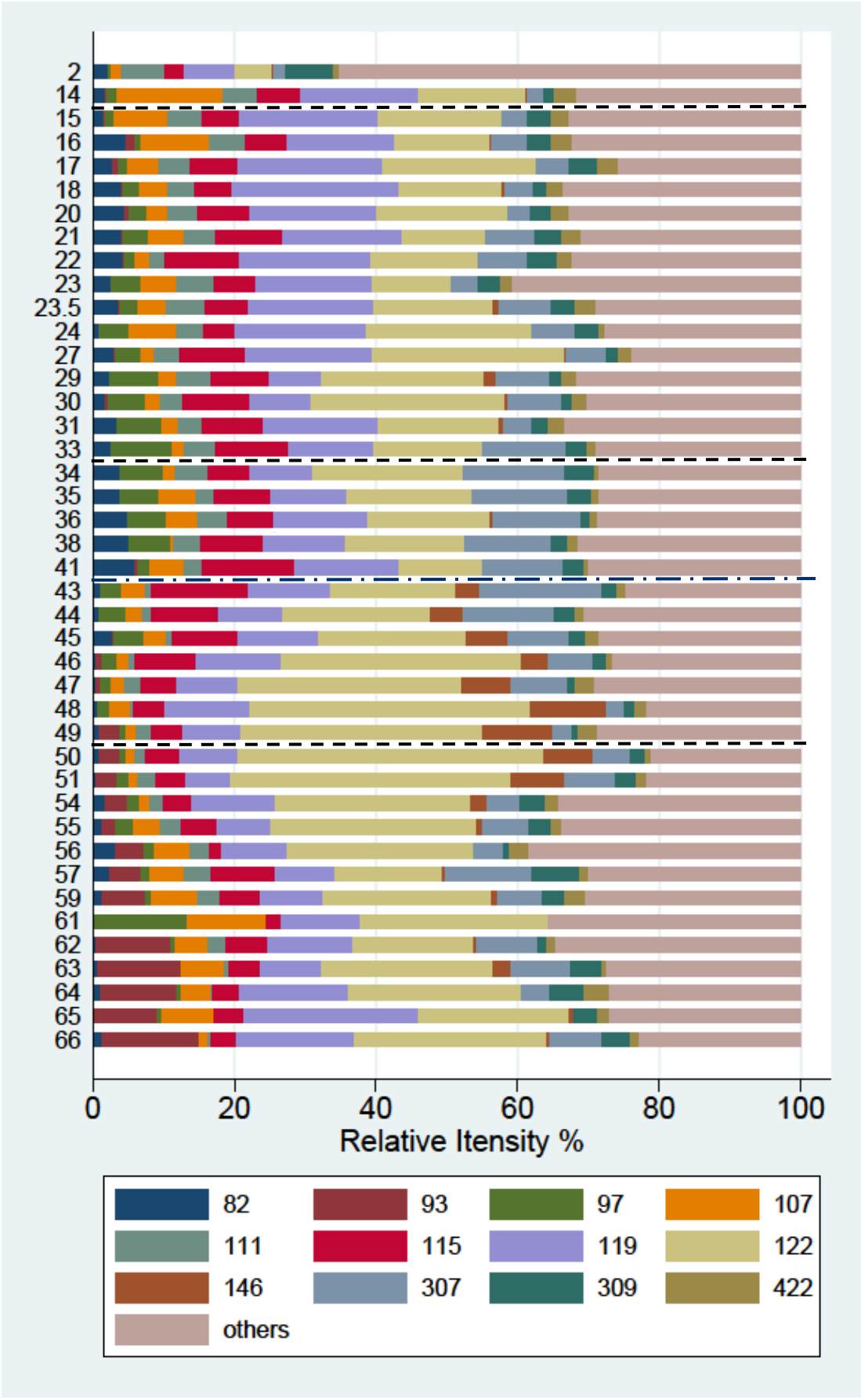


Fig 5. 3 Relative intensity analysis of T-RFLP profiling of Run 3

5.2 Pyrosequencing Results

Pryrosequencing data was treated by OTUMAMI to get OTU intensity of each samples. OTU numbers are automatically assigned by QIIME, and major OTU was selected by compare their mean fraction with the threshold (>0.5% herein). In details, for sample i, fractions $f_{i,j}$ is defined as OTU j's read count percentage:

$$f_{i,j} = \left(\frac{C_{i,j}}{\sum_i C_{i,j}}\right) \times 100 \ [\%] \quad \text{(a)}$$

And mean fraction $\bar{f}_j$ were calculated as follows:

$$\bar{f}_j = \sum_i f_{i,j} \times \frac{100}{N} \ [\%] \quad \text{(b)}$$

where $C_{i,j}$ indicates the read count of OTU j in sample i, and N is the total samples number.

Those OTUs which had mean fraction $\bar{f}_j$ values larger than 0.5% are defined as "major OTUs". OTU intensity is showed with color gradient; the deeper the color the more intensified bacteria (red-yellow-white).

5.2.1 Strains related with function of EBPR and aerobic granule sludge

In EBPR process there are two main groups of bacteria: polyphosphate (poly-P)-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) (Crocetti et al., 2000; Seviour et al., 2008; Zilles et al., 2002). Phosphorus removal is achieved by PAOs which are mainly consisting of the Betaproteobacteria species, while GAOs compete for carbon but do not contribute to phosphorus removal (Crocetti et al., 2002; Oehmen et al., 2007). GAOs are classed with Gammaproteobacteria and Alphaproteobacteria. And Alphaproteobacteria are further ordered as Sphingomonas and Defluvicoccus vanus (Meyer et al., 2006; Wong et al.,2004). PAOs outcompeted by GAOs is one of the explanation for EBPR failure.

For aerobic granule sludge, three classes of Proteobacteria (including Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria) and two classes of Bacteroidetes (including Flavobacteria and Sphingobacteria) was reported to have a high propensity for self-aggregation and thus is common in acetate-fed granules (Williams et al., 2006; Jiang et al., 2007; Liu et al., 2010). Specially, the study of Juang et al (2009) showed that Burkholderiales under Betaproteobateria has close relationship with granulation. Finding the abundance of class Sphingobacteria and Flavobacteria under Bacteroidete was low in hydrophilic phase but high in

hydrophobic phase, researcher also observed main species of Bacteroidetes changed from class Sphingobacteria to Flavobacteria during the granulation. (Guo et al., 2011).

The species usually involved in the functions of EBPR and granular sludge and their special feature was summarized and listed in Table 5.1.

Table 5. 1 species related with EBPR and granular sludge

Phylum	Class	Order	EBPR	Granulation
Proteobacteria	Alphaproteobacteria,	Sphingomonas	GAOs	Hydrophilic Self-aggregation
		Defluvicoccus vanus	GAOs	
	Betaproteobacteria,	Burkholderiales	PAOs	
	Gammaproteobacteria		GAOs	
Bacteroidetes	Flavobacteria			
	Sphingobacteria			

5.2.2 Pyrosequencing data of Run 2

For Run 2, 22 samples were analyzed as shown in Table 5.2.

