学　位　論　文

Doctoral Dissertation

Activated Sludge Bacterial Population Changes Caused by the Addition of Crude Extracts from Activated Sludge

（活性汚泥から得られた粗抽出液を添加することにより生じる活性汚泥中の微生物群集構造変化）

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ABSTRACT

Activated sludge processes are widely used in wastewater treatment. It is very important to understand the factors that affect microbial population in activated sludge, because it is microorganisms that play the role to remove pollutants in wastewater. There are a tremendous number of studies conducted on the effects of different factors on activated sludge microbial populations. But they are mostly focused on factors such as pH, dissolved oxygen, and temperature that are given activated sludge microbial population. On the other hand, there also are factors which reside inside microbial ecosystems, such as bacteriophages or viruses hosted by bacteria and bioactive chemicals generated by microorganisms.

The present study focused on the effects of chemicals generated by activated sludge microorganisms on bacterial community in activated sludge. Already, Ogawa (2009) reported that extracts from activated sludge affect bacterial population of activated sludge. This study was conducted further to know more details on the effect of extracts from activated sludge on bacterial population. Following three specific objectives were pursued:

1. To clarify the response time to cause changes of bacterial population.
2. To clarify the effects of the concentrations of the extracts on bacterial population change.
3. To identify bacterial species affected by the addition of the extracts.

To achieve these objectives, the author adopted the method developed by Ogawa (2009). Activated sludge was obtained from different sources including laboratory sequencing batch reactor and wastewater treatment plants for sewage treatment, extracts were obtained from the activated sludge samples with ethanol as the solvent, and activated sludge was incubated with and without extracts in microplate wells. The ethanol-soluble extracts or pure ethanol was added to microplate wells, the solvent was dried, fresh activated sludge was inoculated, substrate was added, and then incubated for predetermined time. After that, incubated sludges were collected and bacterial population changes of them were analyzed by reverse-transcription polymerase chain reaction (RT-PCR)/restriction fragment length polymorphism (RFLP). In some of the experiments, PCR/RFLP and RT-PCR/pyrosequencing were also conducted.

In Chapter 4, experiments reported in this chapter were conducted rather try and error

to improve the incubation conditions on microplates by 12 separate experiments. Activated sludge samples were obtained from the same laboratory sequencing batch reactor (SBR1), and factors such as F/M ratio and incubation time were studied with different extracts obtained from SBR1 activated sludge sampled on different days. The effects of the extracts on the activated sludge bacterial populations were not clear but the extracts on the 137^{th} day of the operation of SBR1 showed significant effect on bacterial population. The extract was named E137, and was mainly used in experiments reported in chapter 5.

In Chapter 5, experiments were conducted to achieve the objectives previously stated. In Section 5.3.1, the effects of F/M ratio and incubation time were investigated. Bacterial population change was clearly observed only with biomass growth of 20 to 30% by the addition of extracts within less than 24 hours incubation. Then in Section 5.3.2, the sensitivity of bacterial population of activated sludge from SBR1 and a wastewater treatment plant treating real sewage were compared. The results showed the WWTP activated sludge was much more sensitive than that from SBR1.

Further, in Section 5.3.3, the activated sludge bacterial population change affected by the crude extracts with time up to 24 hours was investigated by monitoring the samples every two hours by RT-PCR/RFLP analyses. Bacterial population changes were observed mainly in the initial 2 to 6 hours. It was also suggested that the crude activated sludge extracts not only promoted some of the species but also killed or eliminated other bacterial species. Meanwhile, in Section 5.3.4, the dose-response pattern was investigated by utilizing the extract with different concentrations. The effect of crude extracts followed sigmoid curve in general. At last, in Section 5.3.5, the effects of extracts from different activated sludge were examined.

In Chapter 6, the samples in Section 5.3.3 and Section 5.3.4 were re-analyzed by the pyrosequencing method to know the identity of affected bacterial species. The growth of bacteria in order Pseudomonadales, Aeromonadales and Flavobacteriales were basically promoted by the addition of crude extracts. But in Flavobacteriales, two OTUs were oppositely affected by the extracts. On the other hand, class Sphingobacteria was totally inhibited by the extracts. But this outcome stands only for the present study, and more study would be needed to gain a general picture.

Through above studies, in Chapter 7, following conclusions were obtained: (1)

Bacterial population change by the addition of crude extracts can happen within a couple of hours, which is significantly shorter than sludge retention time that mathematically regulates average retention time of microbial cells. (2) While growth of some of bacteria is promoted, there are bacteria that are killed or eliminated when crude extracts from activated sludge is added. (3) The dose-response pattern is diverse and complicated. (4) Extracts from different activated sludge have different effect. (5) Bacterial species that are affected by crude extracts was clarified, though this aspect of result requires to be examined more.

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DEDICATION

献给我最深爱的妻子（彦杰）和女儿（佳怡）。

ABBREVIATIONS

AHLs	Acyl-homoserine lactones
COD	Chemical oxygen demand
DGGE	Denaturing gradient gel electrophoresis
DO	Dissolved oxygen
DOC	Dissolved organic carbon
F/M ratio	Food-to-microorganism ratio
MLSS	Mixed liquor suspended solids
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse Transcription-Polymerase chain reaction
SBR	Sequencing batch reactor
SRT	Sludge retention time
SVI	Sludge volume index
T-RFLP	Terminal restriction fragment length polymorphism
WWTPs	Wastewater treatment plants

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Chapter 1 Introduction

1.1 Backgound

In many cases, microorganisms are employed to treat wastewater. For example, in Japan, sewage is treated mostly by activated sludge processes, where activated sludge is composed of different microbial species especially bacteria. As bacteria take the role to remove pollutants in wastewater, it is important to study the factors that affect bacterial population in it. There are quite a number of studies that have been conducted to understand the effects of different factors on activated sludge bacterial populations, but they mostly focused environmental factors which are given to bacterial ecosystems such as pH, dissolved oxygen and temperature (Seviour & Nielsen, 2010). Recently, there also are studies which suggest the significance of bacteriophages, viruses that lyse (or kill), bacteria and thus affect bacterial population (Lee, *et al.*, 2006; Lee, *et al.*, 2007).

In this thesis, the author focused on bacterial population changes in activated sludge caused by chemicals that are produced within activated sludge microbial population. Chemicals are known to play important roles in the competition of different organisms. For example, allelopathy has been widely studied in the plant sciences, where "allelopathy" is meant for interactions mediated by chemicals between any types of organisms including microorganisms. It includes both detrimental and beneficial reciprocal interactions (Moore, 1975; Rice, 1979; Inderjit, *et al.*, 1999; Inderjit, *et al.*, 2005). Similarly, it is highly expected that microorganisms in activated sludge are competing, cooperating, or making any kinds of interactions via chemicals. Yet, as will be reviewed extensively in Chapter 2, at this moment, only limited studies have been done on microbial interactions mediated by chemicals in the activated sludge microbial ecosystems.

Here, the author would like to introduce one of the few studies on microbial interactions mediated by chemicals. Ogawa (2009) obtained crude extracts from activated sludge with ethanol as the extractant, and incubated activated sludge with the extracts for 4 days and found bacterial population was significantly different from that incubated without extracts. His study clearly showed that components in the ethanol-soluble extracts caused significant changes of bacterial population. However, his study still remains at a preliminary stage, and many questions were raised such as:

a) Which bacterial species are affected?
b) Which chemicals are involved in the interactions?
c) Who produce the chemicals?
d) How is the intensity of the effect?
e) How fast or slow is the effect?
f) To which extent can it affect activated sludge bacterial population?
and so on.

1.2 Objectives

The author initially tried to answer to question b) above to identify chemical species. As Ogawa (2009) developed a very efficient method using incubation on microplates to detect chemicals that affect bacterial populations, the author thought he can use the technique to isolate bioactive chemicals. That is, initially the author tried to fractionate the contents of extracts from activated sludge by such methods as liquid chromatography, find the fractions that contain the components that affect bacterial populations, and then anyhow purify the contents of the fractions and identify their chemical structures. Yet, though the author tried many experiments to trace the experiments by Ogawa (2009), the author initially could not observe strong chemical interactions. Some of the trials and errors are briefly reported in Chapter 4 of the thesis.

Finally, the author obtained crude extracts that have strong effect to change bacterial populations from the laboratory activated sludge reactor, and then the author conducted many experiments with the crude extracts to investigate following aspects:

1. Response time to cause changes in bacterial population.
2. Effects of the concentrations of the extracts on bacterial population change.
3. Bacterial species that are affected by the addition of the extracts.

In short, the present study was conducted to mainly answer to questions a), d) and e).

The author expects that different kinds of microorganisms in activated sludge are involved in interactions mediated by chemicals, but here, he mostly focused on bacteria. It is because bacteria occupy significant or major part of biomass in activated sludge, and the methods the author employed to analyze microbial population change were intended only for bacterial populations.

1.3 Structure of the thesis

This thesis includes 7 chapters as follows.

Chapter 1: The background, the objective and the structure of this dissertation.

Chapter 2: Literature review which include: 1) Current knowledge on bacterial interactions mediated by chemicals; 2) Current knowledge on bacterial interactions mediated by chemicals in activated sludge, and 3) methods for the analysis of bacterial population changes.

Chapter 3: Descriptions on materials and methods common to all or most of the experiments in this thesis.

Chapter 4: Improvement of the incubation conditions for the detection of bacterial population changes caused by the addition of crude extracts obtained by different operation time of SBR1.

Chapter 5: Experiments were conducted to achieve the objectives. Experiments on (1) response time, (2) effect of dose responses, (3) effects on different sludges, and (4) comparison of the strengths of effects of extracts obtained from different sludges. Here, changes of bacterial population were evaluated by the restriction fragment length polymorphism (RFLP) method.

Chapter 6: By utilizing the pyrosequencing method, the affected bacterial species by the components in the crude extract was studied.

Chapter 7: Conclusions and recommendations.

Chapter 2 Literature Review

In this chapter, reports related to the present study are reviewed. In section 2.1, studies on interactions between organisms mediated by chemicals are reviewed. There are two streams of studies in this regard: allelopathy and quorum sensing. Following the general review of two kinds of interaction chemicals, in 2.2, studies on the allelopathy and signal compounds in activated sludge are reviewed. At last, a brief review on bacterial population analysis method is presented in section 2.3.

2.1 Microbial interaction mediated by chemicals

2.1.1 Allelopathy

2.1.1.1 The definition of allelopathy

According to reviews on allelopathy, it is Molisch who introduced the term "allelopathy" to refer to interactions between all types of plants or plants and microorganisms mediated by chemicals (Rice, 1979). The interaction includes both detrimental and reciprocally beneficial case, the chemicals that mediate the interactions are called allelochemicals" (Moore, 1975; Rice, 1979; Inderjit, *et al.*, 1999; Inderjit, *et al.*, 2005).

Researchers introduced specific terms based on the donor of allelochemicals and affected recipient. For example, "phytoncides" are chemicals produced by a higher plant and affect microorganisms, "marasmins" are those produced by a microorganism and affect higher plants, and "kolines" are those produced by higher plants and affect other higher plants (Rice, 1984). "Antibiotics" are chemicals produced by a microorganism and kill or suppress the growth of other microorganisms. All these compounds are included in allelochemicals.

In some cases, allelopathy positively affect activities of affected species, and in other cases, the effect can be negative (Rice, 1984). In the review by Mallik and Williams (Mallik & Williams, 2005), allelopathy in which the plant growths stimulated by other plants and microorganisms are reviewed: enhancement of soybean growth by the amendment of the soil with shoots of *Solanum nigrum*, improvement of growth and yield

of several crops by triacontanol isolated from alfalfa, stimulation of growth of lettuce by chromosaponin 1 isolated from etiolated pea seedlings, and stimulation of the growth of *Bradyrhizobium japonicum* by unidentified allelochemicals from *Chenopodium album* and *Setaria viridis*, and stimulation of the growth of wheat by *Pseudomonas cepacia* and *P. putida*. There also is a report on interaction between bacterial species. Tanaka et al.(2005) reported that a *Sphingomonas* species isolated from activated sludge produced unknown growth factors which significantly enhanced the growth rate and cell yield of *Catellibacterium nectariphilum*, which was also isolated from the same activated sludge (Tanaka, *et al.*, 2005).

2.1.1.2 Complexity of allellopathic interactions

The biological activity of allelochemicals is concentration-dependent with a response threshold, which is not a constant, but is intimately related to the sensitivity of the receiving species, the plant process, and environmental conditions. For example, the threshold for reducing the growth of grain sorghum seedlings by several cinnamic acids was 1/25 that required to inhibit its germination, and initiation of ferulic acid inhibition of these seedling under high temperatures occurred at about one half the concentration required at moderate temperatures (Einhellig & Schon, 1982; Einhellig & Eckrich, 1984)

Usually, allelopathic interactions are mediated by plural compounds. The allelopathic inhibition typically results from the combined action of plural allelochemcials which have additive or synergistic effects (Rice, 1987; Einhellig, 1996; Macías, 2004). Rasmussen and Einhellig found a mixture of 5×10^{-3} M p-coumaric and 5×10^{-3} M ferulic acids reduced seed germination of sorghum to 34% of the control after 24 hour and 59% after 48 hour. However, with 5×10^{-3} M of either p-coumaric or ferulic acids -treated seeds alone showed up to 69% and 92% germination (Rasmussen & Einhellig, 1977). The combinations of allelochemials of vanillic and p-hydroxybenzoic acids have also been found synergistic effect on growth of radish seeds. A combination of 2.5×10^{-3}M vanillic acid and p-hydroxybenzoic inhibited the germination of radish more than either did individually with the concentration of 2.5×10^{-3} M (Einhellig & Rasmussen, 1978).

The allelochemicals has been reported to be produced at different levels in the lifecycle of a plant. For example, Koeppe *et al*. (1970a) discovered that the concentration of scopolin and total chlorogenic acids decreased with the age of leaves in tobacco. They also found the concentration of chlorogenic acids and iso-chlorogenic acid decreased in

stem and leaf section of *Helianthus* with the age. Woodhead (1981) found the levels of phenolic acids in healthy plants of *Sorghum bicolor* differed depending on the stage of the age of the plant.

According to Rice (1984), an accumulation of allelochemicals can occur within only a short period of time, but it does not always cause significant effects as the susceptibility of recipient plant or microorganisms also changes in their life cycles. Therefore, the allelopathic interaction is dependent on the conditions including growth stages of both producer and recipient organisms.

Each individual also have differences in productivity of and sensitivity to allelochemicals. Individual plants, even of the same species growing close together, have differences in production of allelochemicals (Rice, 1984). Woodhead (1981) found Laboratory and field-grown plants show significant differences in phenolics contents. Putnam and Duke (1974) used two indicator species, a monocot (*Panicum miliaceum* L.) and a dicot (*Brassica hirta* Moench), to investigate the allelopathic effect of cucumber (*Cucumis sativus* L.) accessions from 41 nations of USA, and found the effect of these accessions on two indicator species was diverse.

Allelopathy effect has been reported to be enhanced by the environmental stresses, such as the changes of nutrient, light quality (Koeppe, *et al.*, 1970b), temperature (Koeppe, *et al.*, 1970c), moisture, grazing pressure by insects (Woodhead, 1981) and many other stresses in the environment. For example, sorghum grain and soybean seedlings grown under relatively hot conditions exhibited a ferulic acid inhibition threshold at only one-half the concentration required under moderate temperatures (Einhellig, 1987). The inhibition threshold concentration for ferulic acid to affect seedling growth was reduced with even minor moisture stress or a growth temperature at the higher end of the normal range for a species (Einhellig, 1996). Similarly, lowering the osmotic potential of the growth medium by -0.15 M Pa in concert with exposure to ferulic acid showed a significant inhibitory effect on sorghum growth (Macías, 2004).

Some of allelochemicals have been reported to remain in soil and can affect the recipient for a long period of time, and the duration has been reported to vary depending on conditions. For example, Guenzi *et al.* (1967) found corn and sorghum residues had considerably toxic materials and remained for about 22 to 28 weeks before the toxicity in the water-soluble portion of the residues became nontoxic. Rice residues submerged in

soil have aslo been reported to release phytotoxic substances, which exhibited inhibition on the growth radicle of lettuce and seeds and seedlings of rice. But under greenhouse conditions, phytotoxicity was rapidly degraded within 4 weeks (Chou & Lin, 1976).

After all, allelopathy is quite a complex phenomenon affected by many factors or conditions on both the sides of producer and recipient of the allelochemicals.

2.1.2 Quorum sensing

Bacteria are often regarded to be inferior and have no way to communicate with each other like animals, because of their relatively simple cell. However, many studies have shown that bacteria can communicate with each other by signal chemicals. The phenomenon is widespread in bacteria that employs autoinducing chemical signals to coordinate diverse, often cooperative activities such as bioluminescence, biofilm formation, production of virulence factors or antimicrobials and exoenzyme secretion (Taga & Bassler, 2003; Diggle, *et al.*, 2007; Schuster, *et al.*, 2013; Garg, *et al.*, 2014) , and is often referred to as “quorum sensing”.

Generally, gram-negative bacteria typically communicated with each other by using acyl-homoserine lactone (AHL), which was first discovered by Nealson *et al.* (1970): they found that *Vibrio ficheri*, a bioluminescent marine bacterium, produced light at high cell density but not at low cell density. AHL autoinducers all share a common homoserine lactone moiety and differ only in their acyl side chain moieties (De Kievit & Iglewski, 2000; Taga & Bassler, 2003). AHLs can freely diffuse into and out of the cell and increases in concentration in proportion to cell population density. When the concentration reaches a threshold level, specific genes are activated and their functions are expressed, such as production of luminescent protein, antibiotics, virulence, biofilm formation, and sporulation (Bassler & Losick, 2006).

On the other hand, gram-positive bacteria typically use modified oligopeptides as autoinducers (Taga & Bassler, 2003; Waters & Bassler, 2005). These signals are generally referred to as autoinducing polypeptides (AIPs). AIPs are produced firstly as precursor peptides and then modified. Different from the AHLs used in Gram-negative bacteria, peptide signals are not diffusible across cell membrane but specifically interact with external domains of membrane-bound sensor kinase proteins. Quorum sensing system with AIPs includes sporulation, competence for DNA uptake, and virulence factor

expression (Taga & Bassler, 2003).

In addition, another compounds named as autoinducer-2 (AI-2), derived from the common precursor, 4,5-dihydroxy-2,3 pentandione, has been found to be widespread in the bacterial world, and is considered to mediate interspecies interaction (Taga & Bassler, 2003; Waters & Bassler, 2005; Shrout & Nerenberg, 2012).

Other quorum sensing compounds like natural small molecule (NSM) in *Bacillus subtilis* (López, *et al.*, 2009), diffusible signal factor (DSF) in *Xanthomonas campestris* (He & Zhang, 2008) and Pseudomonas quinolone signal (PQS) in *Pseudomonas aeruginosa* (Toyofuku, *et al.*, 2008) have been reported.

Originally, these signal compounds especially AHLs were reported to affect cells of the same species, but recently they are reported also to affect cells of different species (Manefield & Whiteley, 2007).

2.1.3 Autointoxification

Autointoxication is another phenomenon in which chemicals mediate interactions. Autointoxication is within the same species, and inhibitory to growth of the cells of the same species. Autointoxication is a condition in which organisms are inhibited by excessive accumulation of their own products (Muller, 1969; Dommergues, 1978; Putnam & Tang, 1986). Young and Chou (1985) found the root and stem extracts strongly inhibited the development of asparagus seedlings in the seed bioassay and revealed the autotoxicity effect of extracts from the root and stem extracts. Autointoxication is the direct evidence of the competition of the plant of the same species.

As for microorganisms, the effect of bacteriocin is similar to autointoxificaiton. Bacteriocins are a kind of proteinaceous toxic substances, that have a lethal consequence for the producing organisms and the effect is restricted to closely related bacterium (Ivanovics, 1962; Klaenhammer, 1988). Bacteriocins are wildly existent in bacteria: according to Klaenhammer (1988), 99% of them may make at least one bacteriocin. Recently researchers are finding that while Gram-negative bacteria has bacteriocins with narrower killing spectrum(Riley & Wertz, 2002; Balciunas, *et al*., 2013), those of Gram-positive bacteria, due to the dedicated transport mechanism to release the bacteriocin, have a wider range of killing spectrum (Gillor, *et al*., 2008).

2.2 Microbial interactions mediated by chemicals in activated sludge

2.2.1 Suspected allelopathy in activated sludge

Although allelopathy has been widely studied in plant sciences, reports on allelopahty in activated sludge microbial ecosystems are very limited. But there are not a few reports which support the existence or significance of allelopathy in activated sludge.

Soluble microbial products are the pool of organic compounds that are released into the bulk in the processes of substrate metabolism and biomass decay (Azami, *et al.*, 2012). There are reports that suggested the ecological toxicity of SMPs to the microorganisms. For example, Rappaport *et al*. (1979) reported the mutagenic activity of SMPs in the effluent of a wastewater treatment plant employing the activated sludge process. Chudoba (1985) reported inhibitory effect of SMPs on nitrification. These reports are highly possibly thought to be allelopathy.

Ichihashi *et al*.(2006a) operated two activated sludge reactors in parallel, one with normal hydraulic retention time (HRT) and another with extended HRT in an intention to obtain supernatant enriched with allelochemicals, and performed batch experiments by exchanging the supernatant. They reported that the components in the supernatant of activated sludge with extended HRT affected the microbial activity such as nitrification and anaerobic acetate uptake of the activated sludge (Ichihashi, *et al.*, 2006a). Again, Ichihashi *et al.* (2006b) conducted another series of batch experiments in which they mixed two activated sludges, one with higher activity of enhanced biological phosphorus removal (EBPR) and another with deteriorated EBPR, at different mixing ratios. They reported that the EBPR-related metabolisms were not linear to the mixing ratios and attributed the possible cause to chemicals that affect bacterial metabolisms (Ichihashi, *et al.*, 2006b).

As has been reviewed in 2.1.1.1, Tanaka *et al*. (2005) reported that the growth of *Catellibacterium nectariphilum* isolated from activated sludge was markedly promoted by unknown growth factor(s) produced by another activated sludge bacterium in genus *Sphingomonas* (Tanaka, *et al.*, 2005).

One of the difficulties on the study of allelopathy in activated sludge is the limitation of effective method. To ease studies, Ogawa (2009) introduced microplate to incubate

activated sludge with possible alleopathic components. He obtained extracts from activated sludge with ethanol, ethyl acetate, and acetone. He applied these extracts to wells of the microplate, dried, and then incubated activated sludge in the wells for 4 days. He also incubated activated sludge in wells to which pure solvent was applied and dried. He reported that microbial population in the activated sludge incubated with extracts was significantly different from that incubated without extract addition, and the ethanol extracts had the most significant effect. His results suggested some allochemicals in the ethanol-soluble extracts could significantly affect the activated sludge bacterial population changes. His study has been published as Satoh *et al*. (2009).

The papers cited above have following differences from methodological point of view.

Tanaka *et al*. (2005) worked on chemicals excreted by an isolated bacterium from activated sludge. Ichihashi *et al*. (2006a, 2006b) studied on chemicals that are in the supernatant and in activated sludge, respectively. Ogawa (2009) worked on whole chemicals that are extractable by such solvent as ethanol from activated sludge.

Ichihashi *et al*. (2006a, 2006b) incubated activated sludge at a beaker scale (a couple of hundred milliliters). Running many conditions in parallel is not easy when incubation is done at a beaker scale. On the other hand, Ogawa (2009) introduced a microplate for incubation which allows running many conditions in parallel.

Tanaka *et al*. (2005) and Ogawa (2009) examined microbial population change while Ichihashi *et al*. (2006a, 2006b) examined change in water quality to detect the effects of chemicals. Thanks to the recent development of analytical methods, both microbial population change and water quality can be analyzed with a small amount of sample. Especially, Ogawa (2009) introduced the sonication-dilution method (Satoh et al., 2008) to extract DNA as the template for polymerase chain reaction (PCR) from small volume of samples in microplate wells.

2.2.2 Signal compounds in activated sludge

Up to now, signal compounds related with quorum sensing has been widely and deeply studied in pure cultures and biofilms. Especially, Acyl homoserine lactones (AHLs) are the most studied signal compounds. But the production and the role of AHLs in activated sludge have not been studied so much. The author summarized these reports as follows.

Agrobacterium tumefaciens NTL4 is often employed as the reporter strain of AHLs produced by bacterial strains isolated from activated sludge. For example, based on the reporter strain, Morgan-Sagatume *et al.* (2005) isolated 10 strains (8 *Aeromonas* spp. and 2 *Pseudomonas* spp.) producing AHL-like autoinducers from one municipal activated sludge sample. Yeon *et al.* have identified at least three different AHLs in the membrane bioreactors (Yeon, *et al.*, 2008). Kim *et al.* (2009) reported that 60% of bacterial species on the fouled membrane surface contribute to biofilm formation through the active intraspecies as well as interspecies communication.

Recently, the effect of AHLs on the formation of granular activated sludge has also been widely studied. For example, Liu *et al.* (2010) operated two SBRs and established one stable aerobic granule SBR. He found that quorum sensing compounds (AHLs) are more generated in granular activated sludge than in the conventional activated sludge. Zhang S-H *et al.* (2011) found aerobic granulation can be stimulated by the addition of (R)-4,5-dihydroxy-2,3-pentanedione (DPD), which is a precursor of quorum sensing signal AI-2. Tan *et al.* (2014) studied links between quorum sensing, organization and composition of complex microbial communities, and they found conversion of floccular biomass to highly structured granules was strongly and positively correlated with AHLs (Tan, *et al.*, 2014).