As shown in Figure 5.4, 24 Major OTUs were selected to present the main change in microbial community of Run2. A3 data was deleted because it only gave 83 reads, which is too less to give confident result.

Table 5. 2 Sample name, the related day and the total reads of Run 2

Sample name	f.OuA1	f.OuA2	f.OuA3	f.OuA4	f.OuA5	f.OuA6	f.OuA7	f.OuA8
day	4	9	14	18	28	35	40	46
Total reads	1042	492	83	1383	735	1064	874	788
Sample name	f.OuB1	f.OuB2	f.OuB3	f.OuB4	f.OuB5	f.OuB6	f.OuB7	f.OuB8
day	51	57	63	67	79	85	91	99
Total reads	574	734	410	488	696	700	776	988
Sample name	f.OuC1	f.OuC2	f.OuC3	f.OuC5	f.OuC7	f.OuC8		
day	102	112	119	123	126	130		
Total reads	941	617	1038	717	1318	1122		

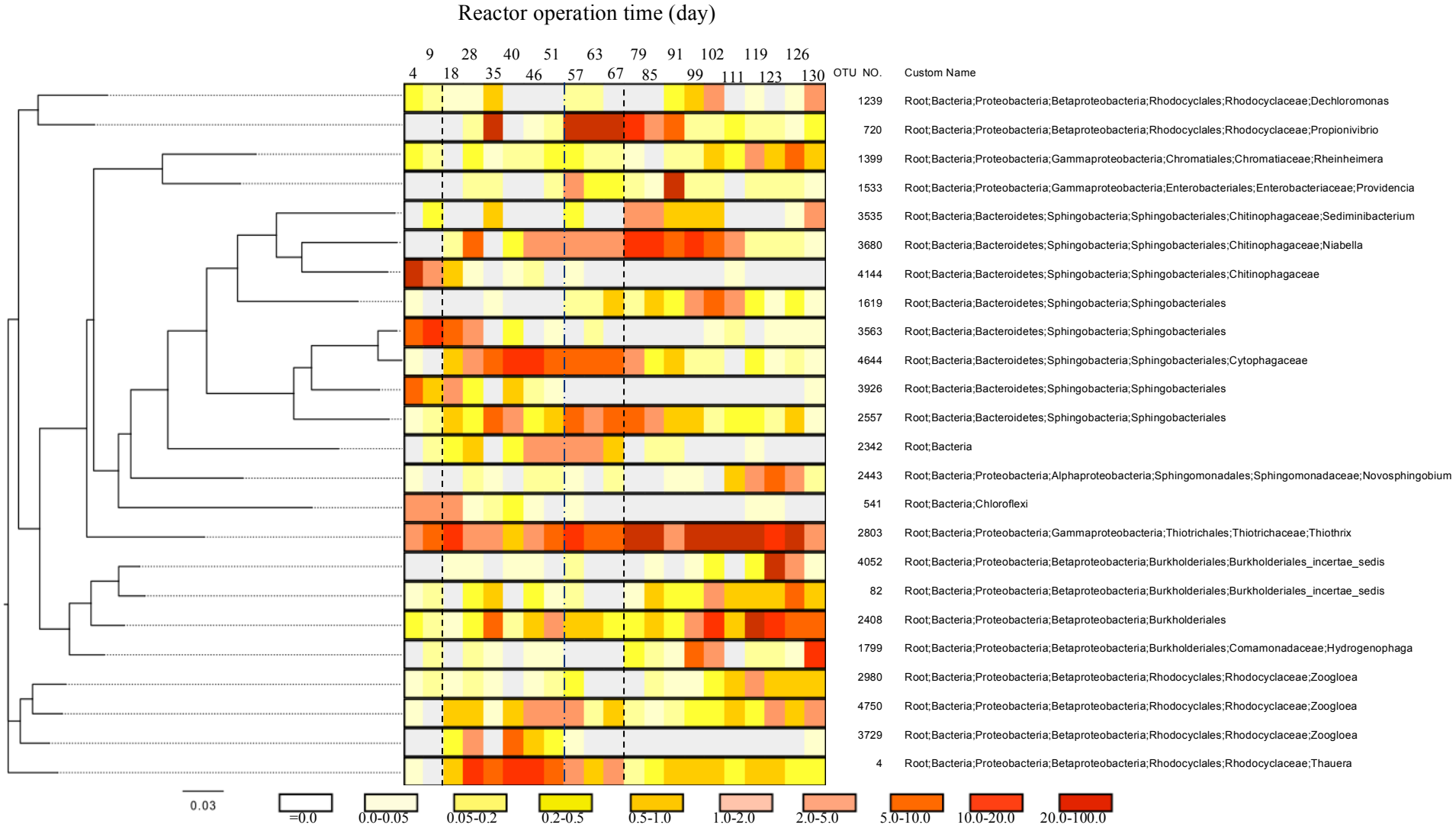


Fig 5. 4 Partial tree and heatmap distribution of major OTUs of Run 2

The three lines in the heatmap is stand for day 11, day63, and day69 which are the changing point as shown in chapter4. Before day 11, there was little granule, and from day 11 to day 69 was the granule predominant period, then after day69 granule deteriorated. Similarly, before day 63 EBPR performance was good and after that EBPR collapse. The major OTU shows close relationship and shifted accordingly.

The OTUs abundant between day 11 to day 69 and disappear latter should stand for species related with granulation, such as OTU4614 (*Cytophagaceae* sp.) and OTU4 (*Thauera* sp.).

And similarly, the OTUS abundant before day 63 and disappear latter should stand for species related with EBPR. OTU720 (*Propionivibrio* sp.) and OTU2557 (Sphingobacteriales, unclassified sp.) for example, is thought to be related with EBPR performance.

OTUs those were little before day 63 and 69 but become dominant in the latter days, such as OTU2433 (*Novosphingobium* sp.), OTU2408 (Burkholderiales, unclassified sp.)and OTU 1799 (*Hydrogenophaga* sp.)should be species compete with EBPR AGS and have bad effect.

The compositions of reads were summarized at phylum and class levels, as shown in Figs. 5.5 and 5.6. The sequence reads were assigned to 18 Phyla and 37 Classes in total, with combining the minor ones together as others.

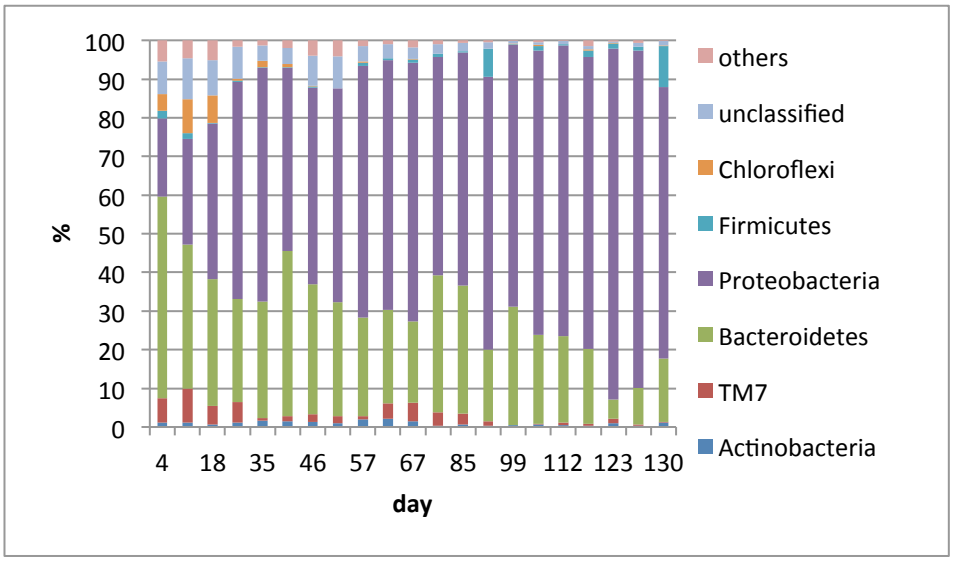


Fig 5. 5 Taxonomy Assignments of reads at Phylum level of Run 2

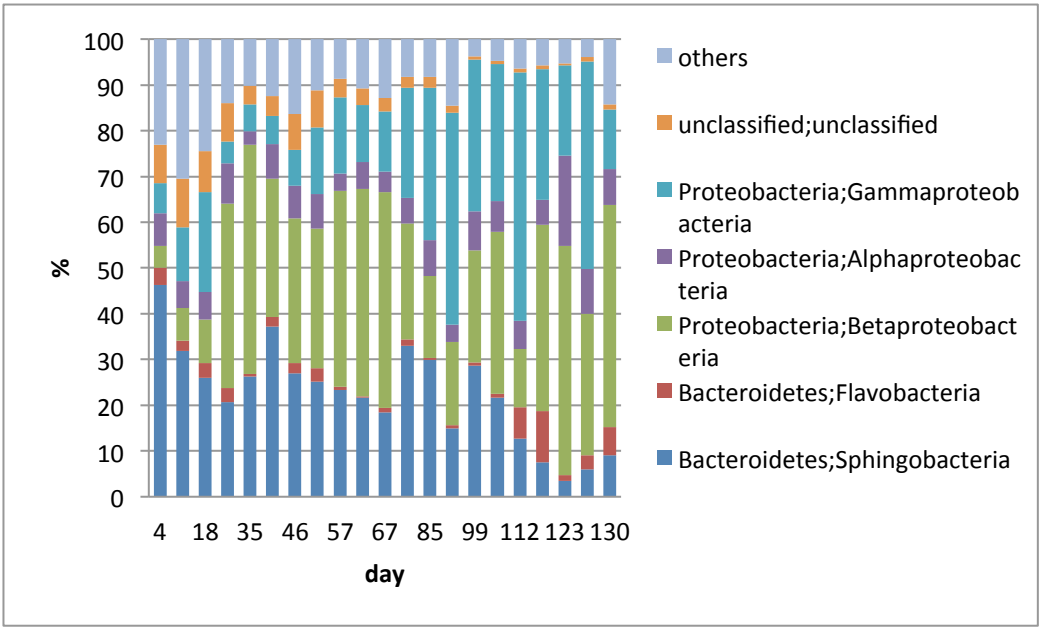


Fig 5. 6 Taxonomy Assignments of reads at Class level of Run 2

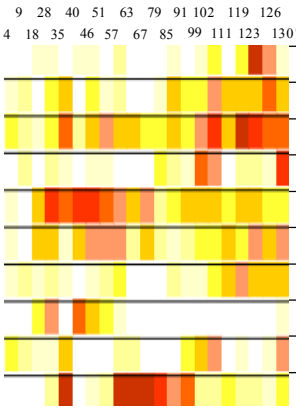
From Fig 5.5, the whole microbial population was mainly Proteobacteria and Bacteroidetes. Main species changed from class Bacteroidetes to Proteobacteria with the former decrease while the latter increase. And from Fig 5.6, the composition of Proteobacteria was not stable, changed from Betaproteobacteria domain to Gammaproteobacteria domain gradually.

Checking of major OTUs to see the detail of the community dynamic was done. Different OTUs of the same class showed different changing patterns during monitoring. According to their phylum, the author divided the major OTUs into three different groups to analyses, as shown in Fig5.7 to Fig 5.10.

To compare the pyrosequencing results with T-RFLP patterns, expected T-RF sizes were calculated for each major OTU.

1) Group A: Proteobacteria.

Figure 5.7 shows the assignments of T-RFs and major OTUs in Betaproteobacteria.

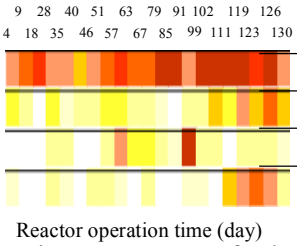


OTU.	Custom Name	T-RF
4052	Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiales_incertae_se	418
82	Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiales_incertae_se	465
2408	Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales	
1799	Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Hydroge	
4	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Thauera	421
4750	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Zoogloea	120
2980	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Zoogloea	120
3729	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Zoogloea	120
1239	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Dechloromo	118
720	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Propionivibri	420

Fig 5. 7 OTUs of Betaproteobacteria changing and their calculated T-RFs of Run 2

The population of *Burkholderiales* kept growing during the whole observation, indicating the progress of granulation. However, the candidates of PAOs of floc-forming Rhodocyclaceae showed different pattern, while *Thauera*, *Propionivibri* and some kind of *Zoogloea* reached maximum in the middle term of the observation. Anyhow for *Zoogloea* of OTU2980 reached maximum in the last period when EBPR deteriorated.

Figure 5.8 shows the assignments of T-RFs and major OTUs in Alphaproteobacteria and Gammaproteobacteria.



OTU	Custom Name	T-RF
2803	Root;Bacteria;Proteobacteria;Gammaproteobacteria;Thiotrichales;Thiotrichaceae;Thiothrix	484
1399	Root;Bacteria;Proteobacteria;Gammaproteobacteria;Chromatiales;Chromatiaceae;Rheinheimera	407
1533	Root;Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;Providencia	425
2443	Root;Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;Novosphin	422

Fig 5. 8 OTUs of Alphaproteobacteria and Gammaproteobacteria changing and their calculated T-RFs of Run 2

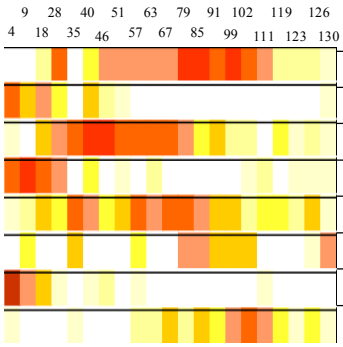
Filamentous-type *Thiothrix* sp. bacteria was predominant right from the beginning granules were formed till the end, with the population of it kept on increasing. This dominance is quite stable. Theories for the formation of anaerobic and aerobic granules suggest that filamentous microorganisms serve as backbones for granular structure and the population of it should be controlled with selective pressure of settling time. (Beun J. J., et al. 1999). However, in my observation here, Thiothrix sp. kept growing and eventually caused bulking of the sludge.