Activated sludge processes with solid-liquid separation by membrane (so called membrane bioreactors or MBRs) are also studied in relation with signal molecules. Biofouling or biofilm formation on membrane surface is one of the major problems of MBRs. On the other hand, in bacterial biofilm studies signal compounds have been suggested to play an important role in biofilm formation. Therefore, to disrupt the intercellular communication is expected to be one of the strategies to solve the problem. Kociolek (2009) extensively reviewed the potential of quorum sensing inhibitors (QSIs) on controlling the formation of biofilms. Xu and Liu (2010) reduced the production of AI-2 by disruption of ATP synthesis, and found the attachment of suspended microorganisms on both nylon membrane and glass slide surfaces was significantly suppressed. These studies offered insights into the control of biofouling by preventing the bacterial communication.

The research group of Nomura and his colleagues at Tsukuba University has done a systematic study on the cell-to-cell communication molecule of

2-heptyl-3-hydroxy-4-quinolone, referred to as the Pseudomonas quinolone signal (PQS). They found PQS controls the activity of extracellular antimicrobial chemicals (Tashiro, *et al.*, 2013), the activity of denitrification (Toyofuku, *et al.*, 2012) and the production of membrane vesicles in *Pseudomonas aeruginosa* (Tashiro, *et al.*, 2010). They demonstrated the improvement of nitrification activity by the addition of AHLs (Inaba, *et al.* 2010a; Inaba, *et al.*, 2010b).

The research groups of Ikeda and his colleagues at Utsunomiya University have done many works on inhibition of the quorum sensing because it has been found very popular in many of the pathogens (Morohoshi, *et al.*, 2007). Specially, Ochiai *et al.* (2013) isolated a total of 672 bacterial strains from activated sludge obtained from seven sewage treatment plants, and screened for AHL-producing and degrading strains. They found the most dominant AHL-producing were assigned to genus *Aeromonas*, while the most dominant AHL-degrading strains belonged to genus *Acinetobacter*. Then they found the *amiE* gene as an AHL-degradative gene. They reported that AmiE had a high level of activity to degrade AHLs with longer acyl chains, but not with acyl chains shorter than eight carbons (Ochiai, *et al.*, 2014).

Although the effects of AHLs on bacterial cultures isolated from activated sludge have been widely studied, their effects on the bacterial community structure are yet limited. Valle *et al.* (2004) incubated activated sludge with and without the addition of AHLs and monitored the change of bacterial community. They found change of bacterial community structure after 10 days incubation with 2 μM and 20 μM AHLs addition. This was the first which reported the bacterial community structure of activated sludge was affected by the signal compounds. Tan *et al*. (2014) also reported change of bacterial community structure by AHLs in their study to correlate AHLs with granulation. They found low concentrations of AHLs quantified were sufficient to activate AHL bio-reporters in situ in complex granular communities and suggested AHL-mediated quorum sensing may be a common feature in many natural and engineered ecosystems.

2.3 Method for microbial community analyses

In the present study, the effect of chemicals in extracts from activated sludge was studied in terms of the changes of bacterial population. Here, methods to analyze bacterial population change are reviewed.

To detect change of bacterial population, so called molecular methods such as denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), terminal restriction fragment length polymorphism (T-RFLP), and pyrosequencing based approaches are often used now.

These methods target differences in the sequences of 16S or 18S ribosomal RNA (rRNA) or genes corresponding to them. 16S or 18S ribosomal RNA is a component of the small subunit of ribosomes, and are also called small subunit RNA (ssu rRNA). The gene coding for it is conserved and variable region (Weisburg, *et al.*, 1991). The sequence of ssu rRNA is thought to reflect evolutionary path (Woese & Fox, 1977), and now bacterial species are often identified not on phenotypically but on phylogenetically based on ssu rRNA sequences.

These methods often start with polymerase chain reaction (PCR) targeting the whole or partial sequence of ssu rRNA gene using so called universal primers. The universal primers are designed to target the conserved regions of ssu rRNA sequence. The PCR products from environmental samples have similar lengths, but their sequences are different because each of the PCR product molecules is from different microorganisms. These methods, DGGE, RFLP, T-RFLP, and pyrosequencing can resolve the PCR product from different microorganisms.

These methods are also used in combination not with PCR but with reverse transcription PCR (RT-PCR). As PCR works on DNA, in the case of ssu rRNA sequence analysis, its original gene ssu rRNA gene is analyzed. Analysis of ssu rRNA gene may reflect dead or non-active cells which still maintains ssu rRNA gene. On the other hand, by introducing RT-PCR, ssu rRNA, which is thought to more directly reflect activity of cells can be analyze (Revetta, *et al.*, 2010, Blazewicz, *et al.*, 2013).

Microbial population changes have been studied not only based on ssu rRNA sequences but also based on other chemical fingerprinting such as quinone profile and fatty acid profile. In these methods, after pretreatment of samples such as extraction and purification, finally, target compounds are separated and quantified by either gas chromatography or liquid chromatography. But these methods are laborious, and the resolution is lower. On the other hand, recently, simpler and more rapid mass spectrometric methods are being reported. One of the promising methods is based on matrix assisted laser desorption ionization time of flight mass spectrometry

(MALDI/TOFMS) targeting whole molecules or proteineous molecules in cells or environmental samples. The MALDI/TOFMS can observe mass spectrum of molecules which can easily be ionized: some of the methods target ribosomal protein molecules (Pineda, *et al.*, 2003), while the other target whatever molecules that can be ionized (Satoh & Mino, 2010). While ribosomal protein typing is getting to be established, and even commercialized, specialized to identify isolated bacterial strains. The use of MALDI/TOFMS on mixed microbial samples has only been reported by Satoh and Mino (2010), as far as the author reviewed, and is still under development.

In this section, from 2.3.1 to 2.3.4, molecular methods which are well established and widely used to compare microbial population are reviewed.

2.3.1 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE), established by Muyzer *et al.* (Muyzer, *et al.*, 1993), is a kind of electrophoresis in which electrophoresis is performed in a gel which has gradient of denaturant. While the normal gel electrophoresis cannot separate DNA molecules with the same size, in DGGE, DNA molecules can be separated based on the differences in denaturing points. While gradient gel was originally introduced by Fischer and Lerman (Fischer & Lerman, 1979), Muyzer established the method as a method to separate PCR products from mixed culture systems especially from such genes as 16S rRNA gene. Muyzer introduced the GC-clamp on one of the ends of the PCR product so that when the molecules start denaturing, only one of the ends remains un-denatured, and as a result the whole molecules become half-denatured. As the half denatured molecules have bigger physical length, they stop migration in the gel.

DGGE has ever been widely used to analyze the microbial community structure in the wastewater treatment processes (Liu, *et al.*, 2000; Onuki, *et al.*, 2000; Schuler, *et al.*, 2002; Valle, *et al* 2004) and many other environmental microbial studies.

2.3.2 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) is another way to analyze DNA molecules with similar sizes. Molecules of DNA are digested with restriction enzymes, or enzymes that cut DNA molecule at unique sequence and the digested products are run on gel electrophoresis.

When the method is applied to amplicons from whole or partial 16S rRNA gene from an isolated bacterium, it will give restriction fragments of their unique sizes. But when the method is applied to 16S rRNA gene amplicon from environmental samples, different bacterial species in the samples will give restriction fragments with different sizes. Thus, the similarities or dissimilarities can be grasped by the similarities or differences of the detected fragment sizes. The profiling of the whole fragment lengths of the restriction digestion products is RFLP.

RFLP has been used to identify or differentiate the microorganisms (Navarro, *et al.*, 1992; Jensen, *et al.*, 1993; Ruiz, *et al.*, 2000) and analysis of microbial communities. The procedure usually includes: 1) amplication of special DNA sequence (Function DNA sequence or 16S rDNA), 2) restriction enzyme digestion, and 3) gel electrophoresis.

Typically gel electrophoresis in RFLP is performed on agarose gel. But resolution can be improved by using acrylamide gel. Capillary chip gel electrophoresis by using such instruments as 2100 Bioanalyzer (Agilent, USA), Experion (BioRad, USA), and MultiNA (Shimadzu, Japan) also enables relatively high-resolution analysis with reproducibility much higher than normal agarose gel electrophoresis.

2.3.3 Terminal restriction fragment length polymorphism

Terminal restriction fragment length polymorphism (T-RFLP), firstly described by Liu *et al.* (1997), is similar to RFLP but there are following differences: one of the ends of the target DNA molecules is marked with fluorescent marker and the only the fragments with the fluorescent marker are detected. To introduce fluorescent dye to the DNA molecules, PCR is performed with one of the two primers (forward primer and reverse primer) labeled with fluorescent marker at the 5' end. By using gel electrophoresis systems with high resolution such as those for DNA sequencing, the sizes (or the numbers of bases in the fragments) of the fluorescently labeled fragments are determined up to 500 bases or more.

Similar to DGGE, T-RFLP analysis is also widely used for the analysis of environmental microbial samples, because of its simplicity (Schütte, *et al.*, 2008). DGGE and T-RFLP have different strengths and weaknesses. In DGGE, bands of interest can be excised, and sequence of DNA in the excised band can later be determined. The

equipment needed for DGGE is in general cheaper than that for T-RFLP. But the preparation of gels with denaturing gradient requires high skill, and it is often difficult to directly compare electropherograms run on different gel plates. On the other hand, T-RFLP does not require special skill, the results are given as the number of bases and their intensities, and it is easy to compare results from different experiments or from different laboratories. It is also possible to narrow-down possible related species based on the size of the fragment, as it is possible to *in silico* calculate the expected fragment sizes. But usually, in order to identify species relevant to a band, cloning study needs to be done separately: each of targets DNA fragment is isolated and introduced to *E. coli* cells, and are separated as plural colonies each of which contain clone of originally single molecule of target DNA.

T-RFLP have been widely used to analyze the microbial community structures in activated sludge (Liu, *et al.*, 1997; Felföldi, *et al.*, 2010; Wells, *et al.*, 2011; Li, *et al.*; 2012; Yang, *et al.*, 2013; Evans, *et al.*, 2014).

2.3.4 Pyrosequencing

From around 2005, different high-throughput DNA sequencing technologies are being developed. Many of them utilize luminescence reaction neatly arranged to occur when pyrophosphate is generated as a result of an extension reaction in DNA sequencing, and thus they are often referred to as pyrosequencing. The principle of pyrosequencing was developed by Mostafa Ronaghi and Pål Nyrén at the Royal Institute of Technology in Stockholm in 1996 (Ronaghi, *et al.*, 1996; Ronaghi, *et al.*, 1998; Nyrén, 2007).

In pyrosequencing, pyrophosphate generated as a result of an extension reaction is utilized for ATP synthesis in the presence of adenosine 5’-phosphosulfate and ATP sulfurylase, and the resulting ATP is further used by luciferin for luminescence emission. In combination with the present video imaging technology with a high resolution and high sensitivity, a tremendous number of pyrosequencing reactions can be monitored simultaneously. For example, a GS FLX pyrosequencer, one of the 454 sequencer models from Roche (USA) perform and monitors around 10^6 sequencing reactions simultaneously. On the other hand, another technology is required to arrange as many as 10^6 target DNA samples for sequencing. In the case of 454 technologies, each of target molecules is separated by dilution and binding each of them to a small bead, the beads are encapsulated as micelles in emersion oil, PCR is performed inside each micelle, oil is

removed, and the beads are placed on a picotiterplate on which pyrosequeincing reactions are performed and luminesce is monitored. It can now read up to 1,000 bp, which is satisfactorily long enough to read partial 16S rRNA gene amplicons.

There also are pyrosequencing instruments manufactured by different companies: they have differences in the maximum lengths of reads and in the number of reads analyzed in one experiment. There also are high-throughput sequencing machines which are not based on pyrosequencing. Ion Torrent utilizes a semiconductor chip which has a large number of pH detectors, and it directly detects pH change in each extension reactions. These technologies are now often called "next generation sequencers". Many of these are used for medical purposes, but are also utilized in environmental microbial studies. Already, about activated sludge microorganisms, following studies have already been reported: Sanapareddy, *et al.*, 2009; Zhang, *et al.*, 2009; Kwon, *et al.*, 2010; Kim, *et al.*, 2011; Park, *et al.*, 2011; Ye, *et al.*, 2011; Ye & Zhang, 2011; Zhang T, *et al.*, 2011; Hu, *et al.*, 2012; Lu, *et al.*, 2012; Wang, *et al.*, 2012; Yu, *et al.*, 2012; Satoh, *et al.*, 2013; Yang, *et al.*, 2013.

2.4 Concluding remark

In 2.1, studies conducted so far on microbial interactions mediated by chemicals were reviewed under three headings: allelopathy, quorum sensing and autointoxification.

In 2.2, reports that support microbial interactions in activated sludge mediated by chemicals were reviewed. Here, some of the studies have been conducted with relatively-well characterized chemicals such as AHLs and AI-2, while other studies were conducted to detect allelopathic effects without knowing chemical species in the activated sludge samples. Both of these two approaches can lead to deepen understanding on microbial interactions in activated sludge mediated by chemicals. While the use of specific chemicals can give clearer ideas on the effect of the chemicals used, there may still be other unknown chemicals that are involved in microbial interactions in activated sludge. Studies on such unknown chemicals are worth to be performed more extensively.

The present study took the latter approach: instead of focusing and clarifying the effects of specific chemicals, the author attempted to approach the general characteristics of microbial interactions mediated by chemicals. Especially, the method developed by Ogawa (2009) was strongly adopted in this study.

Chapter 3 Materials and Methods

3.1 Introduction

As has been mentioned in the concluding remark of Chapter 2, in the present study, the method developed by Ogawa (2009) was adopted. It is because whole effects of bioactive chemicals that are extractable from activated sludge with solvents can be studied by his approach. According to his study, ethanol extract had the biggest effect.

The protocol Ogawa (2009) employed was as follows. He first operated an activated sludge reactor with synthetic sewage. He obtained extracts from the activated sludge using different solvents: ethanol, ethyl acetate and acetone. Then, he added the extract to wells of a microplate, evaporated solvent, the wells were inoculated with activated sludge and then with substrate and incubated for certain time. In control wells, instead of extract solution, pure solvent was added and dried. After incubation, culture in the wells was recovered, DNA was extracted and a partial 16S rRNA gene was amplified by PCR with fluorescently labeled primers, and resolved by T-RFLP. He found that the addition of extracts with all the solvents caused change in bacterial population, and ethanol extract was found to have the highest effect.

The present study took similar approach. Activated sludge was obtained from a laboratory scale reactor or a full scale wastewater treatment plant, ethanol extracts was obtained from it, the extract (or pure ethanol as control) was added to microplate wells, solvent was evaporated, the wells were inoculated with activated sludge and added with substrate and incubated. Up to here, the differences of the present study from Ogawa (2009) is the activated sludge used for extract preparation and incubation, the amount of extract addition, the amount of substrate fed to the biomass, and incubation time. For the bacterial population analyses, in the present study, instead of PCR/T-RFLP, RT-PCR/RFLP was mostly used. The reasons why T-RFLP was not used is for rather technical reason: the analytical equipment in the laboratory used for T-RFLP went obsolete, and it was found that RFLP can resolve change of microbial population as efficiently as T-RFLP when Agilent 2100 Bioanalyzer capillary chip electrophoresis was employed. Rather, when required time and labor are considered, RFLP with Bioanalyzer was found to be more efficient than T-RFLP. As for PCR or RT-PCR, with an expectation that RT-PCR can more directly monitor active bacterial species, the author used RT-PCR mainly in this study.

The details of the source of activated sludge used in the present study are described in 3.2. Activated sludge samples were obtained mainly from one laboratory sequencing batch reactor (SBR1) and one wastewater treatment plant (WWTP1). In addition, some experiments were conducted with activated sludge from another SBR (SBR2), and another wastewater treatment plant (WWTP2).

Some of the activated sludge samples were used to obtain crude extracts which are supposed to contain chemicals that affect bacterial population. The method to obtain crude extract is described in 3.3.

With the crude extracts prepared beforehand and the fresh activated sludge samples obtained on the day of sampling, a series of incubation experiments were conducted, as listed in Table 3.1. In these experiments, activated sludge was incubated in the wells of microplates. Common procedures and conditions about the incubation method are described in 3.4, and intentions of each experiment are explained in Chapter 4 for Exp. 1–Exp. 12 and in Chapter 5 for Exp. 13–Exp. 17.

The analytical methods of the performance of SBR1 are described in 3.5.

Bacterial population analysis methods are described in 3.6. Primers used in pyrosequencing experiments are described in Appendix 1.

Table 3.1 List of experiments conducted.

Exp. No.	Exp. date	Incubated activated sludge (AS)	Source of crude extract	Incubation time (hour)	F/M ratio *
1	2013/07/26	Day 3rd AS from SBR 1	E001 (Day 1st AS from SBR 1)	8 h	3.8
2	2013/0730	Day 7th AS from SBR 1	E001	120 h.	0.5
3	2013/0801	Day 9th AS from SBR 1	E001	12 h	6.6
4	2013/08/14	Day 22nd AS from SBR 1	E020 (Day 20th AS from SBR 1)	10 h	6.8
5	2013/08/24	Day 32nd AS from SBR 1	E029 (Day 29th AS from SBR 1)	12 h	0.4
6	2013/09/03	Day 42nd AS from SBR 1	E035 (Day 35th AS from SBR 1)	8 h	0.5
7	2013/09/07	Day 46th AS from SBR 1	E035	24 h	0.5
8	2013/10/02	Day 72nd AS from SBR 1	E035 & E052 (Day 52nd AS from SBR 1)	48 h	0.5
9	2013/10/15	Day 85th AS from SBR 1	E052	48 h	0.5
10	2013/10/29	Day 97th AS from SBR 1	E052	48 h	0.5
11	2013/11/06	Day 107th AS from SBR 1	E105 (Day 105th AS from SBR 1)	48 h	0.5
12	2013/12/11	Day 142nd AS from SBR 1	E137 (Day 137th AS from SBR 1)	24 h, 48 h, 96 h	0.5 and 2
13	2013/12/17	Day 148th AS from SBR 1	E137	24 h, 48 h, 96 h	0.5 and 2
14	2014/0/05	Day 198th AS from SBR 1	E137	24h	0.5
		As from WWTP			
15	2014/02/13	Feb.13th AS from WWTP1	E137	1h, 3h, 5h, 16h, 19h, 21h, 24h	0.5
16 (1)	2014/02/21	Feb. 21st AS from WWTP1	E137	2h~24h, Each 2h	0.5
16 (2)	2014/02/21	Feb. 21st AS from WWTP1	E137 (dose levels of 0.001×, 0.005×, 0.01×, 0.03×, 0.3×, and 1×)	24 h	0.5
17	2014/03/19	Mar. 19th AS from SBR2	E001, E029, E052, E105, E137 Feb. 5th AS from WWTP1 (EW1), Mar. 12th AS from WWTP2 (EW2) Mar 3rd AS from SBR2 (ES2)	24 h	0.5

*) The unit for F/M ratios is kgCOD/(kgMLSS·d) in experiments longer than 24 h, and kgCOD/kgMLSS in those shorter than 24 h.

3.2 Activated sludge

3.2.1 Laboratory SBRs (SBR1 and SBR2)

Both SBR1 and SBR2 were operated with synthetic wastewater. The seed activated sludge for the SBR1 was obtained from a full-scale wastewater treatment plant of N city of Japan. The seed sludge for SBR2 was taken from a full-scale wastewater treatment plant located within around 1 hour distance from the laboratory.

SBR1 had a working volume of 10L, and was operated as show in Fig. 3.1. In each cycle, 5 L of synthetic wastewater was added during the first 10 min, then reacted for 1 h under anaerobic condition and 2 h under aerobic condition, settled for 55 min, and finally 5L of treated water was discharged in the last 5 min. The hydraulic retention time (HRT) was 8 h. In each cycle, 415 mL of sludge mixed liquor was withdrawn to maintain the sludge retention time of 4 days. The whole process control including influent of synthetic water, discharge of waste water and excess sludge, and dissolved oxygen concentrations were controlled automatically by software named AOSBR3.21 developed in the laboratory and is an application running on LabView (National Instruments, USA). The reactor was not controlled on pH. The reactor was installed in an air conditioned room, in which temperature was around 18˚C in winter, 25˚C in summer, and around 20˚C in spring and fall.

The composition of the synthetic wastewater was: CH_3COONa-$3H_2O$ (180 mg/L), CH_3CH_2COONa (86 mg/L), peptone (160 mg/L), yeast extract (32 mg/L), KCl (67 mg/L), $CaCl_2 \cdot 2H_2O$ (21 mg/L), $MgSO_4 \cdot 7H_2O$ (176 mg/L), KH_2PO_4 (58 mg/L), $FeCl_3 \cdot 6H_2O$ (0.9 mg/L), $CuSO_4 \cdot 5H_2O$ (0.018 mg/L), $MnCl_2 \cdot 4H_2O$ (0.072 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.072 mg/L), H_3BO_3 (0.09 mg/L), KI (0.108 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (0.036 mg/L), $CoCl \cdot 6H_2O$ (0.09 mg/L) and EDTA (6 mg/L).

Another laboratory SBR, SBR2, was operated by my colleague, Ms. Wei SHI, and was operated under the same condition as those for SBR1 with differences as follows:

- The seed sludge was obtained from a full scale wastewater treatment plant of K city in Japan.
- SRT was 7 days.
- Occasionally the extents of the aerobic and anaerobic phases were changed.

Activated sludge samples were obtained from SBR1 and SBR2 as shown in Table 3.1, where some of them were used for incubation, and some to obtain crude extract. These samples were obtained at the end of aerobic phases.

As shown in Table 3.1, incubation experiments were conducted with the SBR1 sludge in Exp. 1– Exp. 13, where date of sampling are indicated as time in operation of the reactor. Crude extracts were obtained from SBR1 sludge samples obtained on days 1, 20, 29, 35, 52, 105, and 137, and are cited as E001, E020, E029, E035, E052, E105 and E137, respectively, as showed in Table 3.1.

SBR 2 sludge sample was used in Exp. 17. As the reactor had been stopped and resumed repeatedly, and the sludge was used only limited time, the sampling day is not indicated in its operation time but as date. Crude extract ES2 was also obtained from SBR2 sample taken on (Mar 3rd, 2014).

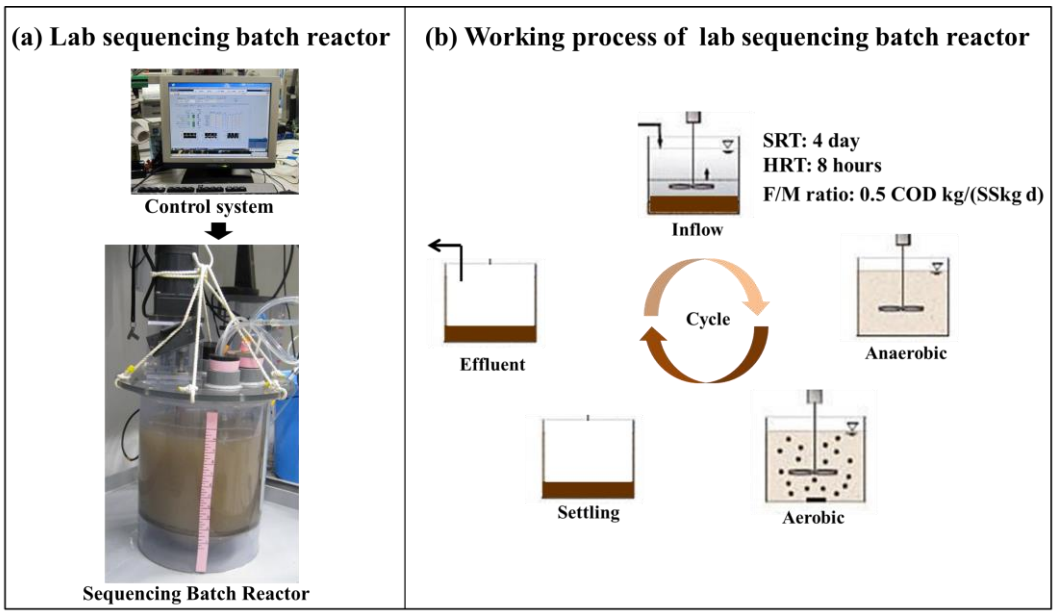

Fig. 3.1 The views and outline of the operation of SBR1.

3.2.2 Activated sludge taken from WWTPs

Activated sludge was sampled on February 5th, February 13th and February 21st, 2014 from WWTP1 with the extended aeration activated sludge process. The plant serves for a population of around 1,000 persons. Crude extract was obtained from the sludge obtained on Feb. 5th, which will be cited as EW1. The location of WWTP 2 is on the coastal line of

Tohoku area and it serves for around 300,000 people. The sludge is from the anaerobic-aerobic activated sludge process for the control of bulking. Activated sludge sample from WWTP2 was obtained on March 12th, 2014, and was used to obtain crude extract. The extract will be referred to as EW2.

3.3 Preparation of crude extracts from activated sludge samples

Crude extract was prepared by the method as was developed by Ogawa (2009). Activated sludge mixed liquor, 100 mL, was centrifuged at 3500 rpm for 5 min, the supernatant was decanted and the sludge pellet was washed with reverse osmosis (RO) water twice, then the activated sludge was further freeze-dried. Then 100 mL ethanol was added, the sludge was re-suspended, and incubated at 15°C for 24 hours on a rotary shaker at 300rpm. Then the mixture was centrifuged at 3500 rpm for 5 min and the ethanol phase was recovered. The ethanol extract was filtered through 0.2 μm membrane filter to remove residual solids, and preserved in –20°C until use.

3.4 Incubation experiments on microplate

3.4.1 Incubation on microplate

A 96-well microplate (Tissue Culture Test Plates Model 92096, Techno Plastic Products AG, Switzerland) was employed for the microplate incubation experiment. The wells were used for four groups of incubation conditions: Group 1: the wells were added with activated sludge, organic substrate and extracts; Group 2: the wells were added with activated sludge, organic substrate and pure ethanol (extract control, or e-control); Group 3: the wells were added with activated sludge and extracts but not substrate (substrate control, or s-control); Group 4: the wells were added with activated sludge only and pure ethanol but not substrate (extract and substrate control, or es-control). Groups 2, 3, and 4 were designed for control: Group 2 as e-control to allow microbial growth without the components in crude extracts; Group 3 as s-control and Group 4 as es-control are to examine microbial growth with the components in added crude extracts for growth substrates.