Rheinheimera sp. and *Novosphingobium* sp. were considered to be members of GAOs which exist through the observation as a competitor. They kept stable at a low populationwhile PAOs working well and began to dominate when EBPR deteriorated.

Providencia sp. found in many EBPR process took a long time to grow more and become most in the middle of the observation.

2) Group B: Bacteroidetes.

Figure 5.9 shows the assignments of T-RFs and major OTUs in Bacteroidetes.



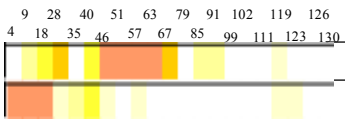
OTU.	Custom Name	T-RF
3680	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Chitinophagaceae;Niabella	114
3926	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales	450
4644	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Cytophagaceae	472
3563	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales	472
2557	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales	97
3535	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Chitinophagaceae;Sediminib	118
4144	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Chitinophagaceae	116
1619	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales	224

Fig 5. 9 OTUs of Bacteroidetes changing and their calculated T-RFs of Run 2

Though Flavobacterial population was not low in the reactor as shown in Fig5.6, no OTU of Flavobacteria was found to be major OTUs. Thus OTUs only contained Sphingobacteria. Some species of Sphingobacteria predominate at the very beginning and latter disappeared. Most of Sphingobacteria dominate when mature granular sludge has formed and granulation was completed. After the day 112, Sphingobacteria lost their dominant position which may indicate granule collapse.

3 Group C: other bacteria which was not dominant

Figure 5.10 shows the assignments of T-RFs and major OTUs in other bacteria.



OTU.	Custom Name	T-RF
2342	Root;Bacteria	370
541	Root;Bacteria;Chloroflexi	426

Fig 5. 10 other OTUs and their calculated T-RFs of Run 2

Chloroflexi is a phylum of photosynthesis bacteria which does not relate too much with wastewater treatment but exist in WWTP seed sludge. Thus it soon disappeared after incubation of the sludge.

The summarized comparison of the virtual digested and experimental T-RF sizes are shown in Table 5.3. In processing T-RFLP profiles, the ±1bp was considered the same species with the several intensified T-RFs. By comparing virtual digested and experimental observed T-RFs size and intensity, major T-RFs were connected with OTUs .

Table 5. 3 Comparison between virtual digested and observed T-RFs of Run 2

	T-RFLP	Virtual Digestion of OTU	Taxonomy
T-RFs	93(92~94)		
	97(96~98)	OTU2557, OTU1916	Bacteroidetes;Sphingobacteria
	107(106~108)	OTU1239	Betaproteobacteria
	115(114~116)	OTU3680, OTU4199,	Bacteroidetes;Sphingobacteria
	117(116~118)	OTU1239, OTU3535,OTU4144	Bacteroidetes;Sphingobacteria
	121(120~122)	OTU4750, OTU2980,OTU3729,	Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Zoogloea
	306(305~307)	OTU82,	Betaproteobacteria;Burkholderiales
	309(308~310)		
	422(421~423)	OTU720,OTU1533,OTU2443, OTU2408,OTU1799,OTU4,OTU2980	Betaproteobacteria; Alphaproteobacteria and Gammaproteobacteria
	431(430~432)	OTU2803, OTU1399,OTU2342	Gammaproteobacteria

5.2.3 Pyrosequencing data of Run 3

For Run 3, 44 samples were analyzed as follows:

Table 5. 4 Sample name, the related day and the total reads of Run 3

Sample name	f.OuD1	f.OuD2		f.OuD4	f.OuD5	f.OuD6	f.OuD7	f.OuD8
day	2	14		15	16	17	18	20
Total reads	935	960		1069	1075	884	877	935
Sample name	f.OuE1	f.OuE2	f.OuE3	f.OuE4	f.OuE5	f.OuE6	f.OuE7	f.OuE8
day	21	22	23	23.5	24	27	29	30
Total reads	1476	818	1319	856	514	915	1328	744
Sample name	f.OuF1	f.OuF2	f.OuF3	f.OuF4	f.OuF5	f.OuF6	f.OuF7	f.OuF8
day	31	33	34	35	36	38	41	43
Total reads	517	468	664	708	714	796	498	616

Sample name	f.OuG1	f.OuG2	f.OuG3	f.OuG4	f.OuG5	f.OuG6	f.OuG7	f.OuG8
day	44	45	46	47	48	49	50	51
Total reads	1202	1015	1062	1063	1189	868	938	1075
Sample name	f.OuH1	f.OuH2	f.OuH3	f.OuH4	f.OuH5	f.OuH6	f.OuH7	f.OuH8
day	54	55	56	57	59	61	62	63
Total reads	1134	1323	644	984	1984	100	783	828
Sample name	f.OuJ1	f.OuJ2	f.OuJ3					
day	64	65	66					
Total reads	714	310	628					

As shown in Figure 5.11, 30 Major OTUs were selected automatically to present the main change in microbial community of Run3. H6 data was deleted because it only gave 100 reads, which is too less to give confident result.

The three lines in the heatmap is stand for day 14, day41, and day49 which are the changing point as shown in chapter4. Before day 14, there was little granule, and from day 14 to day 49 was the granule predominant period, then after day49 granule deteriorated. Similarly, before day 41 EBPR performance was good and after that EBPR collapse. The major OTU shows close relationship and shifted accordingly.

The OTUs abundant between day 14 to day 49 and disappear latter should stand for species related with granulation, such as OTU4750(*Zoogloea* sp.), OTU454 (*Aquiflexum* sp.) and OTU4 (*Thauera* sp.).

And similarly, the OTUS abundant before day 41 and disappear latter should stand for species related with EBPR. OTU1921,OTU3758(*Propionivibrio* sp.)and OTU316 (*Cytophagaceae* sp.) for example, is thought to be related with EBPR performance.

OTUs those were little before day 41 and 49 but become dominant in the latter days, such as OTU1211, OTU2803 (*Thiothrix* sp.), OTU 1399 (*Rheinheimera* sp.),OTU555,OTU1239(*Dechloromonas* sp.) and OTU 2200(*Cloacibacteriu.*sp.) should be species compete with EBPR AGS

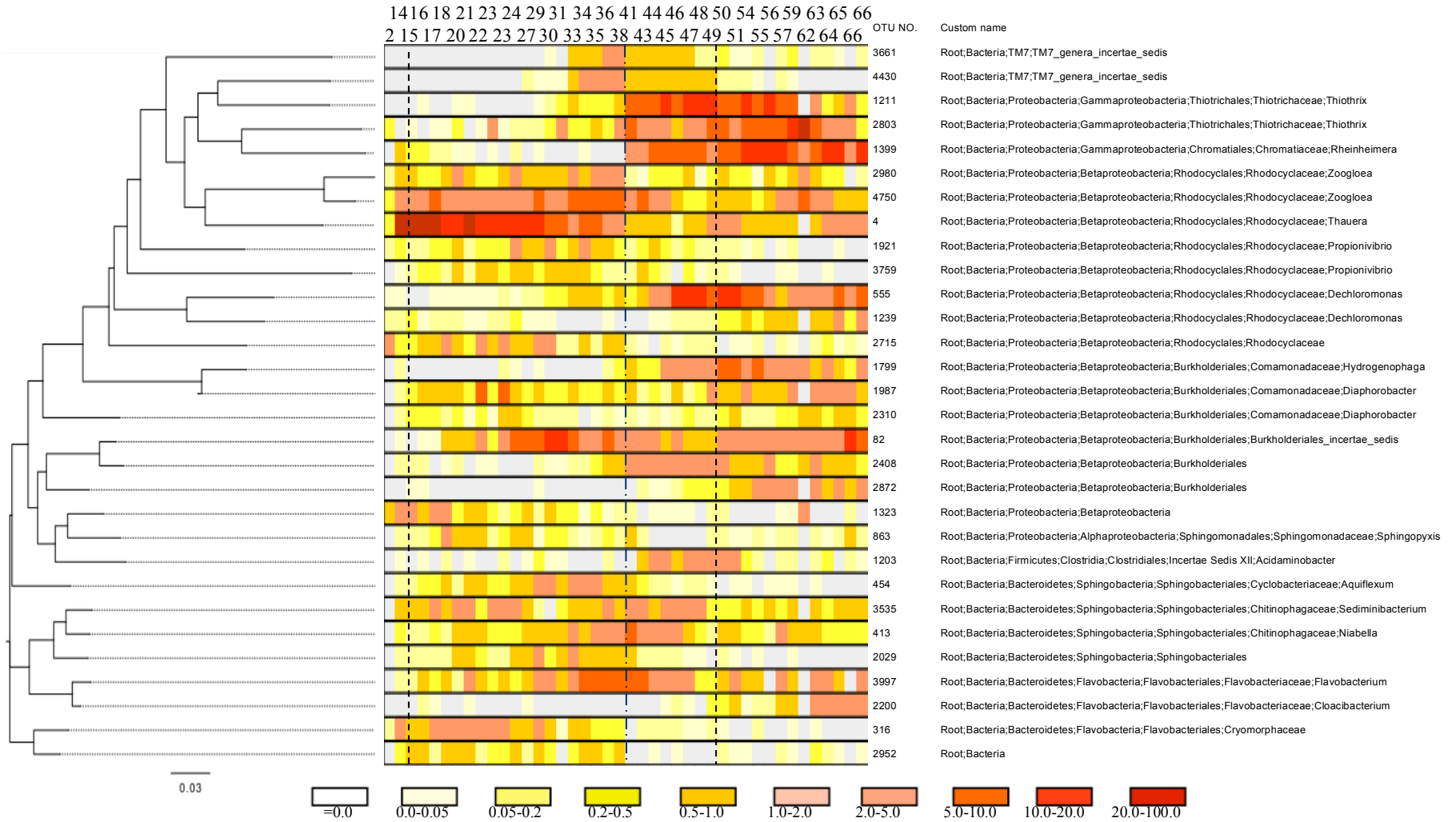


Fig 5. 11 Partial tree and heatmap distribution of dominant OTUs of Run 3

The results are obtained from taxonomic assignment and calculation of microbial community composition. The sequence reads were assigned to 17 Phyla and 42 Classes in total. The reads without assigned to any taxonomic affiliation were grouped as unclassified, and their community percentage was also calculated. To draw the taxonomic dynamic picture of microbial community at Phyla and Class level, the Phylum and Class with minor reads were combined together as others. And the composition change was clearly shown for each sample in Fig 5.12 and Fig 5.13.

As shown in Fig 5.12 Proteobacteria and Bacteroidetes were predominant and consisted about 87% of the total reads. Though fluctuated, Proteobacteria species kept domain during the observation and population Bacteroidetes decreased after day 48 when the settling ability became worse. And from Fig 5.13, the composition of Proteobacteria was not stable though Betaproteobacteria kept domain. Gammaproteobacteria population increased a lot after day 40. Bacteroidetes population decrease largely after day 45.