Before start of the incubation, the microplate wells were pre-conditioned: to each well of Groups 1 and 3, predetermined volume of crude extract was added, to each well of Groups 2 and 4, predetermined volume of pure ethanol was added, then ethanol was

completely dried in an incubator at 15°C for 24 h with rotary mixing at 50rpm. For each group, incubation was performed in duplicate or triplicate by assigning three wells. All the four groups were incubated simultaneously on one microplate in every experiment. If the volume of crude extract or pure ethanol was more than 300μL, the addition of extract or pure ethanol and drying was repeated plural times to dose the predetermined amount.

In some of the experiments especially those reported in Chapter 4, Groups 3 and Groups 4 were omitted.

For incubation, firstly 25mL of activated sludge mixed liquor was obtained from the SBR1, SBR2 or WWTP1. Then the activated sludge was gently sonified, added with 25 mL ultrapure water (Milli-Q Synthesis, Millipore, USA), and coarse solids were removed by settling for 15 minutes. The supernatant was recovered, and was further diluted with ultrapure to make the MLSS concentration to around 300mg/L. Then 290μL diluted activated sludge was loaded onto each of the wells of all the groups on the microplate. To the wells of Groups 1 and 2, predetermined amount of stock organic substrate to achieve the predetermined feed to mass ratio (F/M ratio, the ratio of feed concentration as chemical oxygen demand (COD) to biomass concentration as mass) was added at the beginning and every 24 hours of incubation. The composition of the stock feed was as follows: $CH_3COONa \cdot 3H_2O$ (11.3g/L), CH_3CH_2COONa (5.36g/L), Peptone (10g/L), Yeast extract (2g/L), KCl (4.2g/L), $CaCl_2 \cdot 2H_2O$ (1.32g/L), $MgSO_4 \cdot 7H_2O$ (11g/L), K_2HPO_4 (3.6g/L), $FeCl_3 \cdot 6H_2O$ (113.6mg/L), H_3BO_3 (11.36mg/L), $CuSO_4 \cdot 5H_2O$ (2.272mg/L), KI (13.6mg/L), $MnCl_2 \cdot 4H_2O$ (9.09mg/L), $Na_2MoO_4 \cdot 2H_2O$ (4.546mg/L), $ZnSO_4 \cdot 7H_2O$ (9.09mg/L), $CoCl \cdot 6H_2O$ (11.36mg/L), EDTA (757.6mg/L).

The microplate was incubated under mixing at 1500 rpm on a micro-plate shaker Microplate Genie (Scientific Industries, USA) in an air conditioned room at around 18°C to 20°C. During incubation, the wells were sealed with a gas permeable seal (Abgene AB-0718, Thermo Fisher Scientific Inc., USA) except during feeding and monitoring of optical density.

To grasp microbial growth, optical density at 600 nm in the wells was analyzed at predetermined timings by a microplate reader SH-1000Lab (Hitachi High-technologies Cor., Japan).

The activated sludge mixed liquor in the wells were recovered at the end of incubation or after incubation for a predetermined period of time, mixed with 300 μL of ethanol, and stored at –20°C till use.

3.4.2 Expression of the amount of extract added

In the present study, when components in 300 μL of extract are dosed to a well, it is referred to as 1 time or "1 ×" addition. In the same way, when components in 900 μL of extract are dosed to a well, it is referred to as 3 times or "3 ×" addition.

As described in 3.3, from 100mL of activated sludge mixed liquor, 100mL of ethanol was obtained. And as when activated sludge is incubated with 1 × extract in a microplate well, volumetric concentration of the extract in the original activated sludge and in the well is maintained the same.

3.4.3 Definition of F/M ratio

In this thesis, as an indicator of organic loading rate, food-to-microorganisms (F/M) ratio is used. The F/M ratio is calculated basically as follows, and the unit is kgCOD/(kgMLSS·d).

$$\textbf{F/M ratio} = \frac{\textbf{COD fed to biomass per day}}{\textbf{Initial MLSS}}$$

In the present study, feeding was done every 24 hours. Thus, F/M ratios to the wells incubated 24, 48, 72, 96 or 120 hours are apparent.

But when the wells incubated shorter than 24 hours, the unit is kgCOD/kgMLSS

3.5 Monitoring methods of SBR1

For evaluation of the treatment performance of SBR1, dissolved organic carbon (DOC) concentrations at the end of anaerobic and aerobic phases, mixed liquor suspended solids (MLSS) and sludge volume index (SVI) at the end of aerobic phase were monitored basically every two days. To monitor DOC, a Shimadzu TOCV-csh was used, while the measurements of MLSS and SVI were conducted according to the standard methods (American Public Health Association, 2005).

3.6 Methods in bacterial population analyses

Changes of bacterial population were monitored mainly by RFLP after PCR targeting partial 16S rRNA gene sequence or RT-PCR targeting partial 16S rRNA sequence. While PCR/RFLP, which target 16S rRNA gene, was performed to grasp total bacterial population including less active bacteria, RT-PCR/RFLP, which target 16S rRNA, was performed to monitor active bacteria. In addition, in some of the experiments, RT-PCR products were analyzed by pyrosequencing to identify positively or negatively affected bacterial species.

3.6.1 Extraction of DNA or 16S rRNA for PCR or RT-PCR

The template for PCR and RT-PCR were prepared based on the sonication-dilution method developed by Satoh *et al*. (2008, 2012). For the samples after incubation on microplates, DNA and 16S rRNA were extracted by sonication of the ethanol-mixed samples by a sonifier AD250 (Branson, USA) at an amplitude of 30% (7 W) for 30 s. The extract was then diluted 10 times, and directly used for PCR or RT-PCR reactions.

For the original activated sludge samples taken from SBR1, SBR2 or WWTP1, the mixed liquor was added with the same volume of ethanol, sonified by the sonifier AD250 (Branson, USA) at an amplitude of 30% (7 W) for 30 s, then diluted by 100 times and used as the template for PCR and RT-PCR.

3.6.2 PCR

TaKaRa ExTaq Hot Start Version (Takara Cor., Japan) was used for PCR amplification with 27f (5'- AGA GTT TGA TCM TGG CTC AG -3') and 519r (5'- GWA TTA CCG CGG CKG CTG-3') primers (Lane, 1991). The composition of the reaction mixture was as follows: 2 μL 10×buffer, 1.6 μL dNTP Mixture (2.5mM each of A, T, G and C), 0.4 μL 27f primer (10 pmol/μL), 0.4 μL 519r primer (10 pmol/μL), 0.1 μL TaKaRa Ex TaqHS (5 units/μL), 2 μL template, and 13.5 μL of autoclaved ultrapure water. The PCR reaction was performed by a thermal cycler Dice (TaKaRa, Japan) with the thermal program as follows: 94°C for 10 min, 30 cycles of 94°C for 30 s, 55.3°C for 30 s, 72°C for 30 s, and a final extension step of 72°C for 10 min.

3.6.3 RT-PCR

PrimeScript One Step PCR Kit Version 2 (Takara Cor., Japan) was used for RT-PCR with the primers of 27f and 519r. The composition of the reaction mixture was as follows: 5 μL 2×buffer, 0.4 μL forward primer (10 pmol/μL), 0.4 μL reverse primer (10 pmol/μL), 0.2 μL RNasin (Promega, USA), 0.4 μL prime script enzyme mixture, 1 μL template, and 2.6 μL RNase-free water. The RT-PCR reaction was performed by a thermal cycler Dice (TaKaRa, Japan) with the thermal program as follows: 50°C for 30 min, 94°C for 2 min, 20 cycles of 94°C for 30 s, 55.3°C for 30 s, 72°C for 30 s, and a final extension step of 72°C for 10 min.

3.6.4 Restriction fragment length polymorphism

After PCR or RT-PCR, the product concentrations were determined by using PicoGreen dsDNA Quantification Kit (Invitrogen, USA). The PCR and RT-PCR products from each sample were diluted and adjusted to 25 ng/μL, and digested by the restriction enzyme *Hha*I (TaKaRa Cor., Japan) at 37°C for three hours. The composition of the reaction mixture was as follow: 5μL PCR or RT-PCR product (25 ng/μL), 1μL 10×Buffer, 0.3μL *Hha*I Enzyme (10U/μL), and 3.7 μL of autoclaved ultrapure water. The restriction fragments were analyzed by capillary chip gel electrophoresis by Bioanalyzer 2100 (Agilent Technologies, USA).

3.6.5 Pyrosequencing and sequence data analysis

After confirmation of the quality of PCR products or RT-PCR products by agarose gel electrophoresis, the products were used as template for second PCR with the barcoded and adapter-prepended primers. The barcoded primers, or primers prepended with a short additional sequence, is often used in environmental microbiological studies to identify the source of each reads obtained in sequencing experiments by next generation sequencers. On the other hand, the adapter sequences were used so that the products are directly applicable for pyrosequencing reactions by a Roche 454 GS Junior sequencer.

The forward primers used here were prepended with 8 base barcode (http://pyro.cme.msu.edu/pyro/help.jsp) and further prepended with adapter sequence on the 5’ ends. The reverse primer was not barcoded but prepended with the adapter sequence. The detailed primer information is showed in the Appendix 1.

The second PCR reaction was performed in the same way as described in 3.6.2 with Takara ExTaq Hot Start Version with following differences: primers for pyrosequencing were used, products of PCR or RT-PCR were used as the template, and the number of thermal cycle was 5. The product concentration was checked with a PicoGreen dsDNA Quantification Kit (Invitrogen, USA) and the quality was checked by gel electrophoresis. All the PCR products were mixed together and purified by gel electrophoresis to remove primer dimers. The larger DNA products were recovered by excision of the gel and recovered by utilizing Gel Extraction Kit (Qiaquick, Qiagen, Germany). The recovered DNA was again checked by Agilent 2100 Bioanalyzer (Agilent Technologies, USA) to confirm that the primer dimers had been removed and to confirm the total DNA amount to be enough for pyrosequencing analysis. After this, the samples were analyzed by a GS Junior pyrosequencer (Roche).

Pyrosequencing data was analyzed by utilizing OTUMAMi (Operational Taxonomic Unit Management And Mining, http://www.mwm.k.u-tokyo.ac.jp: 8080/Plone/outcome/OTUMAMi, Satoh et al., 2013), which is dependent mainly on QIIME (Quantitative Insights Into Microbial Ecology, QIIME, Knight Lab in University of Colorado at Boulder, (Caporaso, *et al.*, 2010) for bioinformatic analyses such as to split the reads (or to assign each reads to their original samples based on detected barcode sequences), to formulate operational taxonomic units (OTUs) based on NAST algorithm, and phylogenetic tree calculation based on FastTree algorithm, and taxonomic classification by the RDP Classifier in Ribosomal Database Project (RDP) (Wang, *et al.*, 2007). The reads obtained by pyrosequecing were classified into their operational taxonomic units (OTUs) at 97% similarity. The phylogenetic tree for selected OTUs was calculated by the neighbor-joining method using MEGA 6.0 (Tamura, *et al.*, 2013).

As many OTUs were found, major OTUs were selected by following criteria: For each sample, fractions $f_{i,j}$ and a mean fraction $\bar{f_j}$ were calculated as follows:

$$\mathrm{f_{i,j}} = \left(\frac{\mathrm{C_{i,j}}}{\sum_{\mathrm{i}} \mathrm{C_{i,j}}}\right) \times 100 \ [\%] \tag{1}$$

$$\bar{f_j} = \sum_i f_{i,j} \times \frac{100}{N} \ [\%] \tag{2}$$

where $C_{i,j}$ indicates the read count of OTU j in sample i, and N is the number of samples. In this study, the $\bar{f_j} \geqq 2\%$ was defined as major OTU.

3.7 Principal component analysis

Bands for all RFLP analyses were detected by the Agilent 2100 Expert software (Version B.02.08, Agilent Technologies, USA). Intensity threshold was adjusted so that most of visible bands were detected. The aerial intensities of the bands were standardized against the total band areal intensities in each sample, minor peaks occupying less than 1% of total areal intensities were omitted, and bands at the same positions were carefully assigned manually. The data was further analyzed by MarkerView (Version 2.0, AB Sciex Pte. Ltd., USA) for principal component analysis (PCA) with the Pareto scaling method after logarithmic conversion.

Chapter 4 Improvement of Incubation Conditions by Experiments of Trial and Error

4.1 Introduction

In this chapter, the results of experiments which were conducted before effective outcomes were obtained are reported. The experiments reported in this chapter were conducted rather try and error to improve the incubation conditions on microplate so that bacterial population change can be more clearly be observed. The results of 12 experiments as listed in Table 4.1 conducted during August to December 2013. In these experiments, different efforts were made to observe significant bacterial population caused by crude extracts.

The factors examined in the 12 experiments were as follows, as can be found in Table 4.1.

- F/M ratio: If growth of biomass is small, the whole population is in general expected to be close to that of the original activated sludge. To enhance population change, the author initially thought that more growth is beneficial to detect population change.

- Incubation time: The longer the incubation time, more change can be expected to happen in activated sludge bacterial population. But to improve the throughput of analysis, desirably, population change be detected with shorter incubation time.

Apart from above two factors, each experiment differed in activated sludge used for incubation (that is, the date the sludge was taken from SBR1 differed) and in the source of crude activated sludge extract.

4.2 Materials and methods

In all the experiments reported in this chapter, as listed in Table 4.1, activated sludge used for incubation and the source of the extract used were both from SBR1. The

operational condition of SBR1 has been described in 3.1 and the performance of SBR1 has been showed in Appendix 2. The experiments were conducted by the method as described in 3.2.

Experiment 1, Exp.3 and Exp.4 were conducted mainly to examine the effects of organic loading rate or F/M ratio. While F/M ratio applied to SBR1 was around 0.5 kgCOD/(kgMLSS·d), in Exp. 1, 3 and 4, F/M ratios of 3.8, 6.6, and 6.8 kgCOD/kgMLSS were applied, respectively. Note that F/M ratios in experiments with shorter incubation times than 24 hours are reported with a unit of kgCOD/kgMLSS, while those incubated for longer than 24 hours are reported with kgCOD/(kgMLSS·d). .

In Exp. 1, two types of extract was used, both from SBR1 activated sludge sampled on Day 1: one of them (E001) was extracted from fresh activated sludge right after sampling at the end of the aerobic phase, and the other (E001') from activated sludge aerated for additional 2 hours without feed. E001' was prepared in an intention to have activated sludge enriched with allelochemicals by over aeration. In addition, extract was added at two levels: 1 × and 4 ×.

Exp. 2 was performed to examine the effect of sonication again: as the results of Exp.1, 3 and 4 were not favorable, the author suspected that sonication of activated sludge might be negatively affecting the results. Here the author incubated the sludge with an F/M ratio of 0.5 kgCOD/(kgMLSS·d), which is the same as that for SBR1. In this experiment, to observe differences of bacterial population with a small growth under low feeding, incubation was continued for 5 days.

In Exp. 4, the author used extract not only with ethanol but also with methanol as the extractant.

Exp. 5, Exp. 6 and Exp. 7 were conducted with low F/M ratios of 0.4 or 0.5 kgCOD/kgMLSS with shorter incubation time of 8 to 24 hours. In these experiments, the author focused to maintain original bacterial population during incubation in extract control wells.

Exp. 8, Exp. 9, Exp. 10, and Exp. 11 also were conducted with a low F/M ratio of 0.5 kgCOD/(kgMLSS·d), but with a longer incubation time of 48 hours. The original intention to incubate longer was to enhance bacterial population change by incubating

longer. In Exp. 5 and Exp. 6, the effect of the amount of extract dose was also studied.

Finally, Exp.12 was conducted with two F/M ratios of 0.5 or 2 kgCOD/(kgMLSS·d) and incubated for up to 96 hours. In this experiment, 9 wells were incubated for each condition to destructively take samples at 24, 48 and 96 hours.

In all these experiments, bacterial population changes were analyzed by RT-PCR/RFLP as is described in 3.6.3 and 3.6.4. Group 3 (substrate control) and Group 4 (substrate and extract control) were omitted in the experiments reported in this chapter.

Table 4.1 List of experiments conducted.

Exp. No.	Date of the start of incubation (number of days in SBR1)*)	Crude extract applied **)	Incubation time (hour)	F/M ratio***)	Factor focused
1	Jul. 26th , 2013 (Day 3rd)	E001 & E001'	8 hr	3.8	High loading
2	Jul. 30th , 2013 (Day 7th)	E001 & E001'	120 hr.	0.5	Without sonication
3	Aug. 1st, (Day 9th)	E001 & E001'	12 hr	6.6	High loading
4	Aug. 14th, (Day 22nd)	E020$)	10 hr	6.8	
5	Aug. 24th, (Day 32nd)	E029	12 hr	0.4	Low loading
6	Sep. 3rd, (Day 42nd)	E035	8 hr	0.5	
7	Sep. 7th, (Day 46th)	E035	24 hr	0.5	
8	Oct. 2nd, (Day 72nd)	E035 & E052	48 hr	0.5	Longer incubation time
9	Oct. 15th, (Day 85th)	E052	48 hr	0.5	
10	Oct. 29th, (Day 97th)	E052	48 hr	0.5	
11	Nov. 7th (Day 107th)	E105	48 hr	0.5	
12	Dec. 11th (Day 142nd)	E137	24 hr, 48 hr, 96 hr	0.5 & 2	Different incubation time

*) All sludges incubated were from SBR1.

**) For example, E001 is meant for crude extract obtained from SBR1 activated sludge sampled on Day 1st in the operation of SBR1.

***) The unit of F/M ratio is kgCOD/(kgMLSS·d) for those with incubation times longer than 24 hr and kgCOD/kgMLSS for those with shorter incubation times.

$) Extracts were prepared not only with ethanol but also with methanol.

4.3 Results

(1) Experiment 1

The incubated activated sludge was taken on Day 3rd of SBR1 and incubated with extract from Day 1st sludge (E001 and E001').

The RT-PCR/RFLP results were as showed Fig. 4.1. All the RFLP profiles after incubation were quite similar regardless of the addition or omission of extract, except for the RFLP profile for the original activated sludge.

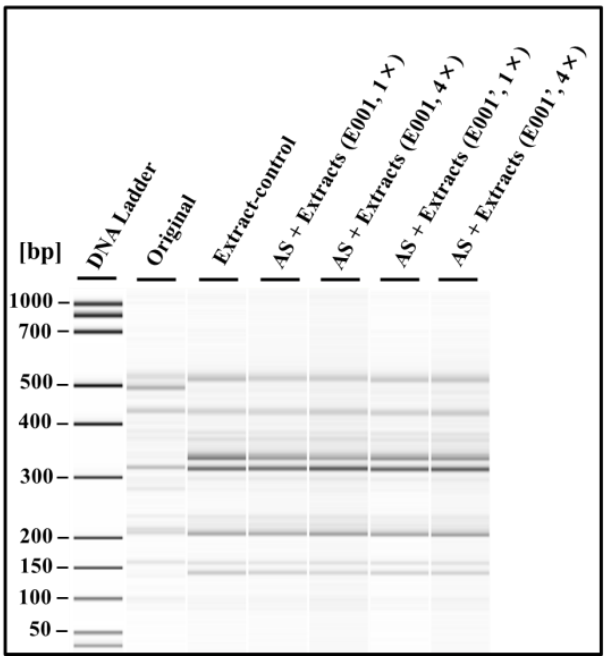

Fig. 4.1 RT-PCR/RFLP results of Exp. 1

(2) Experiment 2

The incubated activated sludge was taken on Day 7th of SBR1 and incubated with extract from Day 1st sludge (E001 and E001').

The RT-PCR/RFLP results were as shown in Fig. 4.2. While other experiments were inoculated with gently sonified activated sludge to improve reproducibility, in this experiment, sonication was omitted.

While the bands for the wells incubated with the same conditions were the same, their intensities differed. And because of this, it is difficult to see if there is any distinct difference between extract control wells and the wells incubated with extract. The cause of the fluctuation of the band intensities was thought to be the omission of sonication which caused significant difference in microbial population of biomass inoculated to the wells.

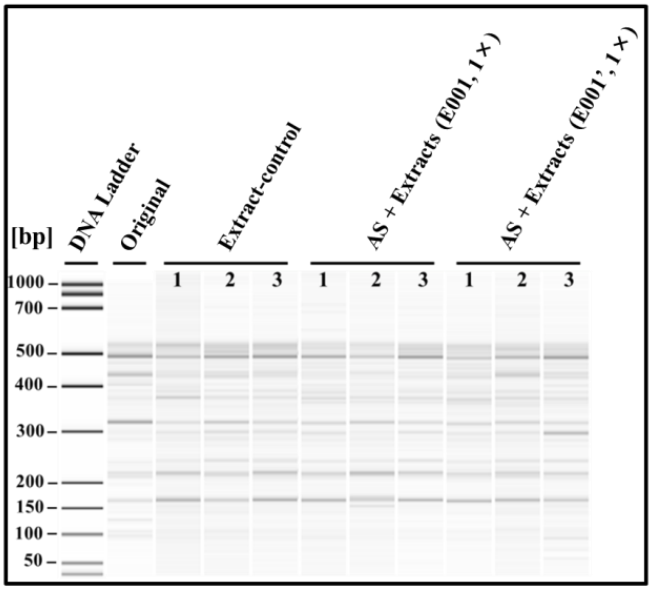

Fig. 4.2 RT-PCR/RFLP results of Exp. 2.

(3) Experiment 3

The incubated activated sludge was taken on Day 9th of SBR1 and incubated with extract from Day 1st sludge (E001 and E001').

This experiment was conducted to re-examine the conditions studied in Exp. 1. The RT-PCR/RFLP results were as showed in Fig. 4.3. In this experiment, conditions applied were similar to that in Exp. 1: F/M ratio applied was close to Exp. 1, and the extract used was the same as that in Exp.1. The results obtained were similar to those in Exp. 1. As the inoculated activated sludge was sonified beforehand, RFLP profiles after incubation were more consistent than those in Exp. 2.

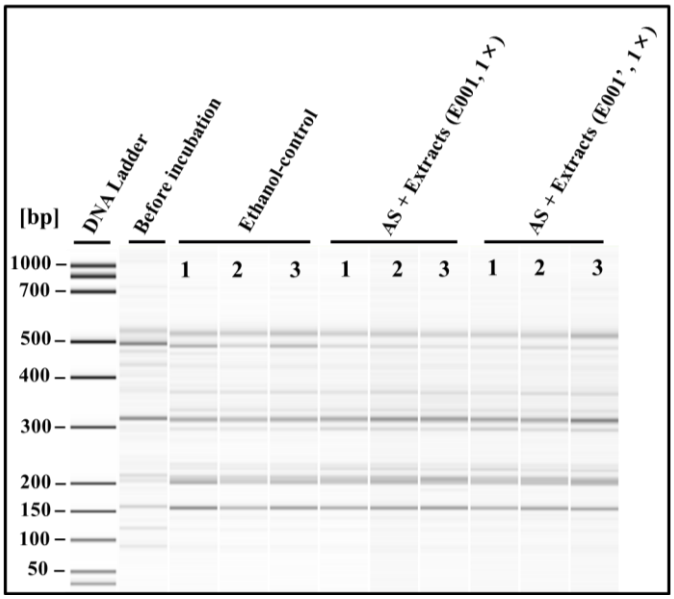

Fig. 4.3 RT-PCR/RFLP results of Exp. 3

(4) Experiment 4

The incubated activated sludge was taken on Day 22nd of SBR1 and incubated with extract from Day 20th sludge (E020).

This experiment was conducted once more again to examine the conditions studied in Exp. 1. In this experiment, not only ethanol extract but also methanol extract was tried.

The RT-PCR/RFLP results were as showed in Fig. 4.4. In this experiment, F/M ratio applied was close to Exp. 1, but the sludge incubated and the extracts used were different. The results obtained were similar to those in Exp. 1, but differences were observed between RFLP profiles of control wells and extract-added wells: bands sized around 320 bp and 370 bp and 520bp were intensified when extract was added. Extract with methanol affected bacterial population to a similar level or a little bit more than ethanol extract did. But difference in band patterns was subtle between control and extract added wells.

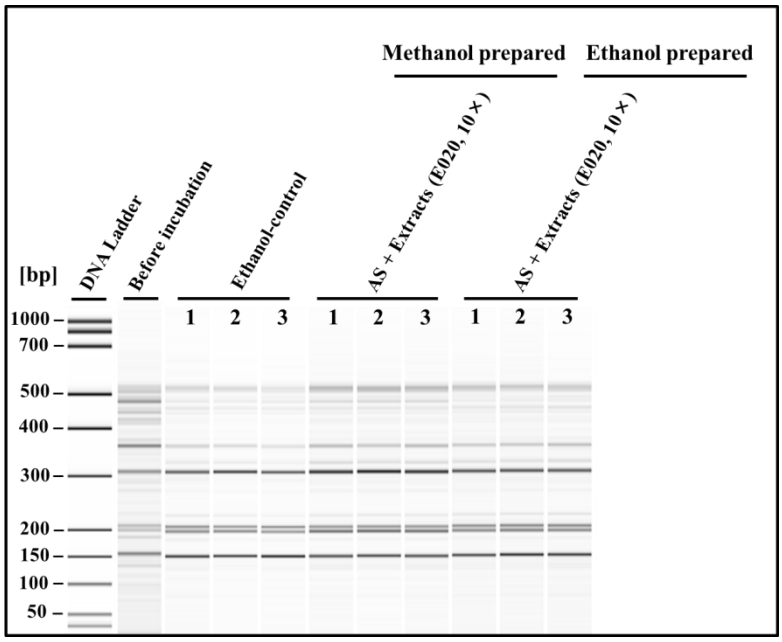

Fig. 4.4 RT-PCR/RFLP results of Exp. 4

(5) Experiment 5

In this experiment, extract was added at three levels: 0.5 ×, 1 × and 4 ×. From this experiment up to Exp. 11, lower F/M ratio of 0.4 or 0.5 kgCOD/kgMLSS·d (for Exp. 5 and 6, kgCOD/kgMLSS) was applied. Incubation time was 12 hours. The incubated activated sludge was taken on Day 32nd of SBR1 and incubated with extract from Day 29th sludge.

The RT-PCR/RFLP results were as shown in Fig. 4.5. The RFLP profiles before and after incubation were quite similar, as was expected. The reproducibility of triplicated wells was satisfactory, though minor differences were observed. The profiles for the extract control wells (Group 2) and the extract-added wells (Group 1) were similar. But the profiles of the wells added with 1 × and 4 × extract looked different from those added with 0.5 × or 0 × (extract control) extract. That is, bands at 320 bp and 520 bp were intensified, 370 bp was weakened, and a new band emerged at around 170 bp by the addition of extract higher than 1 ×.

Still, The change of bacterial population was still smaller .

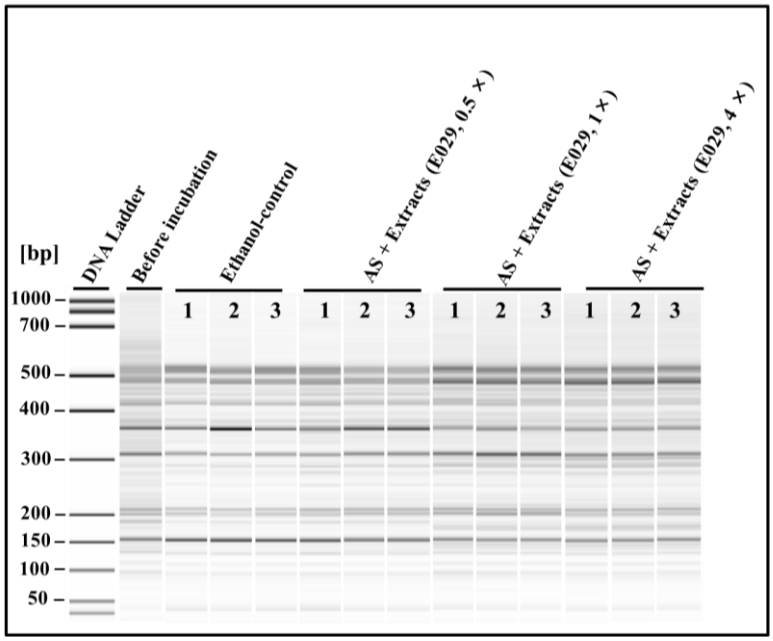

Fig. 4.5 RT-PCR/RFLP results of Exp. 5

(6) Experiment 6

In this experiment, two different levels of extracts, 1 × and 4 ×, were applied. The incubated activated sludge was taken on the 42nd operation day of SBR1, extract obtained from Day 35th sludge (E035), and incubated in microplate for 8 hours at an F/M ratio of 0.5 kgCOD/kgMLSS.

The RT-PCR/RFLP results were as shown in Fig. 4.6. Here, compared with the extract control, the RFLP profiles for wells incubated with 1 × extract addition were similar, but those of wells incubated with 4 × extract addition changed much. Especially, the band of 380 bp and 500 bp became weaker, while the band 205 bp and 320bp became stronger.

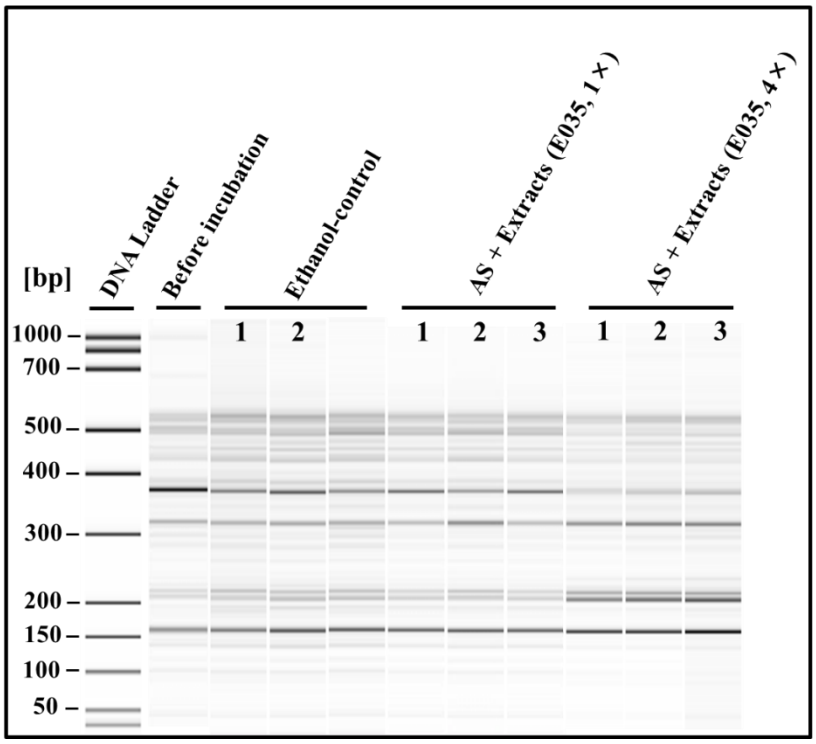

Fig. 4.6 RT-PCR/RFLP results of Exp. 6.

(7) Experiment 7

In this experiment, the extract E035 was again used and activated sludge taken on Day 46th of SBR1 was incubated for 24 hours at an F/M ratio of 0.5 kgCOD/(kgMLSS·d).

The RT-PCR/RFLP results were as showed in Fig. 4.7. When RFLP profiles for the original sludge and the extract control are compared, more differences than that in Exp. 6 can be found, probably due to longer incubation time (Exp. 7 for 24 hours while Exp. 6 for 8 hours) in this experiment. The band around 180 bp disappeared and 410 bp intensified during the 24 hours of incubation in the case of extract control. When RFLP profiles for extract control wells and the 1× extract added wells are compared, bands around 400 bp to 450 bp were weakened in the extract added wells. But the differences were subtle.

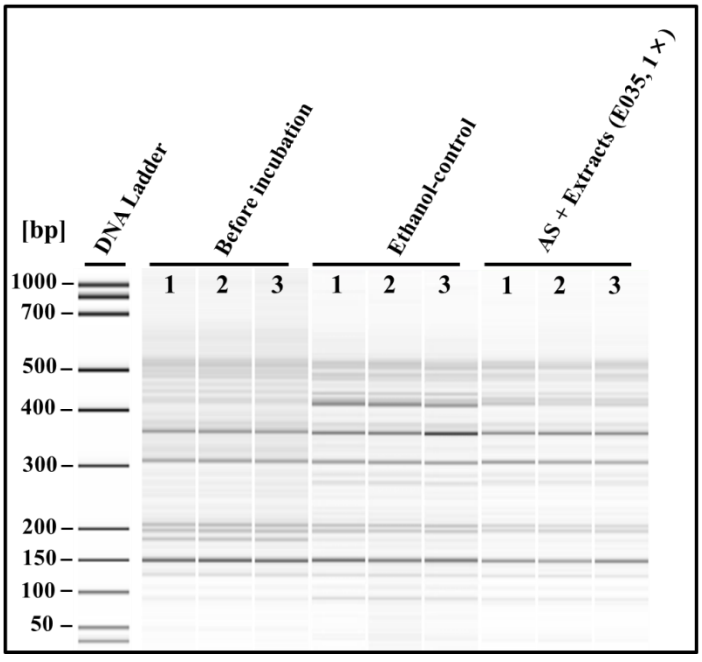

Fig. 4.7 RT-PCR/RFLP results of Exp. 7

(8) Experiment 8

In the results of Exp. 5, 6 and 7, the addition of extract was found to cause subtle but apparent changes in bacterial population in activated sludge. In order to intensify the effects, from this experiment up to Exp. 11, incubation was done for 48 hours with an F/M ratio of 0.5 kgCOD/(kgMLSS·d).

In this experiment, the extracts E035 and E052 were used and activated sludge taken on Day 72nd of SBR1 was incubated.

The RT-PCR/RFLP results were as showed in Fig. 4.8. When compared with the extract control, RFLP profiles for the wells added with extract had weaker intensities for the bands sized around 460 bp and 370 bp. When the effects of E035 and E052 are compared, E052 intensified the bands around 220bp, 290 bp, and 300 bp. But the effects of the extracts on bacterial population change were still smaller than the author was expecting.

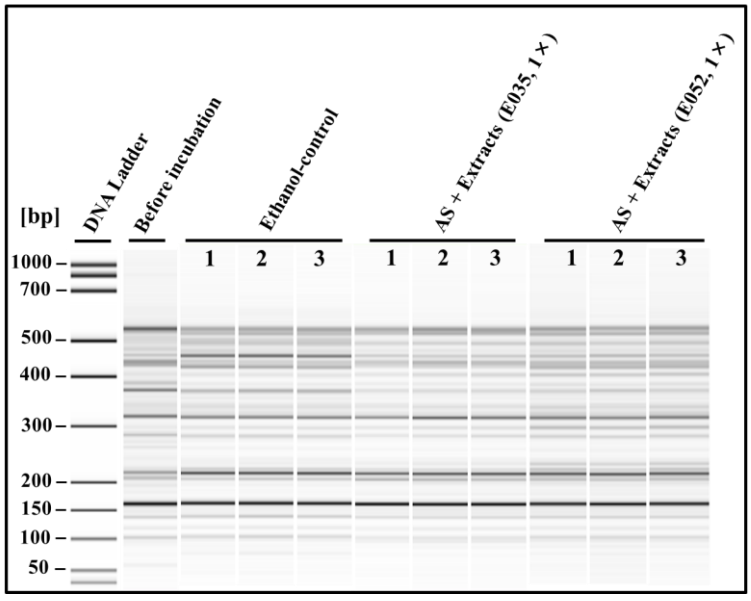

Fig. 4.8 RT-PCR/RFLP results of Exp. 8.

(9) Experiment 9

In this experiment, the extract E052 was used and activated sludge taken on Day 85th of SBR1 was incubated for 48 hours at an F/M ratio of 0.5 kgCOD/(kgMLSS·d).

The RT-PCR/RFLP results were as showed in Fig. 4.9. Anyhow, significant difference of RFLP profiles was found between extract control wells and the original sludge. Difference between the extract control wells and those added with extract were subtle, but following differences were found: a new band around 290 bp emerged, while the bands around 200 bp and 150 bp became weaker by the addition of extract.

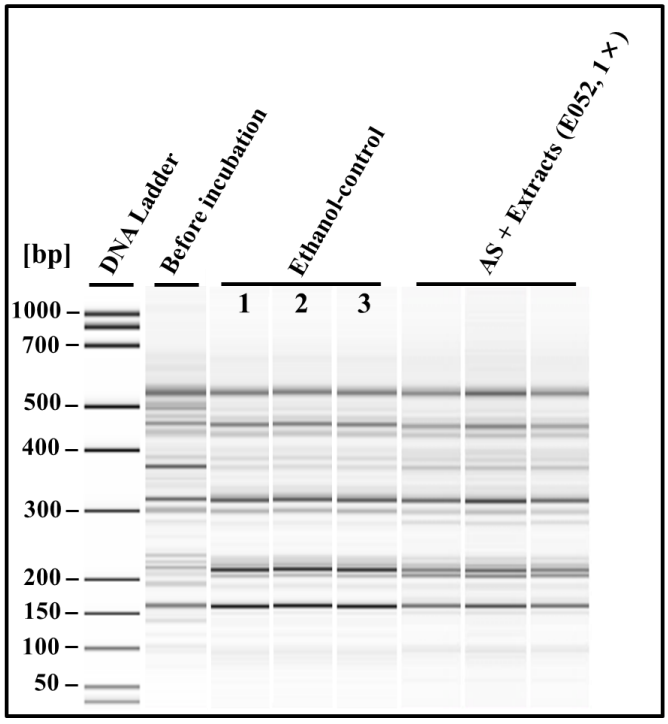

Fig. 4.9 RT-PCR/RFLP results of Exp. 9.

(10) Experiment 10

In this experiment, again, the extract E052 was used and activated sludge taken on Day 97th of SBR1 was incubated for 48 hours at an F/M ratio of 0.5 kgCOD/(kgMLSS·d).

The RT-PCR/RFLP results were as shown in Fig. 4.10. The differences between the extract control wells and extract-added wells were clearer than Exp. 9: a new band around 290 bp and 220 bp emerged, while some bands between 450 to 500bp disappeared, the band around 200 bp and 300 bp became stronger while the band around 380 bp and 150 bp became weaker by the addition of the extract.

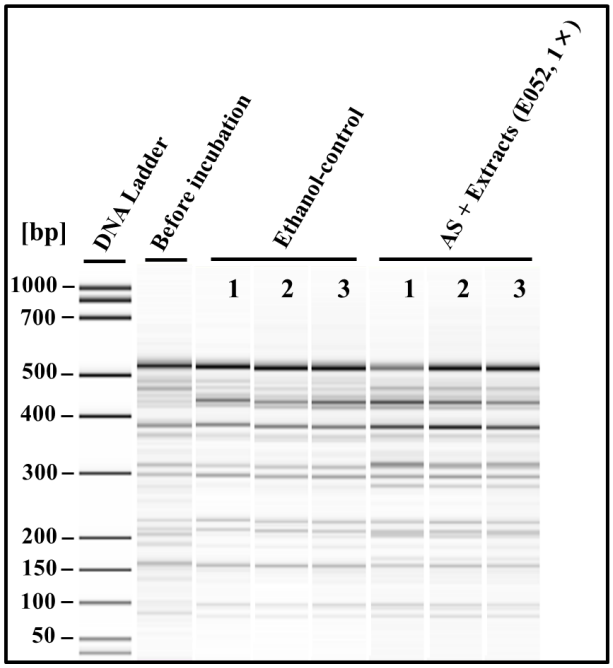

Fig. 4.10 RT-PCR/RFLP results of Exp. 10

(11) Experiment 11

In this experiment, again, the extract E105 was used and activated sludge taken on Day 107th of SBR1 was incubated for 48 hours at an F/M ratio of 0.5 kgCOD/(kgMLSS·d).

The RT-PCR/RFLP results were as shown in Fig. 4.11. The addition of extract caused change in RFLP profiles or change in bacterial population more significantly than in the precedent experiments. The bands around 200 bp and 310 bp became stronger, the bands around 100 bp,380 bp and 450 bp, and minor bands around 500 bp weakened by the addition of extract.

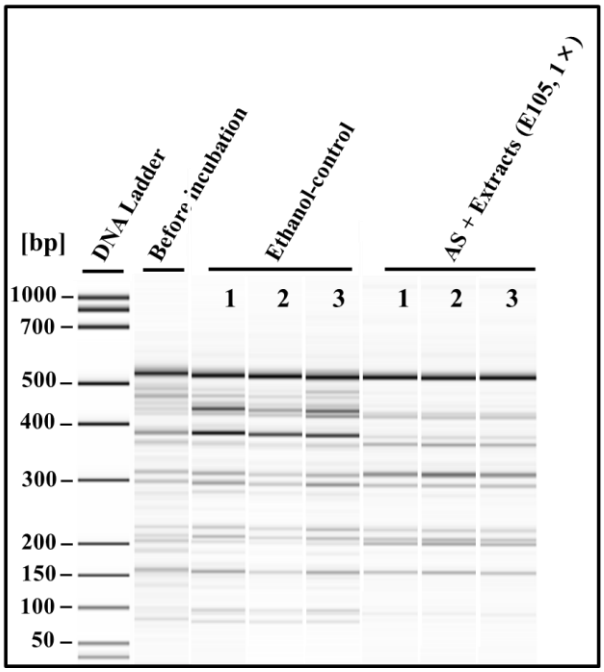

Fig. 4.11 RT-PCR/RFLP results of Exp. 11.

(12) Experiment 12

In the previous experiment, effects of extract on bacterial population were clearly observed. This experiment was conducted to 1) further confirm the observation in Exp. 11, and 2), observe the effects of incubation time (24, 48 and 96 hours), F/M ratio (0.5 and 2 kgCOD/(kgMLSS·d), and the levels of extract addition (1 × or 3 ×). It was also conducted with E137 and SBR1 activated sludge taken on Day 142nd was incubated.

The RT-PCR/RFLP results were as shown in Fig. 4.12. In this experiment, one well only was used for each condition. Even for 24 hours incubation, it was found that the band patterns or bacterial population was significantly changed by the addition of extracts. Bacterial population further changed as the incubation proceeded in wells with extracts addition. On the other hand, even after 96 hours incubation, extract control with F/M of 0.5 kgCOD/(kgMLSS·d) gave similar RFLP profiles to that of the original activated sludge.

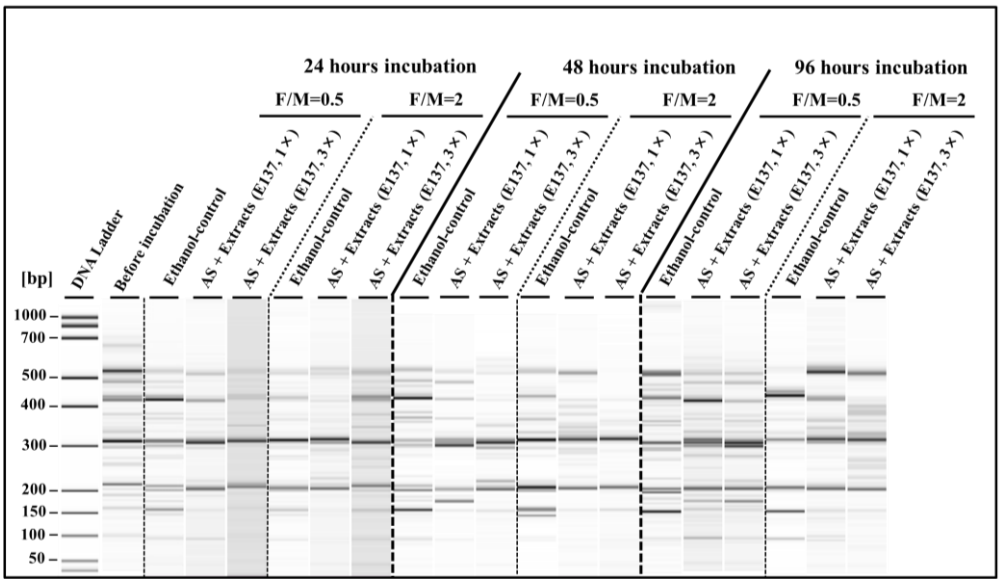

Fig. 4.12 RT-PCR/RFLP results of Exp. 12.

4.4 Discussion and conclusions

In this chapter, 12 experiments were reported which was conducted in an effort to detect bacterial population change caused by activated sludge extract.

Initially, in order to have more growth, higher F/M ratios were applied. But the difference between bacterial population in extract control and that in extract-added wells were not significant. Later, in Exp. 5 and Exp. 6, the author applied an F/M ratio of around 0.5. This time, the bacterial population changes caused by the addition of extract was detected, however, was not very significant. The author considered that the longer incubation time might magnify the effect of extract on the bacterial population changes, and extended the time to 48 hours. Then, the bacterial population changes were more clearly detected in Exp. 9, Exp. 10, and Exp. 11. Then to systematically investigate the bacterial population changes caused by addition of extract, the effects of F/M ratio, incubation time, and the level of extract addition was studied in Exp. 12. In Exp. 12, bacterial population change was detected within 24 hours of incubation clearly.

When the results of Exp. 8, 9, 10 and 11, which were conducted with the same F/M ratio and the same incubation time but were different about the incubated activated sludge and the added extracts, are compared, the changes of bacterial population in Exp. 8 (using E035 and E052), Exp. 9 (E052) and Exp. 10 (E052) were subtle but significant in Exp. 11 (E105). In Exp. 12 (E137), the change of bacterial population was again significant. These results imply that the abundance or the intensities of the chemicals that affect microbial population in activated sludge change during the operation of an activated sludge reactor.

After all, through the experiments in this chapter, the method to detect bacterial population change caused by crude extracts from activated sludge was mostly established, and an extract, E137, which has strong effect on the change of bacterial population, was obtained. In the next chapter, the author performed more experiments to clarify the time scale of the effects of the extract, dose-response patterns, and the effects on different activated sludges, and the comparison of the intensities of extract from different sources.

Chapter 5 Bacterial Population Changes Affected by Addition of Crude Extracts from Activated Sludge

5.1 Introduction

By using the conditions established in Chapter 4, five more experiments, Exp. 13 through Exp. 17, were conducted about the effects of extract on bacterial population in activated sludge to clarify in detail the response time scale, effect of F/M ratios, dose-response patterns, and the effects on different sludge, and the comparison of the intensities of extracts from different sources. Here, the results of four of the experiments, Exp. 13, Exp. 14, Exp. 16 and Exp. 17 are reported. The author omitted the result of Exp. 15 because the objective was similar to Exp. 16 and Exp. 16 was more elaborated. The result of Exp. 15 is reported in the appendix.

In Exp. 13, the effects of different factors were investigated to have an over view. In Exp. 14, activated sludge samples from SBR1 and WWTP1 were compared on their sensitivity to the same extract. Experiment 16 was conducted with two objectives. In Exp. 16 (1), bacterial population changes within 24 hours were monitored every two hours to see the response time. Meanwhile, in Exp. 16 (2), the dose-response patterns were investigated by utilizing the extract at different dose levels. In Exp. 17 the effects of 8 extracts obtained in the present study were compared using the same activated sludge.

5.2 Materials and methods

Activated sludge incubated, the sources of extract, incubation times, and F/M ratios of the experiments are as listed in Table 5.1

The method for chemical extraction, activated sludge incubation, bacterial population analysis and Principal component analysis were as described in Chapter 3.3, Chapter 3.4, Chapter 3.5 and Chapter 3.6 respectively.

Exp. 13 was conducted with almost the same conditions with those in Exp. 12 to have an overview of the effects of different factors: incubation time, F/M ratio, the level of extract addition, and except for following two points: the date of sampling of the

incubated sludge was different, and while Exp. 12 was done with a single well for each condition, this experiment was conducted with triplicate for each condition. As the extract, E137 which was found to have strong effect to change bacterial population was used, while activated sludge incubated was from SBR1 on day 148th.

Exp. 14 was conducted to compare the sensitivity of different sludges to the same extract. Again E137 was used as the extract, and two kinds of activated sludges were incubated: SBR1 sludge obtained on Feb. 5th, 2014 (Day 198th in SBR1) and WWTP1 sludge also obtained on Feb. 5th. The F/M ratio applied was 0.5 kgCOD/(kgMLSS·d), and incubated for 24 hours. To know the differences in sensitivity, extract was added at different levels of 0.3×, 0.6×, and 1×.

Exp. 16 (1) was conducted to examine the response time. Extract E137 was used, and WWTP1 sludge sampled on Feb. 21st, 2014 was incubated for up to 24 hours with an F/M ratio of 0.5 kgCOD/kgMLSS. The samples were taken every 2 hours during incubation for bacterial population analyses by RT-PCR/RFLP.

Experiment 16 (2) was conducted with the same activated sludge and extract to Exp. 16-1. Extract was added at different levels (0.001, 0.005, 0.01, 0.03, 0.3, and 1 ×), and incubated for 24 hours with an F/M ratio of 0.5 kgCOD/kgMLSS.

Experiment 17 was conducted to compare the effects of extracts from different sources. Following extracts were used at the dose level of 1 ×: E001, E029, E052, E105, E137, ES2, EW1, and EW2. While E001 through E137 were from SBR1 sludge, ES2 from SBR2 sludge sampled on Mar. 3rd, 2014, EW1 from WWTP1 sludge on Feb. 5th, 2014, and EW2 from WWTP2 sludge on Mar. 12th, 2014. As SBR1 had been stopped because of bulking, instead SBR2 sludge sampled on March 19th, 2014 was used for incubation. Incubation was done for 24 hours with an F/M ratio of 0.5 kgCOD/kgMLSS.

Table 5.1 Materials and methods outline in Exp. 13, Exp. 14, Exp. 16 and Exp. 17.

Exp. No.	Focus	Conditions
13	Overview of the effects of different factors	Incubated activated sludge: SBR1 (Day 148th) sampled on Dec. 17th, 2013 Extract used and dose level: E137 (1×) Incubation time: 24, 48, 96 hr F/M ratio : 0.5 and 2 kgCOD/(kgMLSS·d) Analysis of bacterial population: PCR/RFLP and RT-PCR/RFLP Controls: all controls were run.
14	Comparison of the sensitivity of different sludge to the same extract	Incubated activated sludge: SBR1 (Day 198th) and WWTP1 both sampled on Feb. 5, 2014 Extract used and dose level: E137 (0.3×, 0.6×, 1×) Incubation time: 24 hr F/M ratio : 0.5 kgCOD/kgMLSS Analysis of bacterial population: RT-PCR/RFLP Controls: only extract control was run.
16 (1)	Response time	Incubated activated sludge: WWTP1 on Feb. 21st Extract used and dose level: E137 (1×) Incubation time: every 2 hours up to 24 hours F/M ratio : 0.5 kgCOD/kgMLSS Analysis of bacterial population: RT-PCR/RFLP Controls: only extract control was run.
16 (2)	Effects of extract dose levels	Incubated activated sludge: WWTP1 on Feb. 21st Extract used and dose level: E137 (0.001×, 0.005, 0.01, 0.03, 0.3, 1) Incubation time: 24 hours F/M ratio : 0.5 kgCOD/kgMLSS Analysis of bacterial population: RT-PCR/RFLP Controls: only extract control was run.
17	Effects of extracts from sources	Incubated activated sludge: SBR2 Mar. 19th, 2014. Extract used and dose level: E001, E029, E052, E105, E137, ES2, EW1, and EW2 (all 1×) Incubation time: 24 hours F/M ratio : 0.5 kgCOD/kgMLSS Analysis of bacterial population: RT-PCR/RFLP Controls: extract control and substrate control were run.