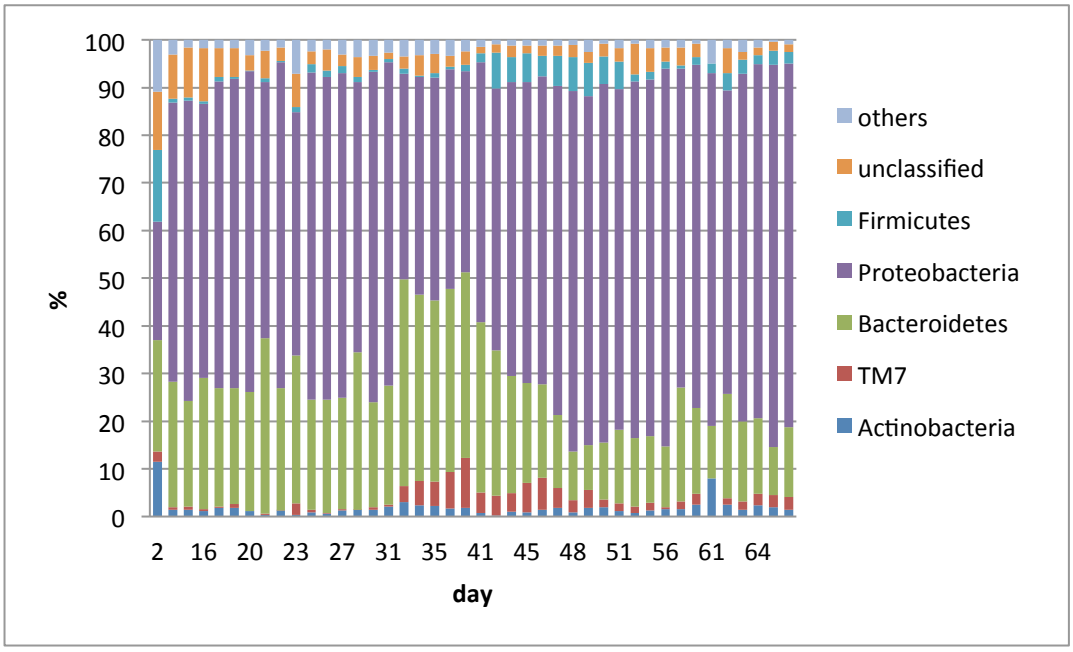


Fig 5. 12 Taxonomy Assignments of reads at Phylum level of Run 3

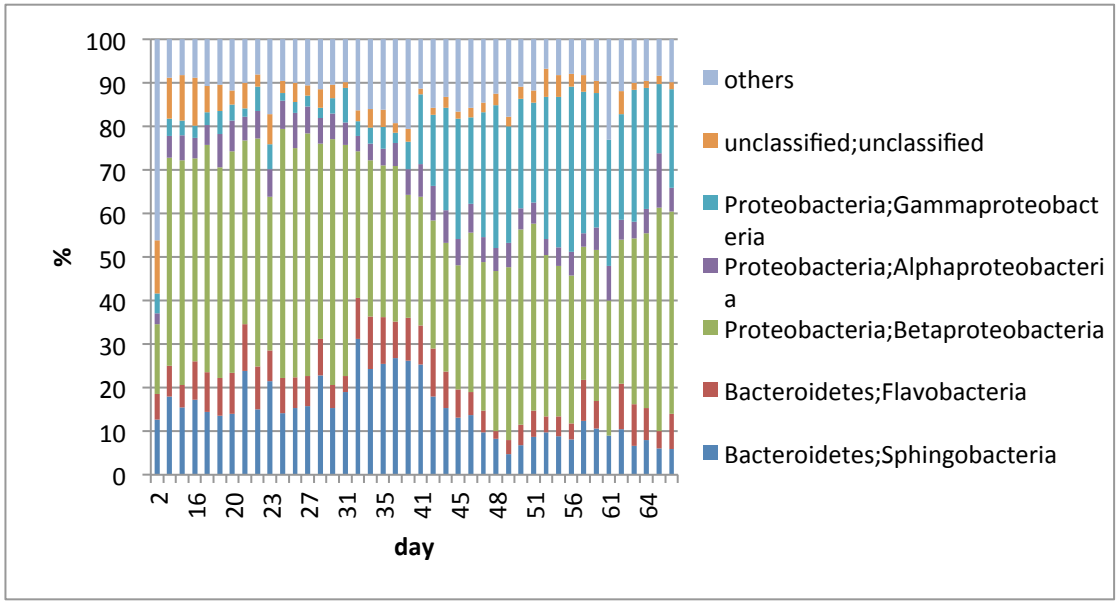


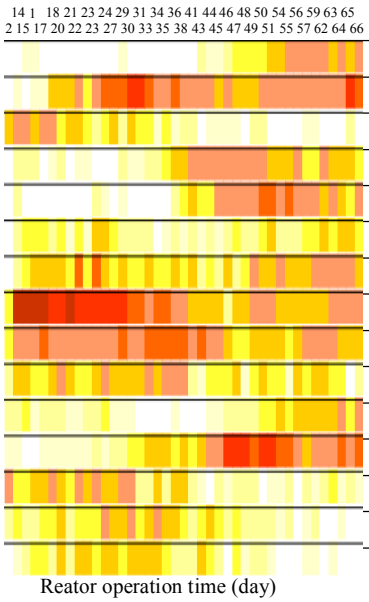
Fig 5. 13 Taxonomy Assignments of reads at Class level of Run 3

Checking of major OTU to see the detail of the community dynamic was done. Different OTU of the same class showed different change patterns during monitoring. According to their phylum, the author divided the major OTUs into three different groups to analyses, as shown in Fig5.14 to Fig 5.18.

To compare the pyrosequencing results with T-RFLP patterns, expected T-RF sizes were calculated for each major OTU.

1 Group A: Proteobacteria.

Figure 5.14 shows the assignments of T-RFs and major OTUs in Betaproteobacteria.

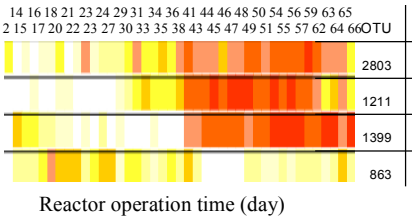


OTU.	Custom Name	T-RF
2872	Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales	418
82	Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiales_incertae_sedis	465
1323	Root;Bacteria;Proteobacteria;Betaproteobacteria	118
2408	Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales	
1799	Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Hydrogenophaga	160
2310	Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Diaphorobacter	417
1987	Root;Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;Diaphorobacter	255
4	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Thauera	421
4750	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Zoogloea	120
2980	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Zoogloea	120
1239	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Dechloromonas	118
555	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Dechloromonas	120
2715	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae	416
1921	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Propionivibrio	418
3759	Root;Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;Propionivibrio	422

Fig 5. 14 OTUs of Betaproteobacteria changing and their calculated T-RFs of Run 3

Though stable as whole class, the population of Betaproteobacteria was shifting between species. Firstly, at the beginning of the operation, *Thauera* sp, *Zoogloea* sp and *Propionivibrio* sp were predominant. And then Betaproteobacteria such as *Burkholderiales_incertae_sedis, Comamonadaceae* sp take the control. Though classified as Rhodocyclales, *Dechloromonas* sp also did not take control until the latter half of observation.

Figure 5.15 shows the assignments of T-RFs and major OTUs in Alphaproteobacteria and Gammaproteobacteria



OTU	Custom Name	T-RF
2803	Root;Bacteria;Proteobacteria;Gammaproteobacteria;Thiotrichales;Thiotrichaceae;Thiothrix	484
1211	Root;Bacteria;Proteobacteria;Gammaproteobacteria;Thiotrichales;Thiotrichaceae;Thiothrix	484
1399	Root;Bacteria;Proteobacteria;Gammaproteobacteria;Chromatiales;Chromatiaceae;Rheinhei	407
863	Root;Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadacea	197

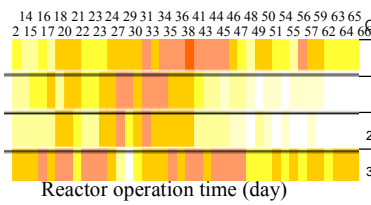
Fig 5. 15 OTUs of Alphaproteobacteria and Gammaproteobacteria changing and their calculated T-RFs of Run 3

Gammaproteobacteria began to grow from the 1/3 of the operation but dominate the latter half stably while Alphaproteobacteria population first fell and then became more.

2 Group B: Bacteroidete

Figure 5.16 shows the assignments of T-RFs and major OTUs in Sphingobacteria

Generally speaking, the Sphingobacteria tooke nearly 18 days to become dominant and stably dominate when the full granulation was achieved. However after day42, all Sphingobacteria decreased and lost their dominant.

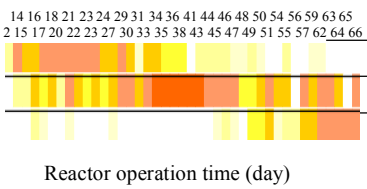


OTU	Custom Name	T-RF
413	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Chitinophagaceae;Niabella	114
454	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Cyclobacteriaceae;Aquiflexum	
2029	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales	
3535	Root;Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Chitinophagaceae;Sediminibact	118

Fig 5. 16 OTUs of Bacteroidete;Sphingobacteria changing and their calculated T-RFs of Run 3

Figure 5.17 shows the assignments of T-RFs and major OTUs in Flavobacteria

The aerobic Flavobacteria, which are yellow-pigmented, seemed usually found in aerobic granular sludge and was thought to be the cause of yellow color of mature granule. Three different species were predominant at the beginning, the middle and the last part of the whole period separately. First, *Cryomorphaceae* sp was at the most and then it decrease while *Flavobacterium* sp. kept on growing and over competed it and took the control. And then after day 45, *Flavobacterium* sp. population also became less while another specie *Cloacibacterium* sp. appear and took the control.
Flavobacteria is widely distributed in natural surface water and some of them presents as pathogens, their roles in granulation are not clear yet.