5.3 Results

5.3.1 Experiment 13

-----Overview of the effects of organic loading and incubation time

5.3.1.1 Growth of activated sludge in the wells

The optical densities of activated sludge in each of the wells fed with substrate increased as incubation proceeded, as shown in Fig. 5.1. Outlier plots in gray resulted most probably from the inhomogeneous growth of bacteria such as in bigger flocs and growth attached to the wall. The optical density was around 0.15 initially, and it linearly increased to around 0.3 and 0.7 in the wells with low and high organic loadings respectively after 96 hours of incubation. The addition or omission of the extract did not cause significant change in the increase of the optical densities.

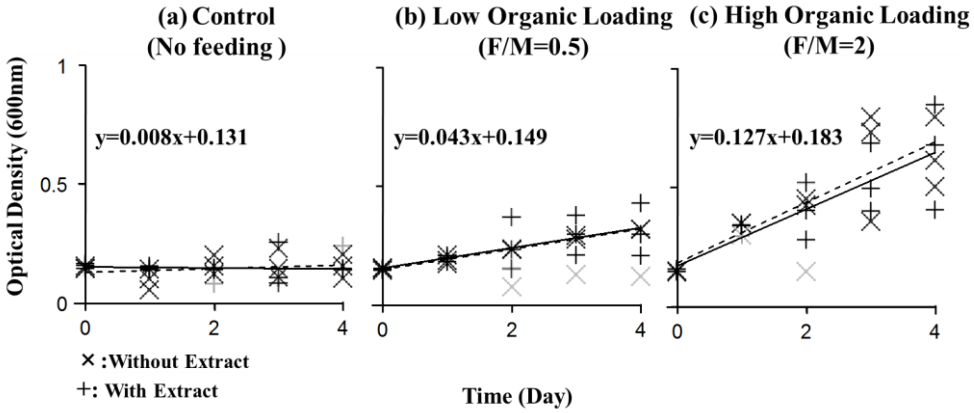

Fig. 5.1 Optical densities of activated sludge in the microplate wells in Exp. 13. The plots in gray are those that were rejected by the Grubb's test. The solid and broken lines are the regression lines for the accepted plots in each condition.

5.3.1.2 Comparison of bacterial population of activated sludge after incubation

Bacterial population in the microplate wells was analyzed by PCR/RFLP and RT-PCR/RFLP, and the results were as shown in Fig. 5.2. Three wells incubated under the identical conditions gave similar band patterns with each other. When band patterns are compared between with and without extract addition, significant difference was observed.

To more clearly compare the band patterns, the results of the PCR/RFLP analyses were further processed by PCA. As Fig. 5.3 shows, the band patterns were divided into three groups as indicated by the dotted circles. The lower left group and the higher left group were for the samples with high organic loading and low organic loading without extract addition, respectively. On the other hand, the middle right group was the samples with extract addition with both high and low organic loadings. The original activated sludge sample was in the higher left group suggesting that bacterial population in the control with lower feeding did not change so much and population structure resulted from the organic loading. The plots for the higher loading without extract addition at 24 hours incubations were located a bit closer to the group with extract addition.

The results of the PCR/RFLP and RT-PCR/RFLP were compared by PCA as shown in Fig. 5.4. The plots were grouped into three: the RT-PCR/RFLP results without extract addition (middle right), the PCR/RFLP results without extract addition (lower left) and the RT-PCR/RFLP and PCR/RFLP results with extract addition (upper left).

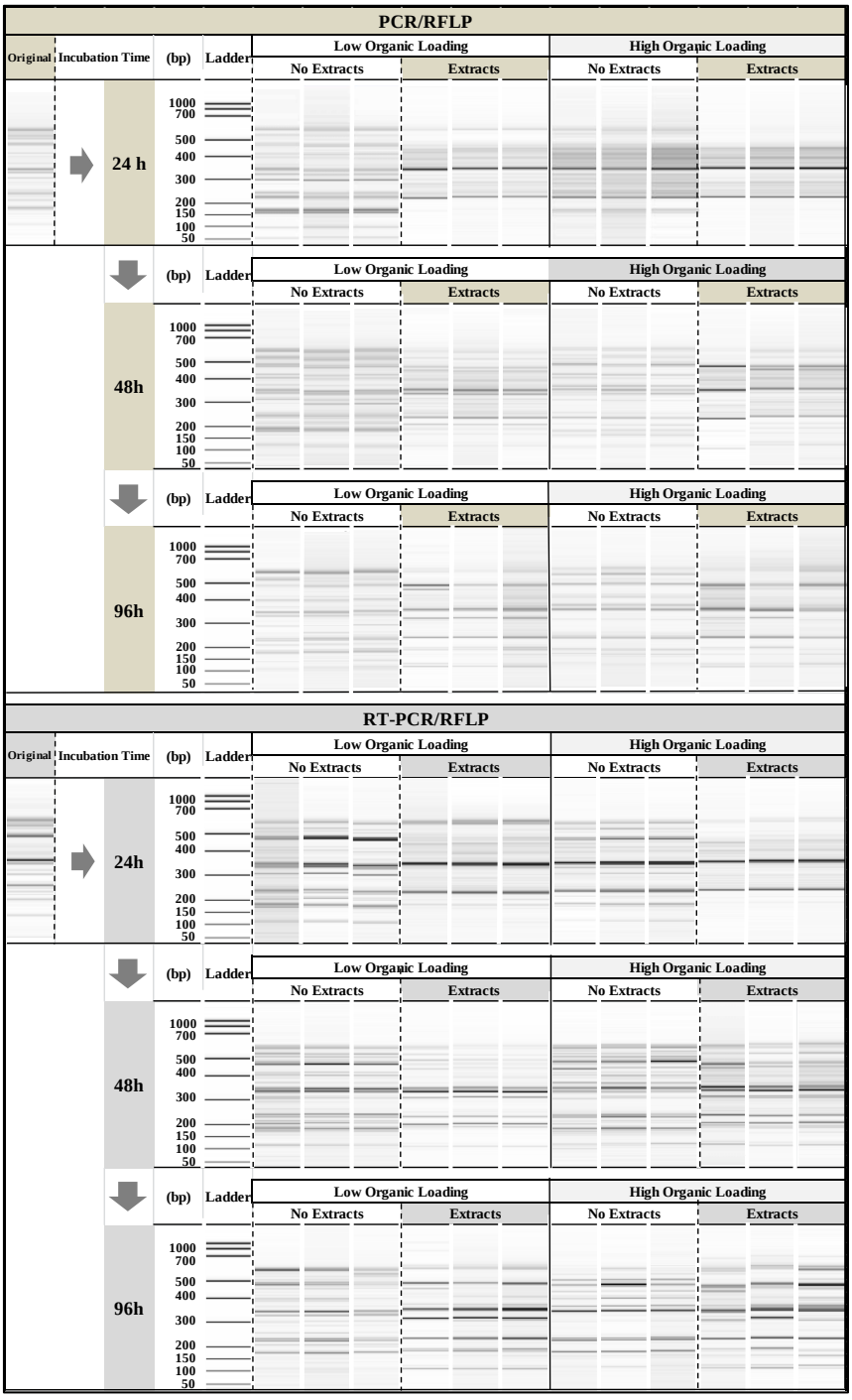

Fig. 5.2 PCR/RFLP and RT-PCR/RFLP results obtained in Exp. 13.

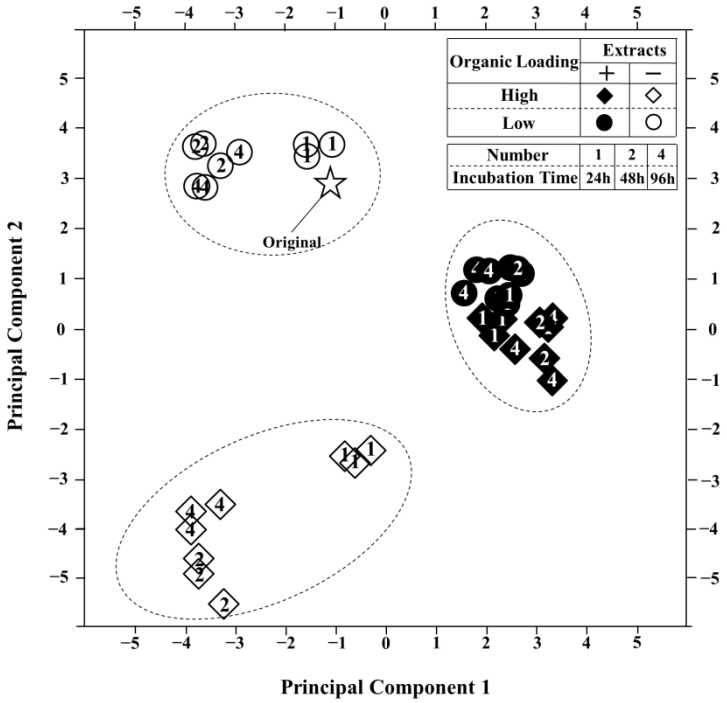

Fig. 5.3 Comparison of PCR/RFLP band patterns by PCA in Exp. 13. The contributions of the first and second principal components were 22.0% and 20.9%, respectively.

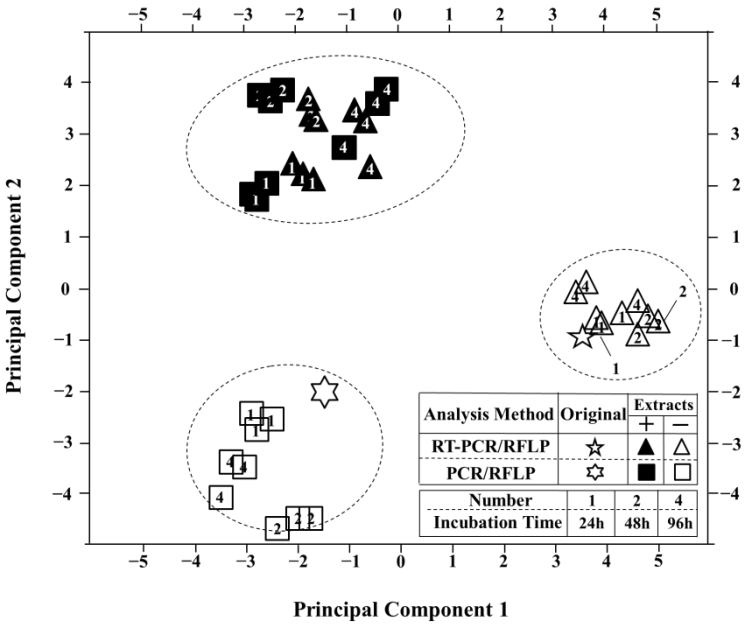

Fig. 5.4 Comparison of the PCR/RFLP and RT-PCR/RFLP for low loading incubation condition in Exp. 13. The contributions of the first and second principal components were 21.6% and 17.1%, respectively.

5.3.2 Experiment 14

----- Comparison of sensitivity of activated sludge to the same extract

5.3.2.1 Growth of activated sludge

The optical densities in the wells were as shown in Fig. 5.5. The optical density was around 0.15 initially, and it increased to around 0.2 in the wells after 24 hours of incubation. The increase of optical densities was at a similar extent regardless of the level of extract addition.

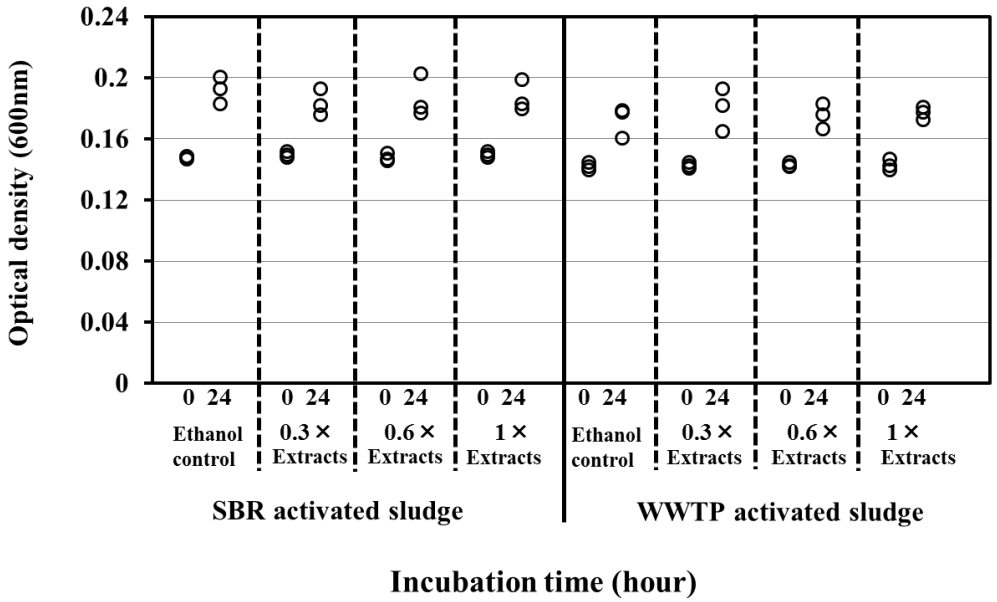

Fig. 5.5 Optical densities of activated sludge in the microplate well in Exp. 14.

5.3.3.2 Bacterial population changes

The results of the RT-PCR/RFLP were as shown in Fig. 5.6. While for each condition incubation was done in triplicate (with three wells), biomass in two of the three wells were analyzed by RT-PCR/RFLP.

For SBR1 sludge shown in Fig. 5.6 (a), the band patterns of the original activated sludge and the extract control were basically similar except that the band around 430 bp was intensified and two bands around 450 bp to 500 bp disappeared in extract control. The addition of 0.3 × extract caused following effects in comparison to extract control: 430 bp band was weakened, and 200 bp and 270 bp bands were slightly increased. More

addition (0.6 × or 1 ×) of extract resulted in the elimination of 420 bp band, and 270 bp band was weakened.

For the change of the intensity of 430 bp band, the threshold of the dose of extract was in between 0.3 and 0.6 ×, and the shape of the response seems not linear. The band 270 bp was intensified by the addition of 0.3 × of extract, but was weakened by the higher level of dose.

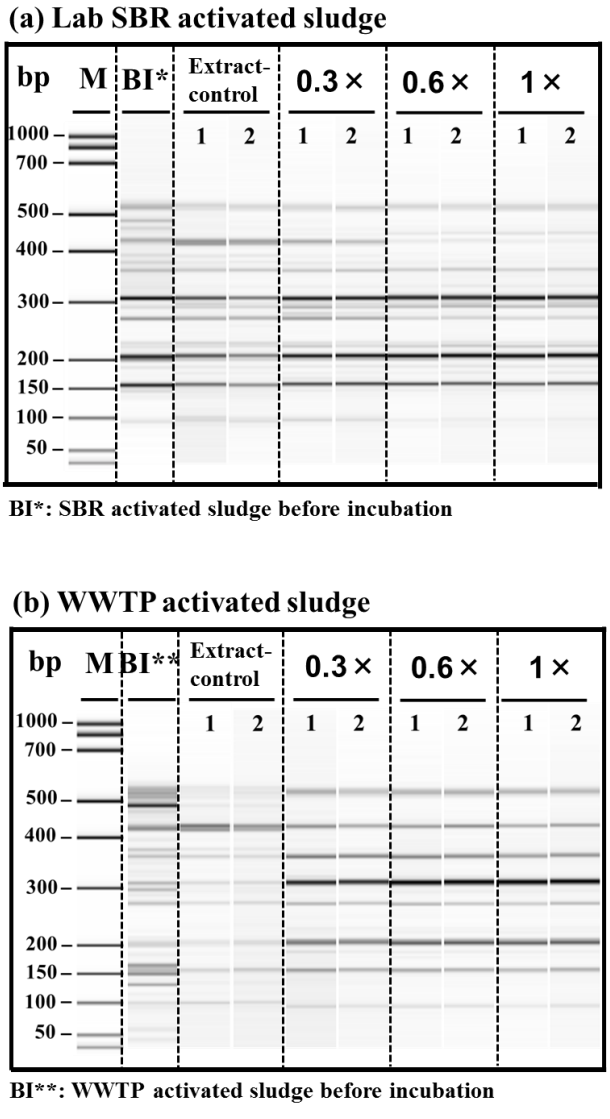

Fig 5.6 RT-RFLP results of Exp. 14.
(a): SBR1 activated sludge; (b) WWTP1 activated sludge.

For WWTP1 sludge shown in Fig. 5.6 (b), the band patterns of the original activated sludge and the extract control were quite different: the RFLP profile of the extract control had one major band of 430 bp and other bands which were clearly seen in the original sludge were faint or disappeared in extract control. The big difference between extract control and the original sludge is apparently coming from the difference in substrate: WWTP1 sludge had been acclimatized with real sewage, but in this incubation synthetic wastewater was used.

Further, RFLP patterns for extract control and the extract added cases were significantly different. In all extract added cases, bands were observed clearly and with similar intensities at following positions: 150 bp, 200 bp, 270 bp, 300 bp, 350 bp, 430 bp, and 530 bp. The threshold of the response was less than 0.3 × for the extract used, and the response curve shape was thought to be sigmoid.

To more clearly compare the band patterns of the samples, the results of the RT-PCR/RFLP analyses were further processed by PCA.

Figure 5.7 shows the PCA plot of RFLP results of lab SBR1 and the plots were grouped into three as identified by the dotted circles. The upper one is the original activated sludge sample before incubation in the microplate. The lower left is the samples with 0.6 ×, 1× extracts addition, while the lower right is the sample without and with 0.3 × extracts addition. This result suggested the bacterial community structure did not change so much compared with the samples without addition of extracts, but changed significantly with 0.6 and 1 × extract addition.

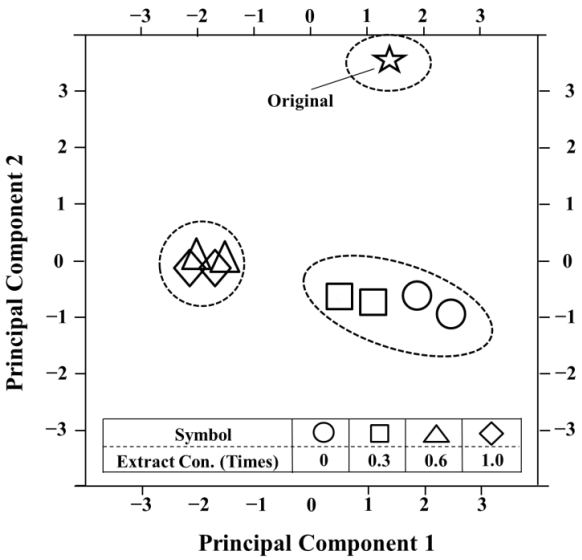

Fig. 5.7 Comparison of RT-PCR/RFLP band patterns by PCA in Exp. 14 for SBR1 activated sludge. The contributions of the first and second principal components are 59.5% and 30.6% respectively.

The band patterns of WWTP1 activated sludge was also analyzed by PCR as shown in Fig. 5.8. The plots are also divided into three groups. The upper one is the original activated sludge sample before incubation in the microplate. The lower left is the samples with extracts addition and the lower right is the samples without extracts addition. The results suggested the bacteria community structure was significant affected when even incubated with as low as 0.3 × extracts addition.

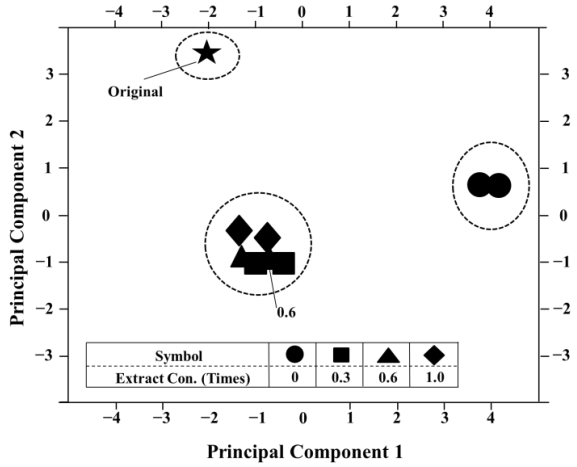

Fig. 5.8 Comparison of RT-PCR/RFLP band pattern by PCA in Exp. 14 for WWTP activated sludge. The contributions of the first and second principal components were 56.7% and 24.6% respectively.

5.3.3 Experiment 16 (1)

-----Response time

5.3.3.1 Growth of activated sludge

The optical densities of activated sludge the wells fed with substrate increased as incubation proceeded, as shown in Fig. 5.9. Incubation was done in duplicate. The optical density was around 0.08 initially, and it increased to around 0.12~0.14 in the wells after 24 hours of incubation.

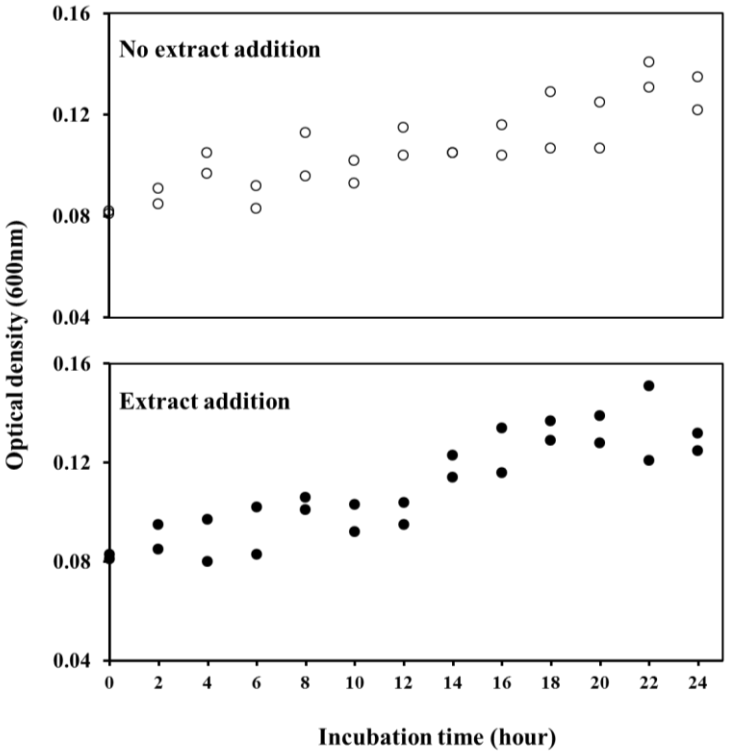

Fig. 5.9 Optical densities of activated sludge in the microplate wells in Exp. 16 (1).

5.3.3.2 Bacteria population changes

The RT-PCR/RFLP profiles of activated sludge without addition of extracts were as shown in Fig. 5.10 (a). While in Exp. 14 the RFLP profiles of the control and the original biomass were significantly different for WWTP1 sludge, in this experiment, the difference was small. The RFLP profiles for the control wells after incubation were similar throughout 2 to 24 hours of incubation. While it seemed as if the growth rate was

constant throughout incubation as shown in Fig. 5.9, in Fig. 5.10 (a), it looks as if population of the original sludge changed slightly during the initial two hours, and then population was maintained almost constant. The bands observed in these lanes for control wells were sized around: 91 bp, 158 bp, 206 bp, 280 bp, 316 bp, and 425 bp, and 534 bp.

The RT-PCR/RFLP profiles for the extract-added wells were as shown in Fig. 5.10 (b) and the results of the two replicates were showed in Appendix 6. The band patterns changed in the initial 6 hours, and then stabilized. And the band patterns between the extract control and the extract-added cases were significantly different. Out of the major bands found in control, those at around 210 bp and 320 bp became stronger and those at around 91 bp and 425 bp were weakened when extract was added. The results suggested that the bacterial population changed within a few hours.

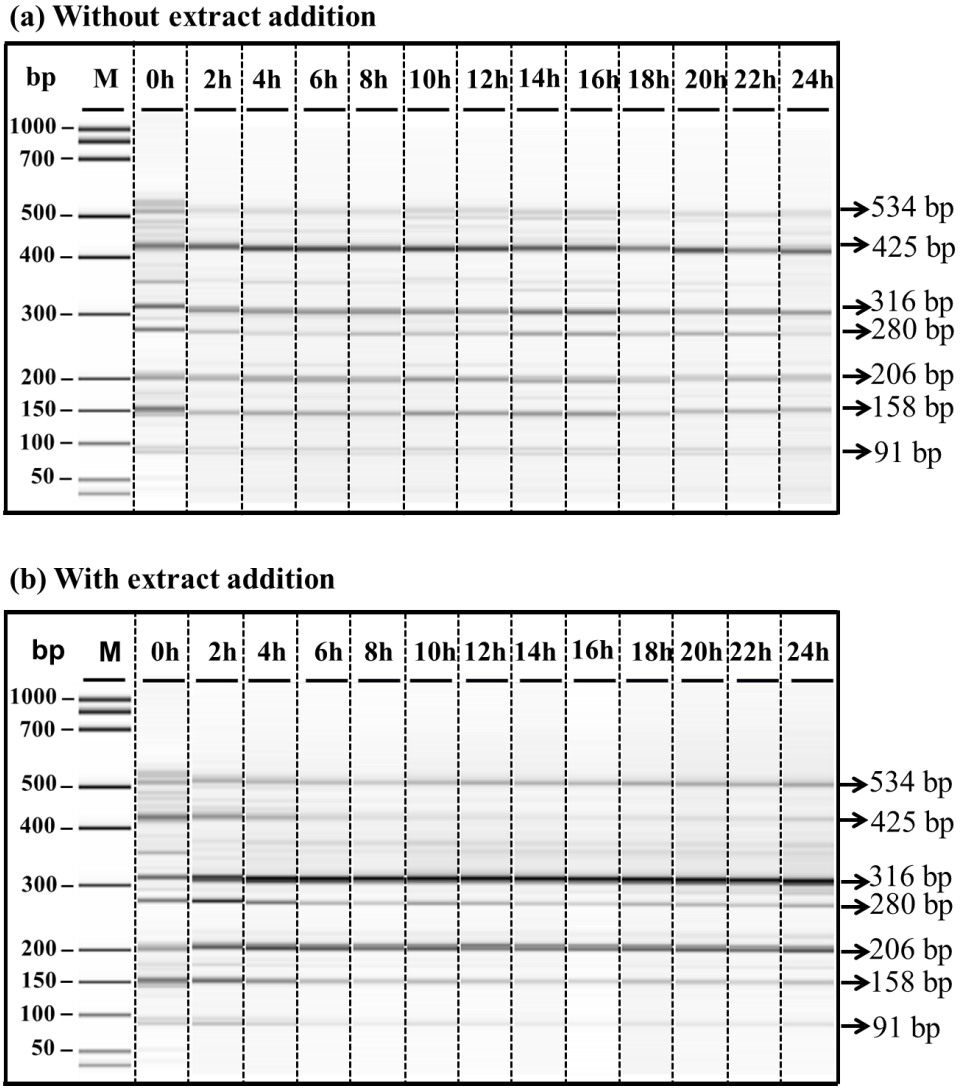

Fig. 5.10 Bacterial population changes with and without extract addition in Exp. 16 (1).

To more clearly visualize the change of the band intensities, the fractions of band intensities (peak area) in total peak intensities of individual wells for extract-added cases were calculated and plotted in Fig. 5.11. It is again clearly seen that the change happened in the initial 6 hours. The dynamics of the bands is thought to reflect the dynamics of their corresponding bacterial species. It is very interesting the bands 316 bp and 206 bp significantly increased within the initial 6 hours and then became almost stable. On the other hand, the band 158 bp significantly decreased within the initial 6 hours and then almost stable. The results suggested that bacteria corresponding to bands 316 bp and 206 bp had gained the competitive advantage to grow in the first 6 hours with the addition of extract, but the bacteria corresponding to band 158 bp was suppressed due to the addition of extracts in the first 6 hours.

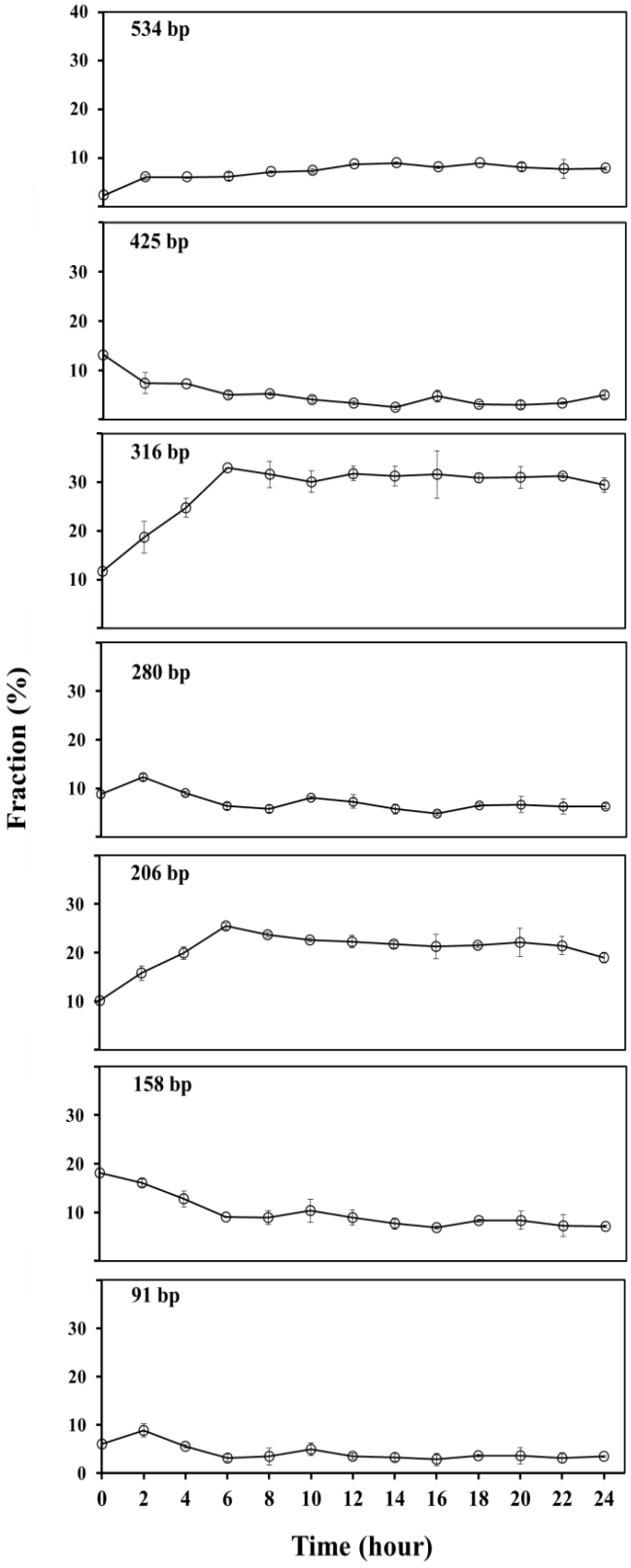

Fig. 5.11 Changes of main RFLP bands with time in Exp. 16 (1).

5.3.4 Experiment 16 (2)

-----Effects of extract dose levels

5.3.4.1 Growth of activated sludge

The optical densities before and after incubation were as shown in Fig. 5.12. Incubation was done in duplicate. The optical density was around 0.08 initially, and it increased to around 0.12~0.14 in the wells after 24 hours of incubation. The addition of extracts at different dose levels did not show significant change compared with the samples without extracts addition. The activated sludge incubated with 1 × extract but without feeding did not show increase in optical density, as shown in the figure, meaning no growth.

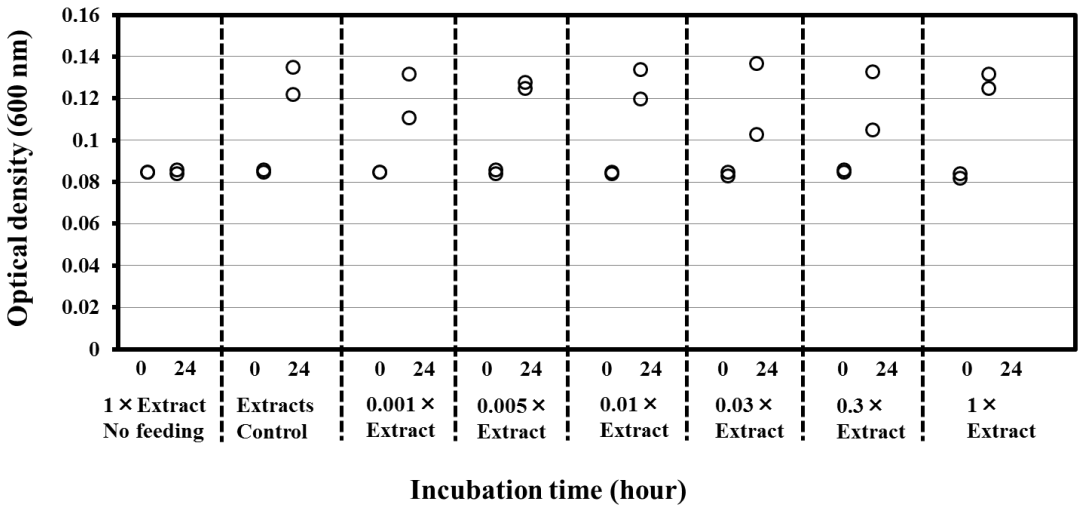

Fig. 5.12 Optical densities of activated sludge at different dose levels in Exp. 16 (2)

5.3.4.2 Bacterial population changes

Figure 5.13 shows the results of RT-PCR/RFLP analysis of the bacterial population. The band patterns of the samples changed with the increase in the level of extract addition, especially more than 0.3 × addition.

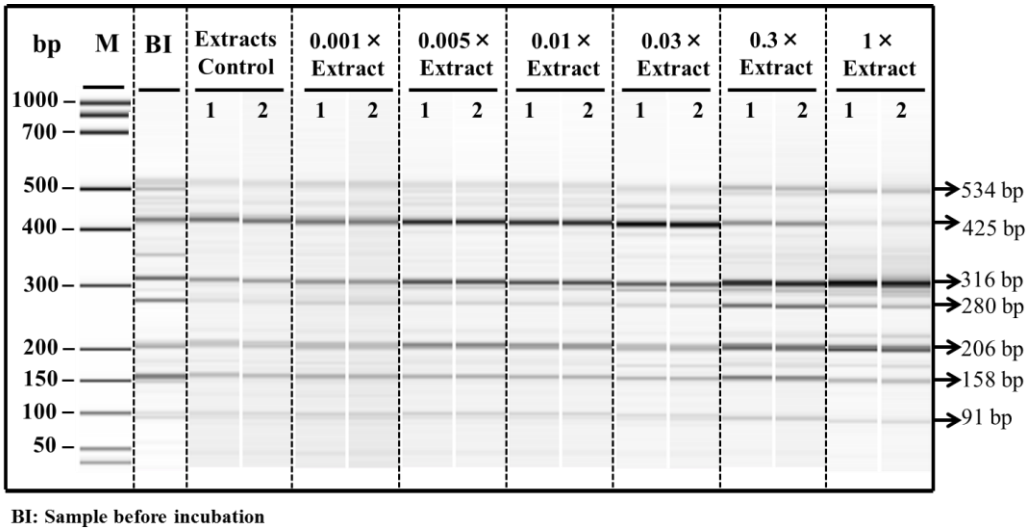

Fig. 5.13 RT-PCR/RFLP analysis of activated sludge sample in Exp. 16 (2).

The fractions of several distinct bands were calculated and plotted against the levels of extract addition, as shown in Fig. 5.14. The horizontal axis of Fig.5.14 (a) is linear, while that of Fig.5.14 (b) is logarithmic. As Fig. 5.14 (b) shows, the intensities of bands changed greatly when the dose level was as high as 0.3 ×, especially the band size of 425 bp, 316 bp and 206 bp. But the bands of 158 bp and 280 bp were different: their intensities increased with the increase of the dose level up to 0.3 ×, then decreased at the dose level of 1 ×. On the other hand, the intensities of bands of 543 bp and 91 bp did not show clear change with the increase of dose level. From Fig. 5.14, bands 425 bp and 316 bp were significantly affected by the higher dose levels, and, they showed opposite changing patterns: while band 316 bp increased, band 425 bp decreased under higher dose levels.

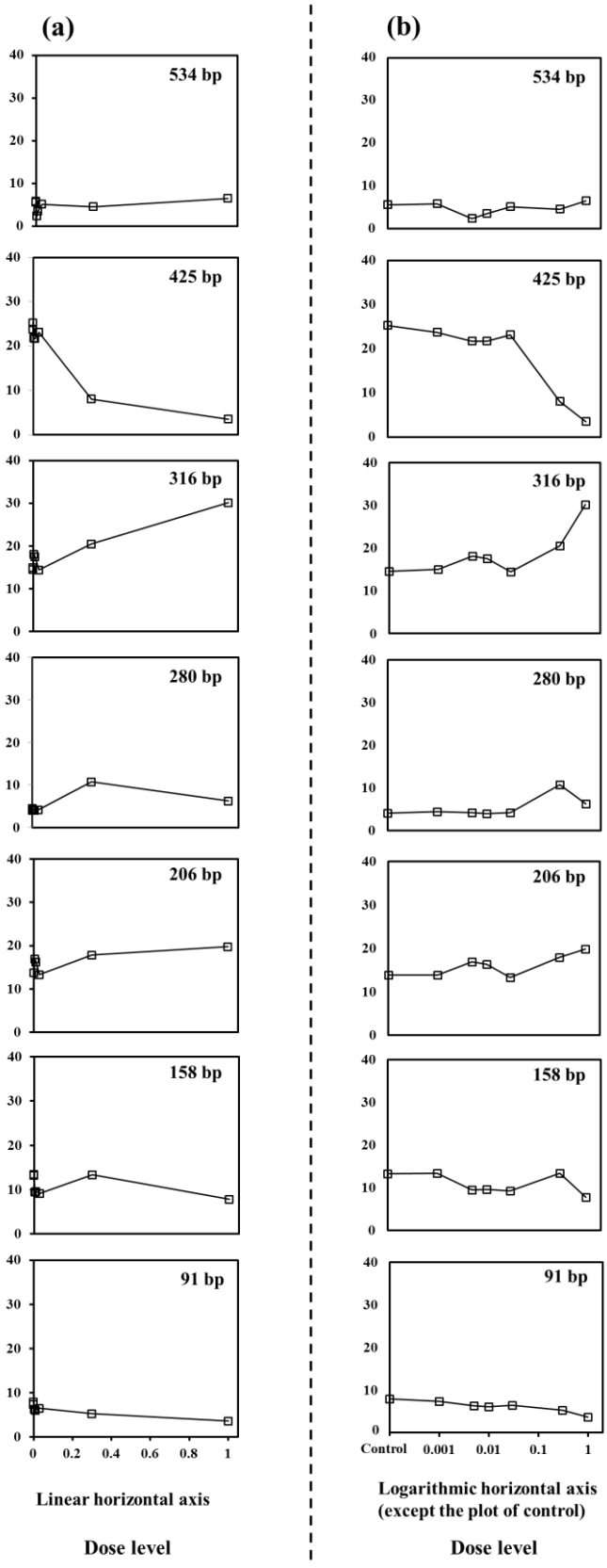

Fig. 5.14 Intensities of RFLP bands under different dose levels in Exp. 16 (2)

To more clearly compare the RFLP profiles, they were further processed by PCA. As shows in Fig. 5.15, the RFLP profiles of the analyzed samples were grouped into three: the upper one is the original activated sludge sample before incubation, the lower left the samples with 0.3 and 1 × extract addition, while the lower right one the samples with lower than 0.3 × extract addition and extract control. The plots of the samples lower than 0.3 × extracts and sample without addition are close to each other, which suggest the bacterial population were similar. However, the plots of the samples with 0.3 × extract and 1 × extract addition are close and far from the other plots. It suggests the big difference of their bacterial population from others. The result of PCA has confirmed the results observable in the gel electropherograms.

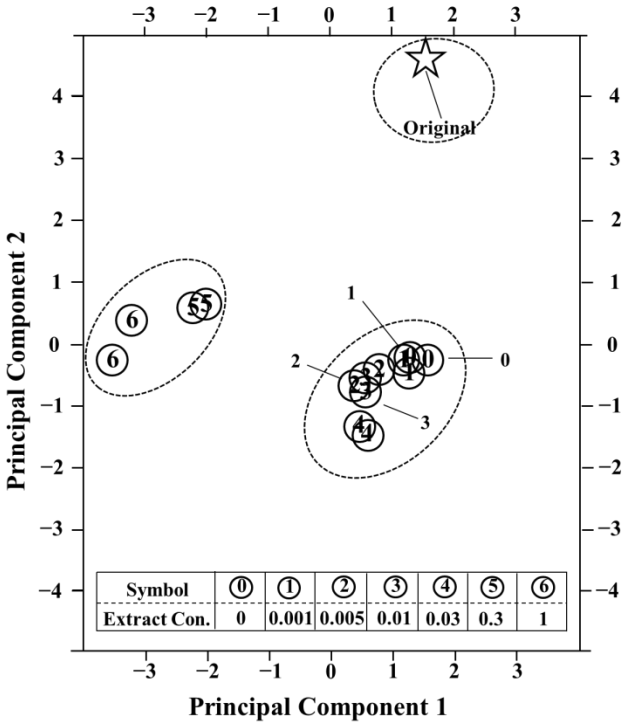

Fig. 5.15 Comparison of RT-PCR/RFLP band patterns of activated sludge samples dosed at different levels by PCA in Exp. 16 (2). The contributions of the first and second principal components were 45.2% and 28.0% respectively.

5.3.5 Experiment 17

-----Effects of extracts from different sources

5.3.5.1 Growth of activated sludge

The optical densities of activated sludge were as shown in Fig. 5.16. Incubation was done in duplicate. The optical densities were around 0.1 initially, and it increased to around 0.16~0.18 in the wells after 24 hours of incubation. The addition of different extracts did not show significant change compared with the samples without extracts addition. However, optical densities in substrate control wells incubated with only extract but without feed did not increase, as shown in Fig. 5.16 (b).

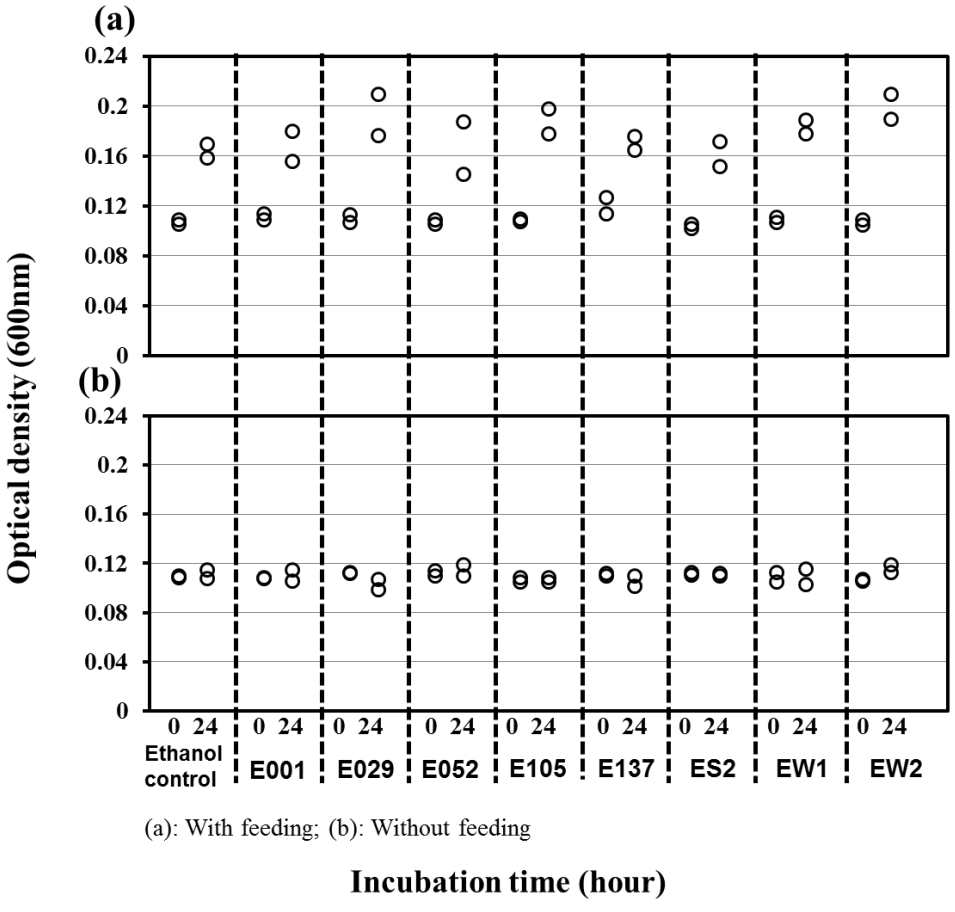

Fig. 5.16 Optical densities of activated sludge in Exp. 17.

5.3.5.2 Bacterial population changes

The results of the RT-PCR/RFLP analysis were as shown in Fig. 5.17. In this experiment, the RFLP profile of the original sludge was omitted. In general, effects of the extracts were subtle, as the RFLP profiles from wells with extract addition were similar to that of control. Yet, when RFLP profiles from the extract-added wells are compared, differences can be found. In E001, the band near 500 bp is visible: this band is found also in control, but not in wells added with other extracts. Bands sized around 240 bp, 270 bp and 280 bp were more intensified when extracts from WWTP1 or WWTP2 were added than when extracts from laboratory SBRs (SBR1 and SBR2) were added. The bands sized 330 bp, 370 bp and 400 bp were significantly intensified by the addition of E137. These bands were also slightly intensified by the addition of E106 and ES2.

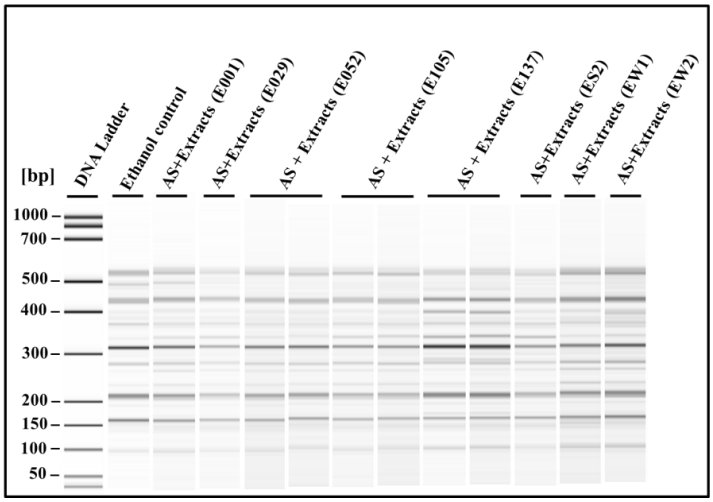

Fig. 5.17 RT-PCR/RFLP results of activated sludge dosed with 8 different extracts.

To more clearly compare the band patterns, the results of the PCR/RFLP analyses were further processed by PCA. As Fig. 5.18 shows, the band patterns were divided into three groups as indicated by the dotted circles. The center group included the samples added with extracts from the lab SBR except E137, the lower left group includes samples added with extracts from WWTP activated sludge, and the lower right group includes samples added with E137. The samples added with extracts EW1 and EW2 were in the left lower group suggesting that the effect of WWTP activated sludge extract on the bacterial population changes were different from the lab SBR activated sludge extracts. In addition, even the extracts from the same SBR, extracts obtained from activated sludge at different timings had differences in their effects.

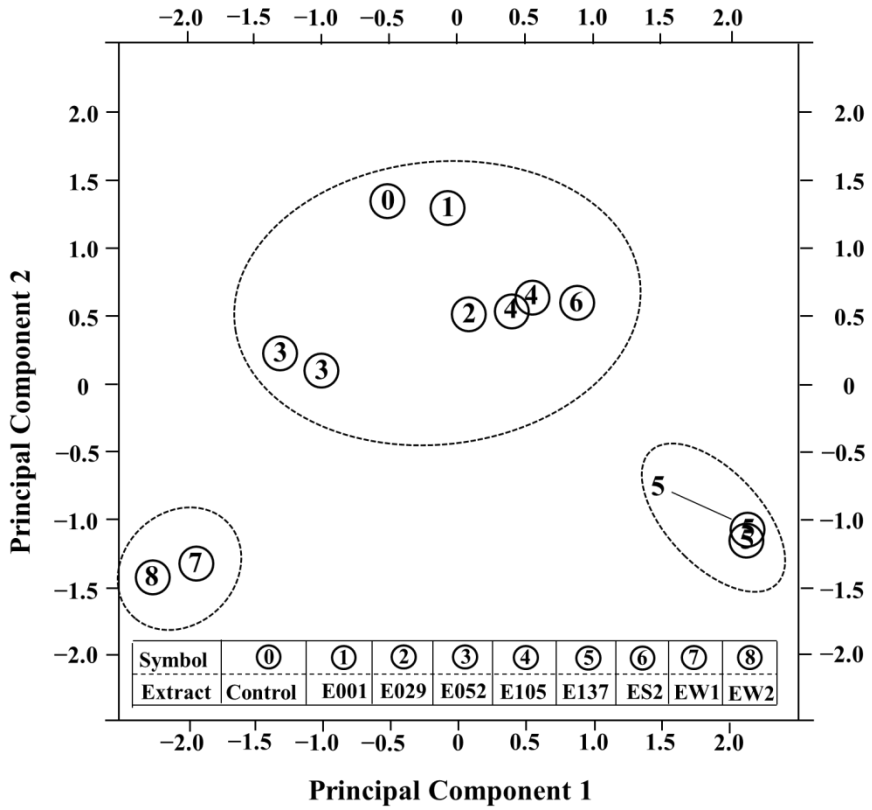

Fig. 5.18 Comparison of RT-PCR/RFLP band patterns of activated sludge samples dosed with different extracts by PCA in Exp.17. The contributions of the first and second principal components were 41.1% and 19.9% respectively.

5.4 Discussion

5.4.1 Effect of organic loading and incubation time

In Exp. 13, the effects of two factors were studied: F/M ratio and time of incubation. The effects of these factors were also studied in Chap. 4 but the methodology was not well organized. Exp. 13 was conducted to confirm the effects of these two factors

The F/M ratio is an important operational parameter affecting organic removal efficiency, sludge properties and microbial composition in activated sludge (Banerji, *et al.*, 1968; Arora, *et al.*, 1985; Liu, *et al.*, 2012).

In this experiment, the sludge incubated was from SBR1, operated with an F/M of around 0.5 kgCOD/(kgMLSS·d). It is expected that applying higher or lower F/M can cause change in microbial population even in control wells without the addition of extract. Indeed, in Exp. 13, significant population change was observed in wells fed with 2 kgCOD/(kgMLSS·d) without extract, but bacterial population in wells fed with 0.5 kgCOD/(kgMLSS·d) remained similar to the original (Fig. 5.2 and Fig. 5.3). And even after 4 days, original population was still maintained, based on RFLP profiles for the extract control.

On the other hand, incubation time is also an important factor that is related to change of population. Even when population change small within short time, in general, population can change after a long time. Indeed, if F/M is 0.5 kgCOD/(kgMLSS·d), and if yield of biomass is about 0.5 kgMLSS/kgCOD (which is around 0.4 to 0.45 kgMLVSS/kgCOD or around 0.6 kgCOD/kgCOD), after one day of incubation, 1kg MLSS of biomass will be 1.25 kgMLSS. The difference of 0.25 kgMLSS is, in general, thought to be new biomass which is directly associated with population change, and microbial population in the remaining 1 kg of biomass is not thought to have changed so much. Thus, population change is expected to be as small as only 25%. But after 4 days, new biomass will occupy almost 50 % of original biomass, which will make easier to see population change.