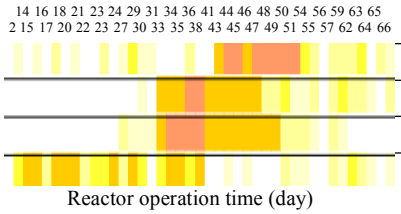
OTU.	Custom Name	T-RF
316	Root;Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Cryomorphaceae	475
3997	Root;Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Flavobacte	306
2200	Root;Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Cloacibact	93

Fig 5. 17 OTUs of Bacteroidetes; Flavobacteria changing and their calculated T-RFs of Run 3

3 Pattern C: other bacteria which was not dominant

Figure 5.18 shows the assignments of T-RFs and major OTUs in other bacteria

Firmicutes make up the largest portion of the mouse and human gut microbiome and can always produce endospores, which are resistant to desiccation and can survive extreme conditions. Thus it is not surprising to find Firmicutes in seed sludge from human night soil treatment plant. It kept in very low concentration while breakout from the day 40 to50.



OTU	Custom Name	T-RF
1203	Root;Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis	146
3661	Root;Bacteria;TM7;TM7_genera_incertae_sedis	78
4430	Root;Bacteria;TM7;TM7_genera_incertae_sedis	118
2952	Root;Bacteria	470

Fig 5. 18 other major OTUs changing and their calculated T-RFs of Run 3

The summarized comparison of the virtual digested and experimental T-RF sizes are shown in Table 5.5. In processing T-RFLP profiles, the ±1bp was considered the same species with the several intensified T-RFs. By comparing virtual digested and experimental observed T-RFs size and intensity, major T-RFs were connected with OTUs .

Table 5. 5 Comparison between virtual digested and observed T-RFs of Run 3

	T-RFLP	Virtual Digestion of OTU	Taxonomy
T-RFs	82(81~83)		
	93(92~94)	OTU2200,	Bacteroidetes; Flavobacteria
	97(96~98)		
	107(106~108)	OTU1239, OTU1323	Betaproteobacteria
	111(110~112)	OTU2408	Betaproteobacteria
	115(114~116)	OTU863, OTU4199,	Alphaproteobacteria
	119(118~120)	OTU4430,OTU3535,OTU1323,OTU4144,OTU1239, OTU555	TM7, Bacteroidetes;Sphingobacteria
	122(121~123)	OTU4750,	Betaproteobacteria
	146(145~147)	OTU1203,	Betaproteobacteria;Burkholderiales Firmicutes;
	307(306~308)		
	309(308~310)	OTU3997,	Bacteroidetes; Flavobacteria
	422(421~423)	OTU3759, OTU2872,	Betaproteobacteria

CHAPTER SIX

6.1 Conclusions

6.1.1 Three runs of EBPR AGS performance were monitored

Fully granulated sludge was formed successfully from conventional activated sludge using anaerobic-aerobic laboratory-scale SBR reactor by shortening of settling time. However with the same operational manner, their treatment performance in EBPR process and capacity of solid-liquid separation showed different patterns with some of them fluctuated and one relatively stable. This difference was related with the microbial community change.

6.1.2 Monitored dynamics of microbial population using T-RFLP

Dynamics of microbial population was monitored by T-RFLP for Run2 and Run3, several dominant T-RFs were selected. The most dominant T-RFs in 2 Runs were generally similar; both were 115bp, 119bp and 122bp. However, the shifting pattern was different. As for bacterial population Run3, T-RFs 119bp dominated all the time, while 115bp, 119bp and 122bp competed severely fighting for dominance in Run 2. T-RFs correlation to granulation and treatment performance was not clearly clarified.

6.1.3 Identified bacteria by OTU and virtual digested T-RFs

Pyrosequencing was applied to further identify bacteria community. Bacteria species, such as *Propionivibrio* sp., *Cytophagaceae* sp., *Thauera* sp.,and *Zoogloea* sp., were thought to have positive effect on EBPR AGS performance. Bacteria species, such as *Thiothrix* sp., *Rheinheimera* sp., *Dechloromonas* sp., *Novosphingobium* sp., *Hydrogenophaga* sp.,and *Cloacibacteriu* sp., were thought to have negative effect on EBPR AGS performance. However, further investigation is needed to clarify why bacteria belong to the same taxonomy affect granulation in different ways. With the most population, bacteria, such as Sphingobacteria, Flavobacteria, Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria, were expecting to be relevant with granulation.

6.2 Recommendation

6.2.1 Improvement on the monitoring of granulation process

In order to clearly understand the correlation between granulation process and microbial population in activated sludge, it is definitely necessary to further apply the developed approach to investigate granulation features other than the treatment ability. For instance, morphological data, robustness against shear force and the distribution of different functioning species inside the granules assessed by fluorescence in situ hybridization (FISH) should also be checked respectively. Therefore, the author suggests that the investigation should be still based on experiments to be conducted in laboratory, and to evaluate the detailed granulation process under different conditions.

6.2.2 Develop of microbial community library for single granule

To further understand the microbial ecosystem inside one granule and to relate morphological data to molecular data of granules, typical granules should be separated and properly galleried all features from macro to micro into a database. This is expected to become an idea source for latter research. For instance, only by comparing the morphology of actual granule with the library, researcher could get rough information about its microbial community compositions without applying any advanced methodologies which is laborious.

6.2.3 Influence on handling of data from pyrosequencing

During this study, pyrosequencing approach was still on its way of development, while data handling and analyzing method was also under development. Thus, in the present study, there is still a large room for improvement of generate profiles and the way to present data. Correlation between bacteria community and EBPR AGS performance should be clarified in more specific way at genus level in the future

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APPENDICES

Appendix 1:

Barcoded primers used in this paper.

519f primer with adapter region only was same for each sample

Primer name	Run2 Adapter region	barcode	519f primer region
519r with adapter	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG	no	GWATTACCGCGGCKGCTG

27r primer with different barcode was used for sample identification.
For Run 2:

Primer name	Run2 Adapter region	barcode	27f primer region
27f_A1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGAGAGAG	AGAGTTTGATCMTGGCTCAG
27f_A2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGAGATGC	AGAGTTTGATCMTGGCTCAG
27f_A3	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGAGCAGC	AGAGTTTGATCMTGGCTCAG
27f_A4	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGAGCATG	AGAGTTTGATCMTGGCTCAG
27f_A5	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGATCATC	AGAGTTTGATCMTGGCTCAG
27f_A6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGATCTGC	AGAGTTTGATCMTGGCTCAG
27f_A7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGATGAGC	AGAGTTTGATCMTGGCTCAG
27f_A8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGATGATG	AGAGTTTGATCMTGGCTCAG
27f_B1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGATGCAG	AGAGTTTGATCMTGGCTCAG
27f_B2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGATGCTC	AGAGTTTGATCMTGGCTCAG
27f_B3	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCAGAGC	AGAGTTTGATCMTGGCTCAG
27f_B4	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCAGATG	AGAGTTTGATCMTGGCTCAG
27f_B5	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCAGCAG	AGAGTTTGATCMTGGCTCAG
27f_B6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCAGCTC	AGAGTTTGATCMTGGCTCAG
27f_B7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCATCTG	AGAGTTTGATCMTGGCTCAG
27f_B8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCATGAG	AGAGTTTGATCMTGGCTCAG
27f_C1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCTCAGC	AGAGTTTGATCMTGGCTCAG
27f_C2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCTCATG	AGAGTTTGATCMTGGCTCAG
27f_C3	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCTGATC	AGAGTTTGATCMTGGCTCAG
27f_C4	CCATCTCATCCCTGCGTGTCTCCGACTCAG	AGCTGCTG	AGAGTTTGATCMTGGCTCAG
27f_C5	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCAGATC	AGAGTTTGATCMTGGCTCAG
27f_C6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCAGCTG	AGAGTTTGATCMTGGCTCAG
27f_C7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCATCAG	AGAGTTTGATCMTGGCTCAG
27f_C8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCATCTC	AGAGTTTGATCMTGGCTCAG