Yet, even when low organic loading of 0.5 kgCOD/(kgMLSS·d) was applied, the addition of extract caused significant change in bacterial population only within 24 hours of incubation. Especially, many of the bands recognized in the original sample were disappeared after 24 hours of incubation with extract. This means that effect of extract added not only promoted selected group of bacteria: it rather killed than suppressed the growth of many other species.

Further, in Exp. 16 (1), population change was observed only within 2 hrs, as shown in Fig. 5.10 when increase of biomass was still very small, as shown in Fig. 5.9.

These results clearly show that components in the extracts affected bacterial population within as short as a couple of hours, causing drastic change in bacterial population by promoting or killing selected bacterial species.

In general, population change in activated sludge is thought to proceed based on the replacement of microorganisms by their growth and withdrawal of biomass, but in this study, addition of extract caused changes in microbial population much faster. For example, when SRT is 4 days, every day, 25% of biomass is removed, and replenished with newly born biomass. After 1 day, 75% of biomass still remains from the start of incubation, and 25% is newly born within the 24 hr of incubation. After 4 days, $0.75^4 \times 100 = 31.6\%$ of biomass is from the original biomass, and the remaining 68.4% is newly grown biomass. But the population change found here is much quicker than this.

It is of interest to compare the scale of the response time. Typically, wastewater engineers consider that bacterial population in activated sludge changes in the order of SRT, which is usually a couple of days or longer. But in this study, the addition of extracts caused bacterial population change in the order of hours. This suggests a possibility that the bioactive chemicals in activated sludge may advance the bacterial population changes more quickly than SRT, nominal doubling time.

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On the other hand, AHLs, the signal chemical, induces the bioluminescence of *Vibrio fischeri* within a few doubling times (Sitnikov, *et al.*, 1995). Basu *et al*. (2004) also reported the induction of green fluorescent protein in genetically modified *E.coli* cell within 20 minutes, a doubling time of *E. coli*. As in this study doubling time cannot be known, it is difficult to compare the present results with these reported response times. It would be necessary to evaluate response time in terms of absolute values and in relative to doubling time.

The rapidness of the bacterial population changes caused by the extracts implies the importance of bacterial interactions in activated sludge mediated by chemicals which might cause change in bacterial population and further in wastewater treatment performance.

5.4.2 Dose-response relationships

In Exp. 14 and Exp. 16 (2), the change of bacterial population was studied with different dose levels of extract E137. In Exp. 14, while bacterial population in SBR1 sludge was affected when dose level was higher than 0.6 ×, that in WWTP1 sludge was more sensitive and population change was apparent even with extract dose as low as 0.3 ×.

Another experiment Exp. 16 (2) was conducted to more carefully study the effects of the addition of chemicals with wider different dose levels using WWTP1 sludge. While in Exp. 14 WWTP1 sludge showed population change with dose level of 0.3 ×, in this experiment, 0.3 × addition was right on threshold. Further, some of the bands showed a non-sigmoid type response: they became the most intense with 0.3 × additions, and more or less resulted in their reduction.

The dose-response patterns of many of allelopathic chemicals have been reported to follow sigmoid curve patterns with a response threshold (Putnam & Tang, 1986). The widely studied signal compounds, AHLs, have also been studied to make effects only when its concentration is higher than a threshold level (Bassler & Losick, 2006). The findings on the dose-response patterns of bacterial population changes caused by the addition of activated sludge extract were also found to be with a threshold basically.

5.4.3 Differences of sensitivity on the side of recipient

In Exp. 16 (2), the author compared the responses of SBR1 activated sludge and WWTP1 activated sludge against extract E137 which was originally obtained from SBR1 sludge. It was found that WWTP1 sludge was more sensitive to E137 than SBR1 sludge.

One of the possible causes may be coming from the growth condition: while SBR1 sludge was incubated under a condition similar to its original condition (the same substrate was used with similar F/M ratio), WWTP1 was exposed to totally new substrate. The response threshold concentration of allelopathic chemical on the recipient has been widely studied in the plant sciences. Allelopathy effect has been reported to be enhanced by the environmental stresses, such as the changes of nutrient, temperature, moisture, pH and so on (Einhellig, 1987; Einhellig, 1996). The stress might have caused higher sensitivity of bacterial population in WWTP1 sludge, because microorganisms were put under totally different condition from their original habitat. Another possible cause can go as follows: bacteria in SBR1 sludge are more or less familiar with chemicals in E137, as E137 was obtained from the same sludge though timing was different. On the other hand, bacteria in WWTP1 sludge might have never experienced the chemical components in extract E137, and reacted with bigger response.

5.4.4 Differences of extracts from different sources

In Exp. 17, the effects of 8 different extracts on bacterial population were studied. The incubated sludge from SBR2 seemed to have had lower sensitivity to the extracts used, even to E137, and the differences in RFLP patterns between extract control and extract added wells were small. Yet, the band patterns for the samples with addition of extracts from WWTPs and laboratory SBRs were significantly different. In addition, extracts from the same lab SBR1 obtained at different timings were found to have differences in their effect. Especially, the effect of extract E137 on the bacterial population changes was the most significant.

Ogawa (2009) reported that the extracts from sludge performing EBPR and extract obtained after EBPR deterioration had different effect on activated sludge bacterial population (Satoh, *et al.*, 2009). Ichihashi *et al*. (2006a, 2006b) reported the fluctuation of chemical-mediated interactions in time. The present study provided more cases showing the differences of the effects of extracts from different activated sludge. The fluctuation of these interaction chemicals needs to be studied more.

5.4.5 Chemicals extracted by ethanol

Ethanol has a polar hydroxyl group and hydrophobic methyl group, and can be used to extract relatively hydrophilic lipids. Ethanol has been used to extract saccharide or carbohydrates (Jennings & Thornton, 1984; Lyaskovskii, 1991a, 1991b), lipid (Carroll, 1961; Burton, *et al.*, 1985; Temelli, 1992), protein (Bilgic & Imre, 1989; Kubiczek, *et al.*, 1990; Ren, *et al.*, 1990; Kubiczek, *et al.*, 1991) and some small molecule including peptide (Bartley, *et al.*, 1972; Poveda, *et al.*, 2006; Wang, *et al.*, 2014), aliphatic acid, ketone, ester, ,indole, hydrocarbon and phenol (Ma, *et al.*, 2010).

Allelochemials or signal chemicals in activated sludge are expected to have the solubility in water and adhesively to flocs with hydrophobicity. It is supposed that a chemical molecule that has bioactivity is excreted from a bacterial cell in activated sludge. If it is fully soluble in water, it will easily diffuse to bulk water, and will be washed away with treated water. On the other hand, if the molecule sticks around the cell from which the molecule was excreted, it will not affect other cells. Thus, for the chemical molecule to effectively affect other cells, it should be mobile in activated sludge flocs, and better not have too much solubility in water. Such chemicals are

expected to have hydrophobic group to stay within flocs and hydrophilic group to adjust mobility in floc. Ethanol is thought to be suitable to extract such chemicals.

Ethanol is thought to extract not only allelochemicals or signal chemicals but also chemicals which are not excreted from microbial cells, such as vitamins. Such chemicals are thought to enhance growth of bacteria, as they are used as nutrient. But in this study, not only enhancement but also elimination of bacterial species (or more exactly, increase or decrease of RFLP bands) were observed.

5.4.6 Bacterial population changes analyzed by RT-PCR/RFLP

In the present study, bacterial population was mostly analyzed by RT-PCR/RFLP, not by PCR/RFLP. Both of these two methods can be used to analyze bacterial population changes, but they give different results. In general, PCR can amplify DNA sequences not only from live cells but also from dead cells (Selenska-Pobell, 1995). On the other hand, RT-PCR targeting 16S ribosome RNA is thought to reflect the active fraction of bacterial population in environmental samples (Selenska-Pobell, 1995; Duarte, *et al.*, 1998; Blazewicz, *et al.*, 2013).

In the present study, to grasp the change of microbial population while emphasizing their activities, the author employed the RT-PCR/RFLP method, which reflected the real active bacterial population changes of activated sludge. However, there is a possibility that cells which have lost activity but are still alive could not be reflected in this study.

5.5 Conclusions

In this chapter, bacterial population changes by the addition of extract were studied by focusing on the effects of F/M ratio, incubation time, dose-response relationships, differences of activated sludge to the same extract, and differences of extracts from different activated sludge. Following conclusions were obtained.

1. Activated sludge population changes caused by the addition of extracts were detected even with around 20%~30% increase of biomass. (Exp. 13)

2. The crude activated sludge extracts not only promoted growth of selected species but also kill or eliminated other bacterial species. (Exp. 13 and Exp. 16-1)

3. The effect of crude extracts mostly followed a sigmoid curve with a threshold. But it also was found a response taking the maximum at a certain dose level, and a higher and lower dose resulted in lower response. (Exp. 14 and Exp. 16-2)

4. The sensitivities of the different activated sludges on the same crude extracts were shown to be different. (Exp. 14)

5. Crude extract from different sources of activated sludge showed differences in their effects on the same activated sludge bacterial community. (Exp. 17)

Chapter 6 Identification of Bacterial Species Affected by Crude Extracts Addition

6.1 Introduction

Here, products of RT-PCR obtained in Exp. 16 (1) and Exp. 16 (2) were analyzed by pyrosequencing with barcoded primers to identify bacterial species that were affected by the addition of extracts. By the way, the samples of Exp. 13 and Exp. 14 were also analyzed by pyrosequencing, these results were placed in Appendix 3 and Appendix 4

6.2 Materials and methods

6.2.1 Samples analyzed by pyrosequencing

The samples analyzed by pyrosequencing were the RT-PCR products of all the samples in Exp. 16 (1) and Exp. 16 (2) described in 5.3.3 and 5.3.4. The information of incubated activated sludge sample and the extracts obtained are showed in Table 6.1.

6.2.2 Pyrosequecing analysis method

The procedure of the sample preparation for pyrosequencing and data analysis is described in Chapter 3.6.5. Primers used were as listed in Appendix Table A1. The reads obtained were filtered and poor quality reads were removed, assigned to their original samples based on the sequence of the barcode region, and analyzed by the data processing method described in 3.6.5. In total 79,164 reads were obtained, and were classified into 6282 OTUs. These OTUs were generated for the mixed data set for Exp. 16 (1) and Exp. 16 (2), so the same OTU number in the following sections mean the same operational taxonomic group.

Table 6.1 Activated sludge taken for incubation and chemical extraction and related incubation time and F/M ratio in Exp. 16

Experiment No.	Incubated AS	Extracted AS	Incubation time (hour)	F/M ratio (kgCOD/kgMLSS·d)
16 (1)	Feb. 21st WWTP	Day 137th of SBR 1 (E137)	2hr, 4hr, 6hr, 8hr, 10hr, 12hr, 14hr, 16hr, 18hr, 20hr, 22hr, 24hr	0.5
16 (2)	Feb. 21st WWTP	Day 137th of SBR 1 (E137) (Concentration of 0.001, 0.005, 0.01, 0.03, 0.3, 1 ×)	24 hr	0.5

6.3 Results

6.3.1 Bacteria population changes with time in Exp. 16 (1)

6.3.1.1 The effects of extract on the bacterial population changes at different hierarchical levels

In total, 26,139 reads with 6,282 OTUs were obtained. Table 6.2 shows read numbers from each sample sequenced by pyrosequencing.

Table 6.2 Number of reads detected from sample of Exp. 16 (1)

	Original	**2hr**	**4hr**	**10hr**	**16hr**	**20hr**	**24hr**
Control	7618	1655	1654	1190	1401	1345	1957
Extract added	7618	1713	1585	1517	1461	1344	1699

At Phylum level showed in Fig. 6.1, Proteobacteria (red color) and Bacteoriodetes (green color) occupied most of the reads.

As a general impression, Proteobacteria increased by the addition of the extract.

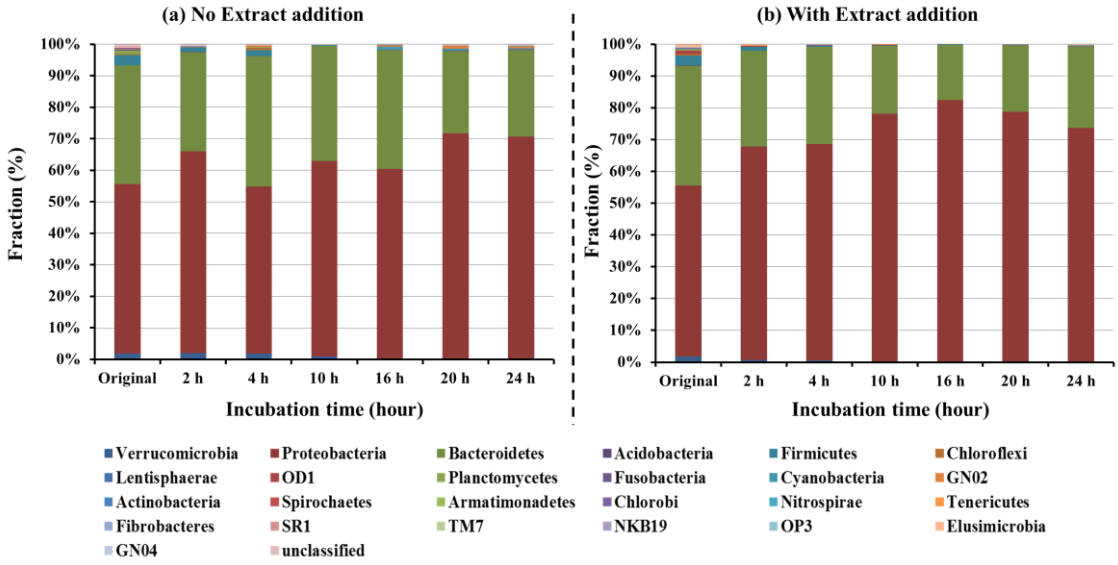

Fig. 6.1 Phylum level bacterial population changes in Exp. 16 (1).

At class level, as shown in Fig. 6.2, compared with the control, three distinct differences were observed as follow:

1. The fraction of Gammaproteobacteria (deeper red, bottom) increased with time and decreased a little towards 24 hours incubation. But its fraction did not change significantly in the control.

2. The fraction of Sphingobacteria (blue) decreased and nearly disappeared after 10 h incubation, while in the control it almost kept stable.

3. The fraction of Alphaproteobacteria (deeper orange) decreased and nearly disappeared (fraction percentage=0.49 %) after 16 h incubation and then increased again toward 24 hours. While in control, it increased largely after 16 hours by 3 times of the original.

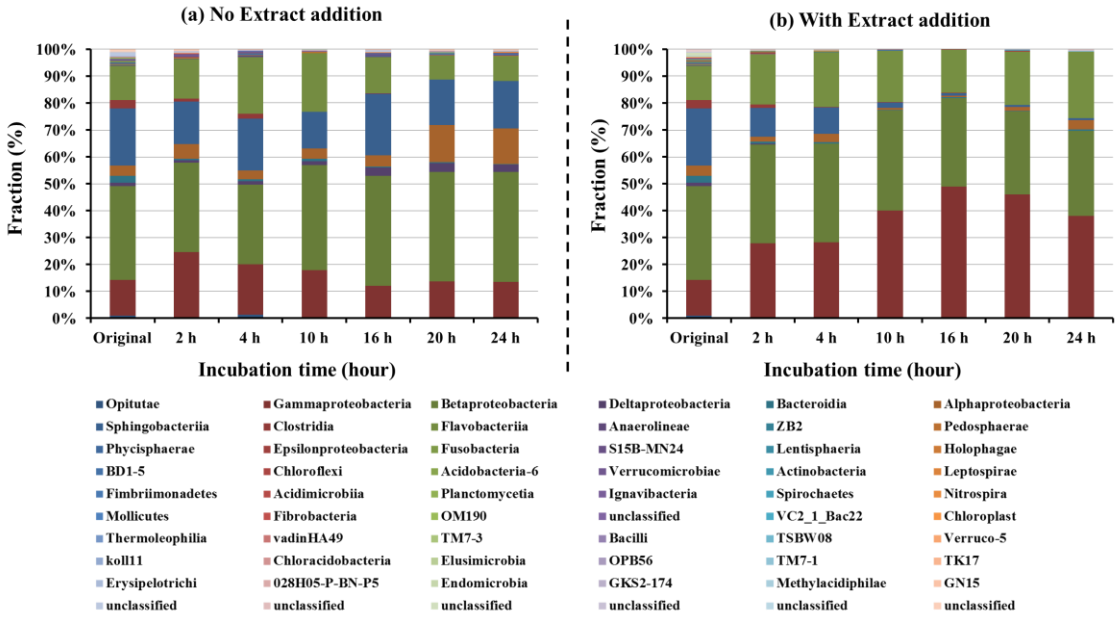

Fig. 6.2 Class level bacterial population changes in Exp. 16 (1).

Bacterial population change at order level was as showed in Fig. 6.3. Compared with the control, there are mainly 4 distinct differences in the sample with the extract addition.

1. The fraction of Pseudomonadales (deep red, bottom) increased, especially its percentage increased about 3.5 times of the original one. While in the control, it did not change so much.

2. The fraction of Sphingobacteriales (upper light green) decreased and almost disappeared after 20h incubation. While it almost kept stable in the control.

3. Flavobacteriales (deeper orange at the lower position) increased with time, while it decreased in the control.

4, The Aeromonadales (light orange at upper position) increased with time and 40 times increase when incubated after 16 h, but it did not increase so much in the control and kept around 1% of fraction percentage.

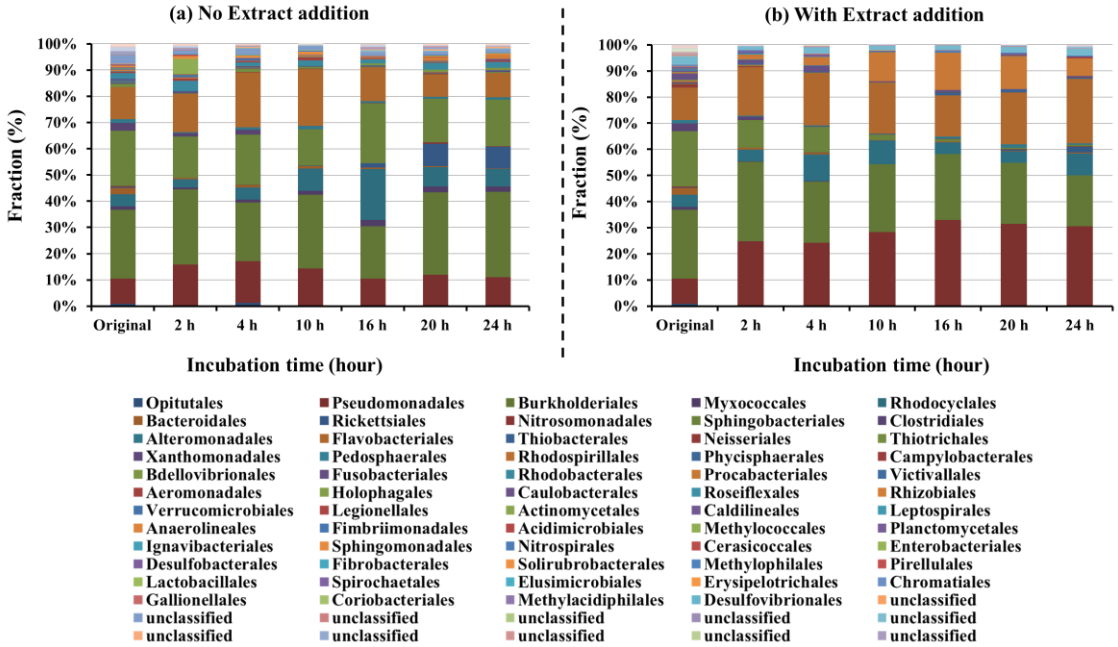

Fig. 6.3 Order level bacterial population changes in Exp. 16 (1).

At family level (Fig. 6.4), 5 distinct changes could be observed:

1. The fraction of Pseudomonadaceae (deep red, bottom) increased with the time, but that it decreased in the control.

2. The fraction of Moraxellaceae (second deep red from bottom) increased from 5% to 15% in the first 2 hours and then kept stable, while it did not changed so much in the control.

3. The fraction of Flavobacteriaceae (third deep red from the bottom) increased regardless of the addition/omission of extract in the first 10 hour, then it continued to increase in the sample with addition of extracts while decreased in the control.

4. Unclassified Sphingobacteriales (peak color) decreased in first 4 hours and then almost disappeared from 16 hour, but it always accounted for a big percentage in the control.

5. The fraction of Aeromonadaceae (deep blue, top) increase from 0.44% in the original sample to 14.2% (fraction percentage) in the first 16 hours, but decrease after 24 hours. However, in control, its fraction kept less than 1% all the time.

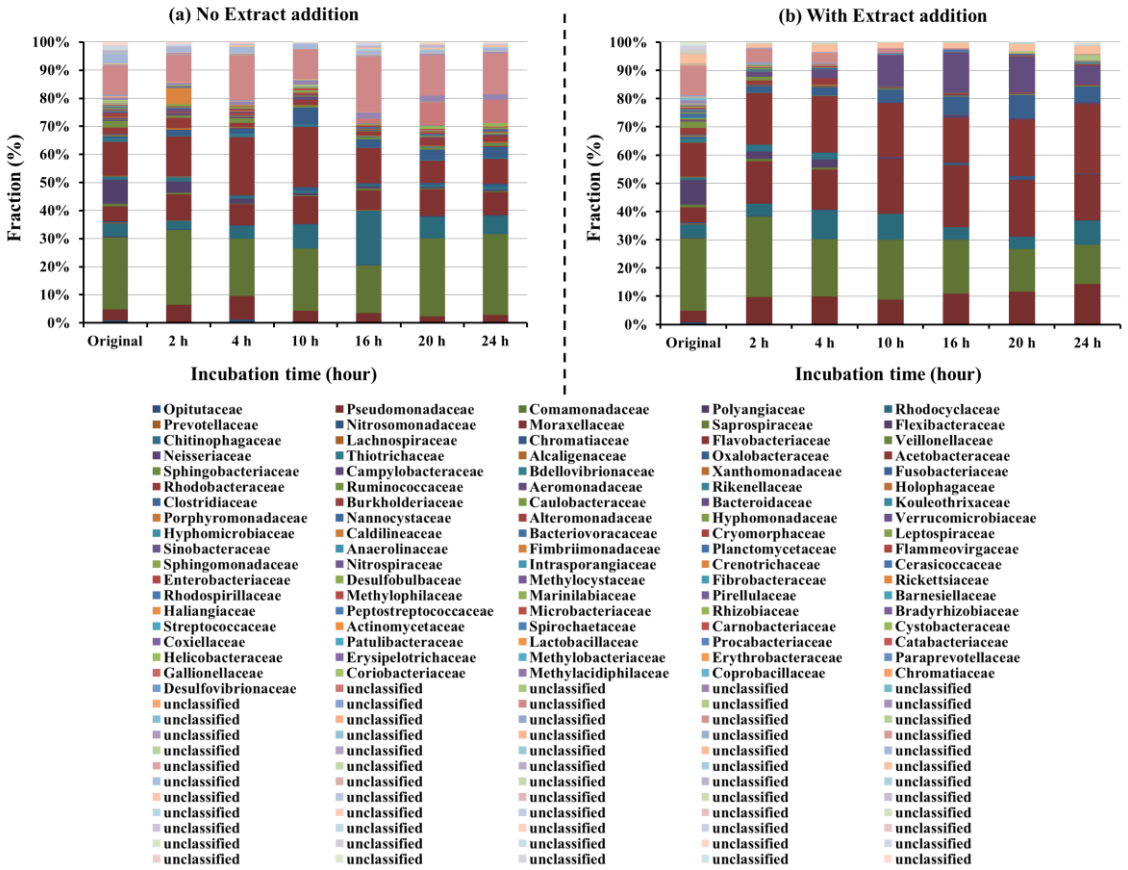

Fig. 6.4 Family level bacterial population changes in Exp. 16 (1).

6.3.1.2 The effect of extract on the bacterial population changes at the OTU level

Figure 6.5 shows OTU level (similarities ≥97%) fluctuations of bacterial population. Here, OTUs which had average fraction value of 0.5% are shown. The sum of the average fraction values of these OTUs is 47.1%, which means that around half of the total reads are grouped into one of these major OTUs. The heatmap is accompanied by a phylogenetic tree on the left to show the phylogenetic relationships of these OTUs. For each OTU, upper row is for control and the lower row is for the case with the addition of extract. And the classified genus level and class level information is shown on the right part.

The OTUs that increased by the addition of extract were : OTU1420, OTU5655 and OTU110. On the other hand, OTU6166, OTU2850, and OTU1332 decreased due to addition of the extracts. The changes of these OTUs suggested they were more sensitive to the chemical components in the extracts.