For Run 3:

	Run3		
Primer name	Adapter region	barcode	27f primer region
27f_D1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCTCATC	AGAGTTTGATCMTGGCTCAG
27f_D2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCTCTGC	AGAGTTTGATCMTGGCTCAG
27f_D3	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCTGAGC	AGAGTTTGATCMTGGCTCAG
27f_D4	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCTGATG	AGAGTTTGATCMTGGCTCAG
27f_D5	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCTGCAG	AGAGTTTGATCMTGGCTCAG
27f_D6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATCTGCTC	AGAGTTTGATCMTGGCTCAG
27f_D7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATGAGAGC	AGAGTTTGATCMTGGCTCAG
27f_D8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATGAGATG	AGAGTTTGATCMTGGCTCAG
27f_E1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATGAGCAG	AGAGTTTGATCMTGGCTCAG
27f_E2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATGAGCTC	AGAGTTTGATCMTGGCTCAG
27f_E3	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATGATCTG	AGAGTTTGATCMTGGCTCAG
27f_E4	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATGATGAG	AGAGTTTGATCMTGGCTCAG
27f_E5	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATGCAGAG	AGAGTTTGATCMTGGCTCAG
27f_E6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATGCATGC	AGAGTTTGATCMTGGCTCAG
27f_E7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATGCTCAG	AGAGTTTGATCMTGGCTCAG
27f_E8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	ATGCTCTC	AGAGTTTGATCMTGGCTCAG
27f_F1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGAGAGC	AGAGTTTGATCMTGGCTCAG
27f_F2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGAGATG	AGAGTTTGATCMTGGCTCAG
27f_F3	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGAGCAG	AGAGTTTGATCMTGGCTCAG
27f_F4	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGAGCTC	AGAGTTTGATCMTGGCTCAG
27f_F5	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGATCTG	AGAGTTTGATCMTGGCTCAG
27f_F6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGATGAG	AGAGTTTGATCMTGGCTCAG
27f_F7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGCAGAG	AGAGTTTGATCMTGGCTCAG
27f_F8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGCATGC	AGAGTTTGATCMTGGCTCAG
27f_G1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGCTCAG	AGAGTTTGATCMTGGCTCAG
27f_G2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CAGCTCTC	AGAGTTTGATCMTGGCTCAG
27f_G3	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CATCTCTG	AGAGTTTGATCMTGGCTCAG
27f_G4	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CATCTGAG	AGAGTTTGATCMTGGCTCAG
27f_G5	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CATGAGAG	AGAGTTTGATCMTGGCTCAG
27f_G6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CATGATGC	AGAGTTTGATCMTGGCTCAG
27f_G7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CATGCAGC	AGAGTTTGATCMTGGCTCAG
27f_G8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CATGCATG	AGAGTTTGATCMTGGCTCAG
27f_H1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCAGAGC	AGAGTTTGATCMTGGCTCAG
27f_H2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCAGATG	AGAGTTTGATCMTGGCTCAG
27f_H3	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCAGCAG	AGAGTTTGATCMTGGCTCAG
27f_H4	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCAGCTC	AGAGTTTGATCMTGGCTCAG
27f_H5	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCATCTG	AGAGTTTGATCMTGGCTCAG

27f_H6	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCATGAG	AGAGTTTGATCMTGGCTCAG
27f_H7	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCTCAGC	AGAGTTTGATCMTGGCTCAG
27f_H8	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCTCATG	AGAGTTTGATCMTGGCTCAG
27f_J1	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCTGATC	AGAGTTTGATCMTGGCTCAG
27f_J2	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTCTGCTG	AGAGTTTGATCMTGGCTCAG
27f_J3	CCATCTCATCCCTGCGTGTCTCCGACTCAG	CTGAGATC	AGAGTTTGATCMTGGCTCAG

Appendix 2:

Detail for each sample.

For Run 2:

		Run2	
Primer name	Sample name	Sample(day)	Read Count
27f_A1	A1	4	1042
27f_A2	A2	9	492
27f_A3	A3	14	83
27f_A4	A4	18	1383
27f_A5	A5	28	735
27f_A6	A6	35	1064
27f_A7	A7	40	874
27f_A8	A8	46	788
27f_B1	B1	51	574
27f_B2	B2	57	734
27f_B3	B3	63	410
27f_B4	B4	67	488
27f_B5	B5	79	696
27f_B6	B6	85	700
27f_B7	B7	91	776
27f_B8	B8	99	988
27f_C1	C1	102	941
27f_C2	C2	112	617
27f_C3	C3	119	1038
27f_C4	C4		
27f_C5	C5	123	717
27f_C6	C6		
27f_C7	C7	126	1318
27f_C8	C8	130	1122

For Run3:

		Run3	
Primer name	Sample name	Sample(day)	Read Count
27f_D1	D1	2	935
27f_D2	D2	14	960
27f_D3	D3		
27f_D4	D4	15	1032
27f_D5	D5	16	1069
27f_D6	D6	17	1075
27f_D7	D7	18	884
27f_D8	D8	20	877
27f_E1	E1	21	1476
27f_E2	E2	22	818
27f_E3	E3	23	1319
27f_E4	E4	23.5	856
27f_E5	E5	24	514
27f_E6	E6	27	915
27f_E7	E7	29	1328
27f_E8	E8	30	744
27f_F1	F1	31	517
27f_F2	F2	33	468
27f_F3	F3	34	664
27f_F4	F4	35	708
27f_F5	F5	36	714
27f_F6	F6	38	796
27f_F7	F7	41	498
27f_F8	F8	43	616
27f_G1	G1	44	1202
27f_G2	G2	45	1015
27f_G3	G3	46	1062
27f_G4	G4	47	1063
27f_G5	G5	48	1189
27f_G6	G6	2	935
27f_G7	G7	14	960
27f_G8	G8		
27f_H1	H1	15	1032
27f_H2	H2	16	1069
27f_H3	H3	17	1075
27f_H4	H4	18	884
27f_H5	H5	20	877

27f_H6	H6	49	868
27f_H7	H7	50	938
27f_H8	H8	51	1075
27f_J1	J1	54	1134
27f_J2	J2	55	1323
27f_J3	J3	56	644

２０１２年度　修士論文　生物学的りん除去廃水処理プロセスにおいて生成したグラニュール汚泥の処理性能と微生物群集構造の経時変

化　欧陽