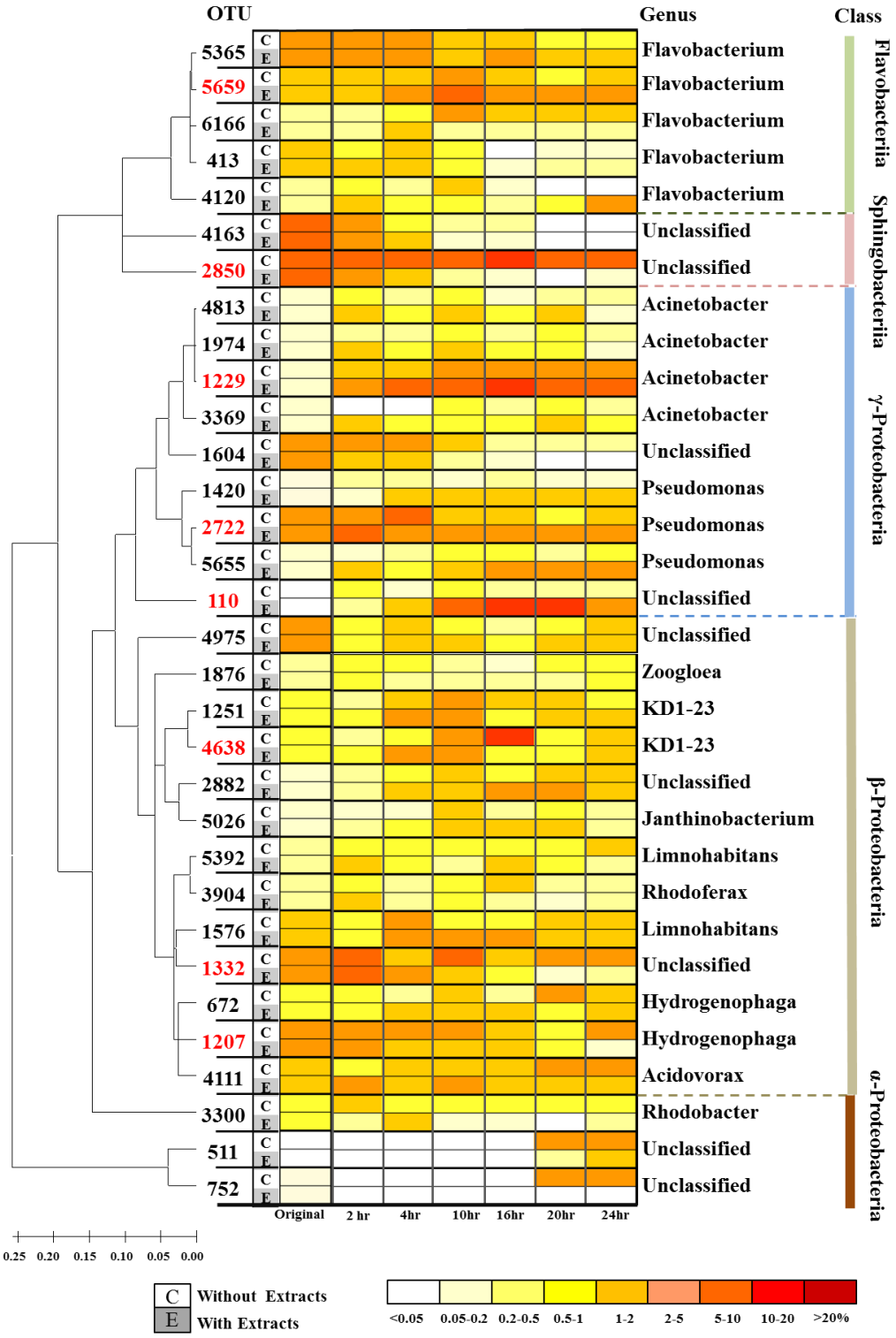

Fig. 6.5 Heatmap showing the fluctuations of OTUs (average fraction higher than 0.5) with and without the addition of extract in Exp. 16 (1). The OTUs with the average fraction higher 2 are shown in red color of the OTU number.

Major OTUs which had average fraction values higher than 2% were selected, and their fluctuations in the control and the extract-added incubation were plotted against incubation time, as shown in Fig. 6.6. The closed circles are for the samples with extract addition while the open ones are the control. Out of these 8 major OTUs, OTU2722, OTU1332 and OUT1207, did not show significant differences by the addition or omission of extract. However, significant differences were observed for OTU2850, OTU5659, OTU1229 and OTU110. Especially, OTU2850 decreased significantly within 10 hours in the sample incubated with extract, but in control, it did not show such significant reduction. On the contrary, OTU110 increased largely due to the extract addition, although at 24 hr it decreased. The behavior of OUT 4638 was strange: it increased only in 16hr sample in control.

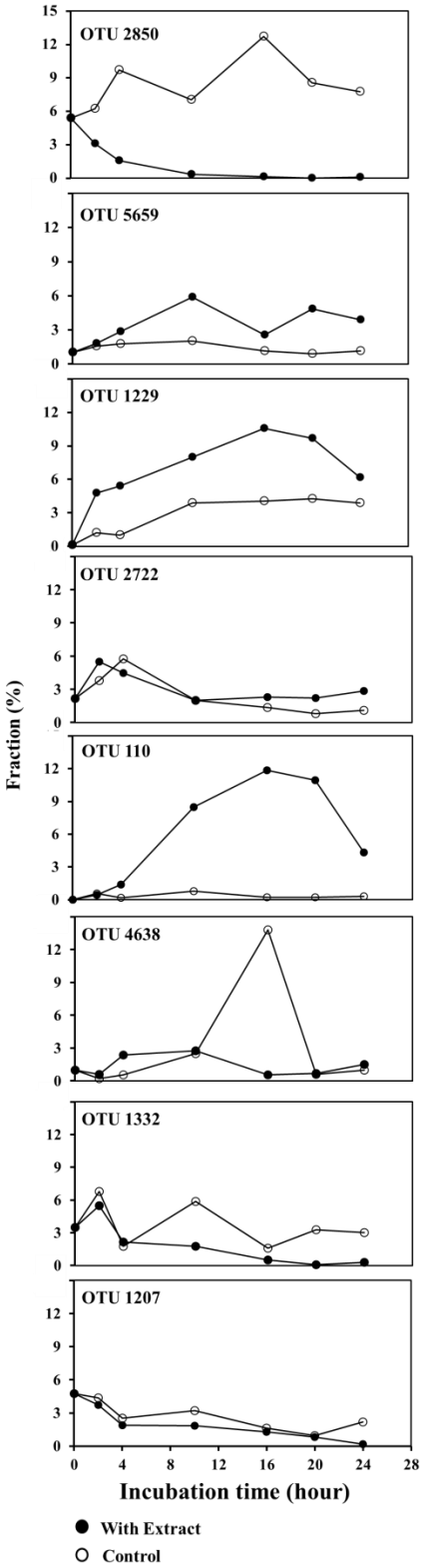

Fig. 6.6 Dynamics of major OTUs (Average fraction higher than 2%) with and without addition of extract in Exp. 16 (1).

6.3.1.3 Comparison of the RFLP results with pyrosequencing results

In section 5.3.3 in Chapter 5, the population change in Exp. 16-1 was analyzed by RT-PCR, while in this thesis it was analyzed by RT-PCR/pyrosequencing. Here, these two results are compared. These two methods should give consistent results. The observed change of major OTUs in pyrosequencing should be correlated with the corresponding changes of band intensities in RFLP.

In the previous section, four major OTUs were suggested to have been affected by extract addition: OTU2850, OTU5659, OTU1229 and OTU110. From the reads of each of these major OTUs, one of the relatively longer reads was selected as the representative sequence, and the expected fragment sizes with the restriction enzyme *Hha*I were calculated. The original DNA sequences of these OTUs were as follows.

OTU 2850: Bacteroidetes; Sphingobacteriia; Sphingobacteriales; unclassified below family level.

5’-AGAGTTTGATCCTGGCTCAGGATGAACGCTAGCGGCAGGCTTAATACATGC
AAGTCGAGGGGCAGCACGAATAGCAATATTTGGTGGCGACCGGCGCACGGG
TGCGTAACACGTATACAACCTACCTATAACAGGGGGATAGCCTTTCGAAAGA
GAGATTAATACCCCGTAATAAATTGAATGGCATCATTTGATTTTGAAAGCTCCG
GCGGTTATAGATGGGTATGCGTCTGATTAGCTAGTTGGTGAGGTAACGGCTCA
CCAAGGCGACGATCAGTAGGGGATCTGAGAGGACTAACCCCCGACACTGGT
ACTGAGACACGGACCAGACTCCTACGGGAGCAGCAGTAAGGAATATGGTCA
ATGGAGGCAACTCTGAACCAGCCATGCCGCGTGCAGGATGACGGCCCTATGG
GTTGTAAACCTGCTTTTGTACCAGAGAAAACCCTTCACGTGTGGGAGTTGAT
AGTATGGTAAGATAAGCATCGGCTAACCTTGTGCCAGCCGCCGCGGTAATTC-
3’

OTU 5659: Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; *Flavobacterium*.

5’-AGAGTTTGATCCTGGCTCAGGATGAACGCTAGCGGCAGGCTTAACACATG
CAAGTCGAGGGGTAGAGGAAGCTTGCTTCCTTGAGACCGGCGCACGGGTGC
GTAACGCGTATACAATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGA
TTAATACCTCATAGTATTTTCGAATCGCATGGTTTGATTATTAAAGTTCCAACG
GTAAAAGATGAGTATGCGTCCCATTAGCTAGATGGTAAGGTAACGGCTTACCA
TGGCAACGATGGGTAGGGGTCCTGAGAGGGAGATCCCCCACACTGGTACTG

AGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAAT
GGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGAAGACGGTCCTATGGAT
TGTAAACTGCTTTTATACAGGAAGAAACACACCCTCGTGAGGGTACTTGACG
GTACTGTATGAATAAGGACCGGCTAACTCCGTGCCAGCCGCCGCGGTAATTC-
3’

OTU 1229: Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; *Acinetobacter*.
5’-AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATG
CAAGTCGAGCGGAGATGAGGTGCTTGCACCTTATCTTAGCGGCGGACGGGCG
AGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATTTCGAAAGGAAT
GCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACCTTGC
GCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTAC
CAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAA
TGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTAGG
TTGTAAAACGTCACTTTTAAAGGCGAGGAGGAGGAGCTCTAGACTAATACTC
TAGATGCTTGGACGTTACTCGCAGAATAACGCACCGGCTAACTCT-3’ (reverse primer region not detected)

OTU 110: Proteobacteria; Gammaproteobacteria; Aeromonadales; Aeromonadaceae; unclassified at genus level.
5’-AGAGTTTGATCCTGGCTCAGGTTGAACGCTGGCGGCAGGCCTAACACATG
CAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGC
GGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGATAACAGTT
GGAAACGACTGCTAATACCGCATACGCCTACGGGGGAAAGCAGGGGACCTT
CGGGCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGAGGT
AATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCA
CACTGGAACTGAGACACGGTCCAGACTCCTACGGGGAGGGCAGCAGTGGGG
AATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGA
AGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTTGGTACGCTAA
TACGTAGCCAGCTGTGACGTTACTCGCAGACGAAGCACGGC-3’ (reverse primer region not detected)

For OUT1229 and OTU110, reverse primer region was not detected in the reads. The reverse primer regions of many of the reads (47%) were not found because sequencing

was done one way from the forward primer end.

The author calculated the sizes of the fragments based on the determined DNA sequence and summarized them in Table 6.3. Then, expected fragments calculated in Table 6.3 were compared with the RFLP results reported in 5.3.3. In RFLP gel electrophoresis figure, it is not easy to distinguish the band size less than 10 bp, so in this analysis, the author classify the expected band sizes and bands observed in real RFLP as showed in Table 6.3.

Table 6.3 The calculated band sizes of OTUs 2850, 5659, 1229 and 110 and their corresponding band sizes in RFLP analysis

	92 bp	96 bp	151 bp	207 bp	214 bp	276 bp	423 bp
OTU2850		√					√
OTU5659	√		√			√	
OTU1229				√			
OTU110					√		
Corresponding band sizes	91 bp		158 bp	206 bp		280 bp	425 bp

Figure 6.7 shows the comparison of these results. These OTUs and their corresponded fragments showed similar increase and decrease patterns.

Thus, fragments 425 bp and 91 bp in RFLP are thought to represent unclassified Sphingobacteriales. Fragments 280 bp, 158 bp and 91 bp are thought to represent *Flavobacterium* in Flavobactreria. Fragment 206 bp is thought to represent *Acinetobacter* in Gammaproteobacteria and unclassified Aeromonadaceae in Gammaproteobacteria respectively.

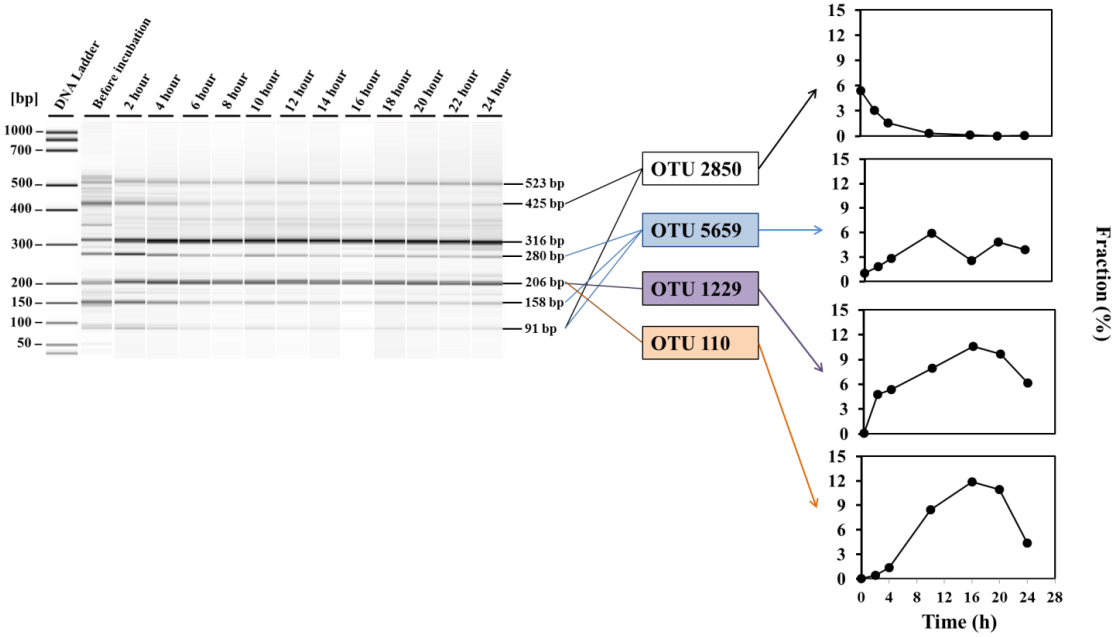

Fig. 6.7 Comparison of OTU2850, OTU5659, OTU1229 and OTU110 with related RFLP band patterns in Exp. 16 (1).

6.3.2 Bacterial population changes with different dose levels in Exp. 16 (2)

6.3.2.1 The effect of extract on the bacterial population changes at different hierarchical levels

For the samples from Exp.16 (2), 16,399 reads with 6,282 OTUs were obtained. Table 6.4 shows the read numbers for the samples. The reads for the original sample and 1 × extract addition are shared with Exp. 16 (1).

Table 6.4 Number of reads detected from sample of Exp. 16 (2)

	Original	0 ×	0.001 ×	0.005 ×	0.01 ×	0.03 ×	0.3 ×	1 ×
Reads	**7618**	**1190**	**1343**	**1334**	**1389**	**1361**	**1107**	**1957**

At phylum level, as Fig. 6.8 shows, Proteobacteria (deep red color) and Bacteoriodetes (blue color) were main bacterial groups. With the increase of concentration of extract addition, Proteobacteria increased and Bacteroidetes decreased accordingly, however the trend was not so clear.

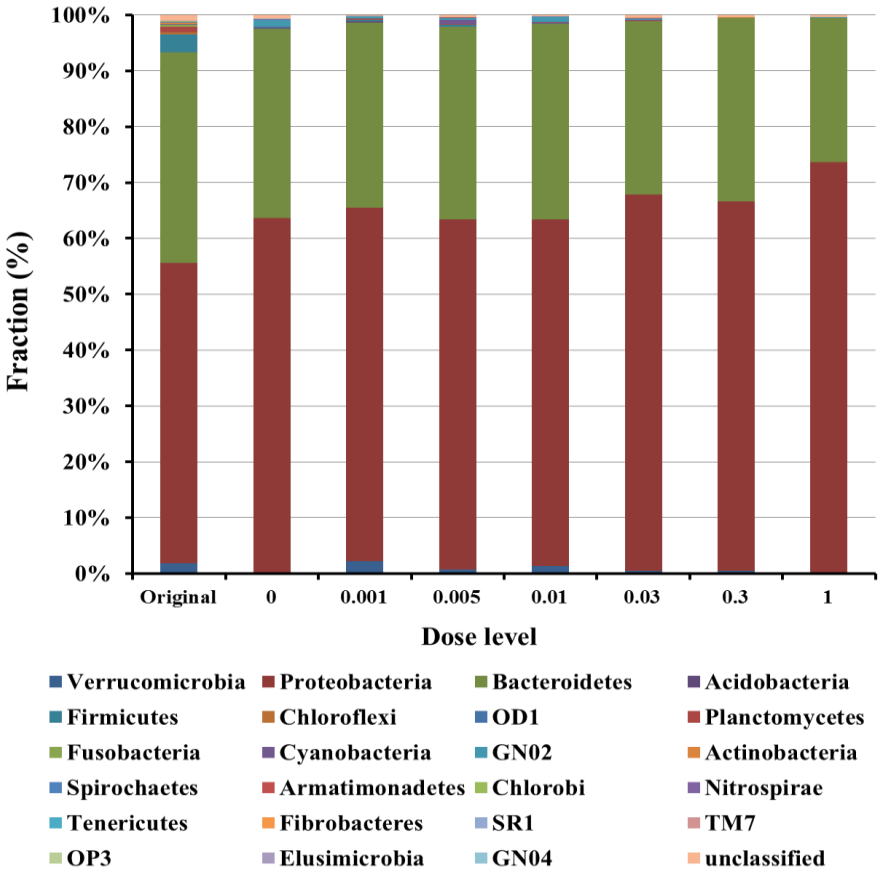

Fig. 6.8 Phylum level bacterial population changes in Exp. 16 (2).

At class level, as shown in Fig. 6.9, with the increase of dose level, Gammaproteobacteria (deep red, bottom) increased while Sphingobacteriia (light blue) decreased, especially when the concentration higher than 0.03 ×, and almost disappeared with 1 × extract addition. Flavobacteria (top green) almost kept stable when dose was lower than 0.3 ×, but increased more than twice either when dose level was 0.3 × or 1 ×. Deltaproteobacteria (deep blue) and Alphaproteobacteria (yellow), with the similar changing trend, first increased and then decrease to around the original.

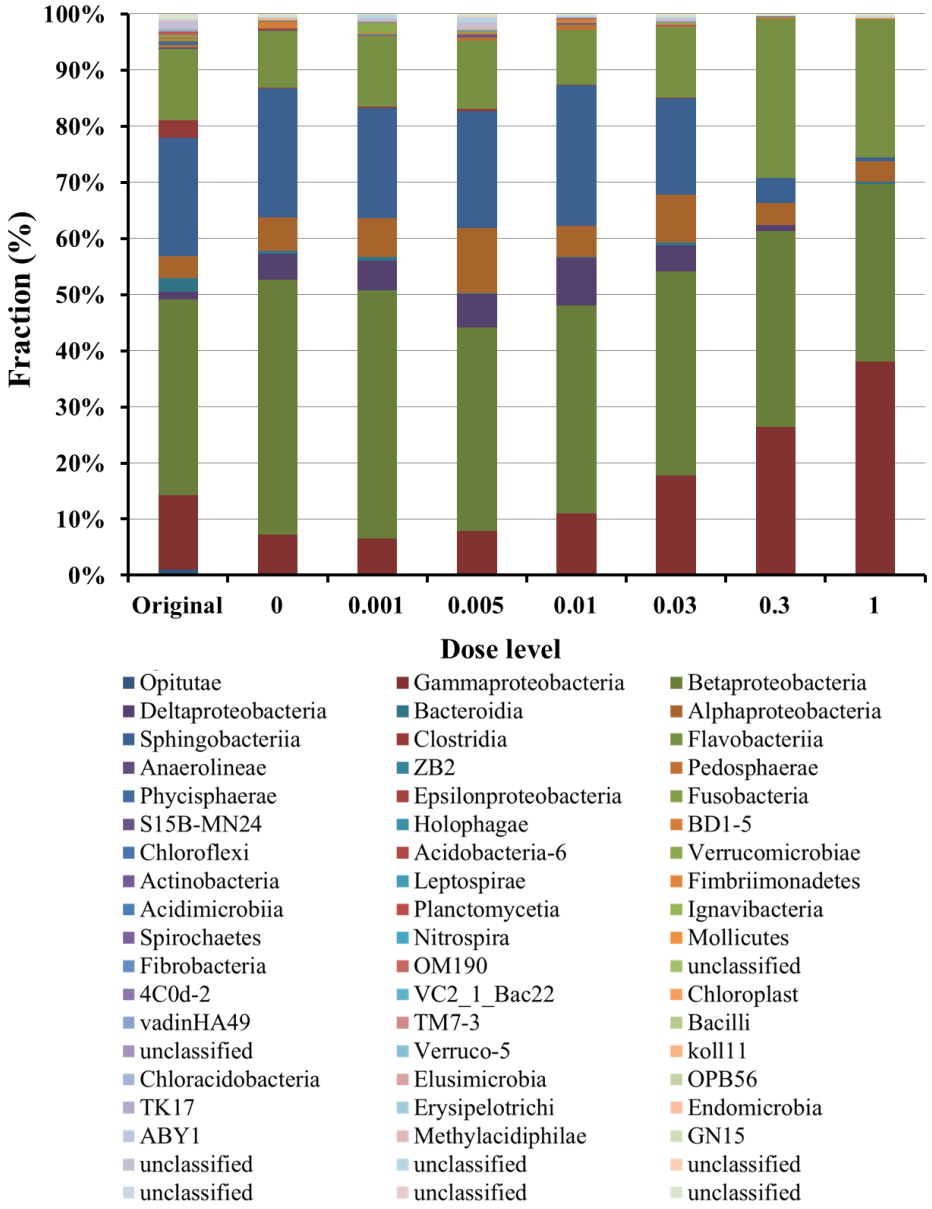

Fig. 6.9 Class level bacterial population changes in Exp. 16 (2).

At order level, as shown in Fig. 6.10, with the increase of dose level, Pseudomonadales (red color, bottom) and Flavobacteriales (deep yellow, top) increased, while the Sphingobacteriales (green, top) decreased especially when more than 0.3 × of extract was added.

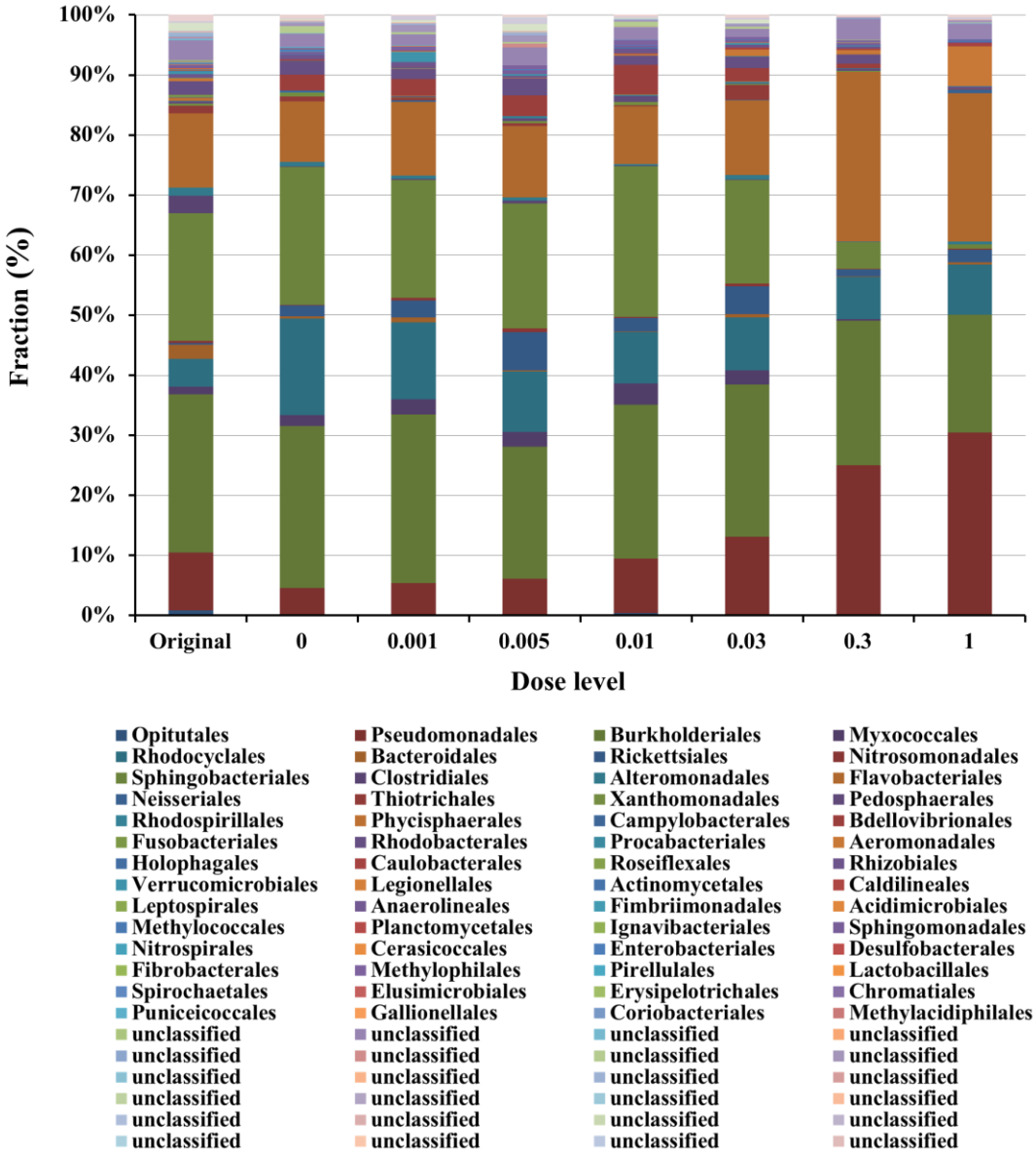

Fig. 6.10 Order level bacterial population changes in Exp. 16 (2).

At family level, as shown in Fig. 6.11, extract addition of more than 0.3 × caused more significant change. Especially, Pseudomonadaceae (first red color from bottom), Moraxellaceae (second red color from the bottom), and Flavobacteriaceae (third red color from the bottom) increased significantly, while unclassified bacteria (pink color) belonging to order Sphingobacteriales, which occupied a big percentage when less than 0.3 × extract addition, almost disappeared when extract addition was more than 0.3 ×.

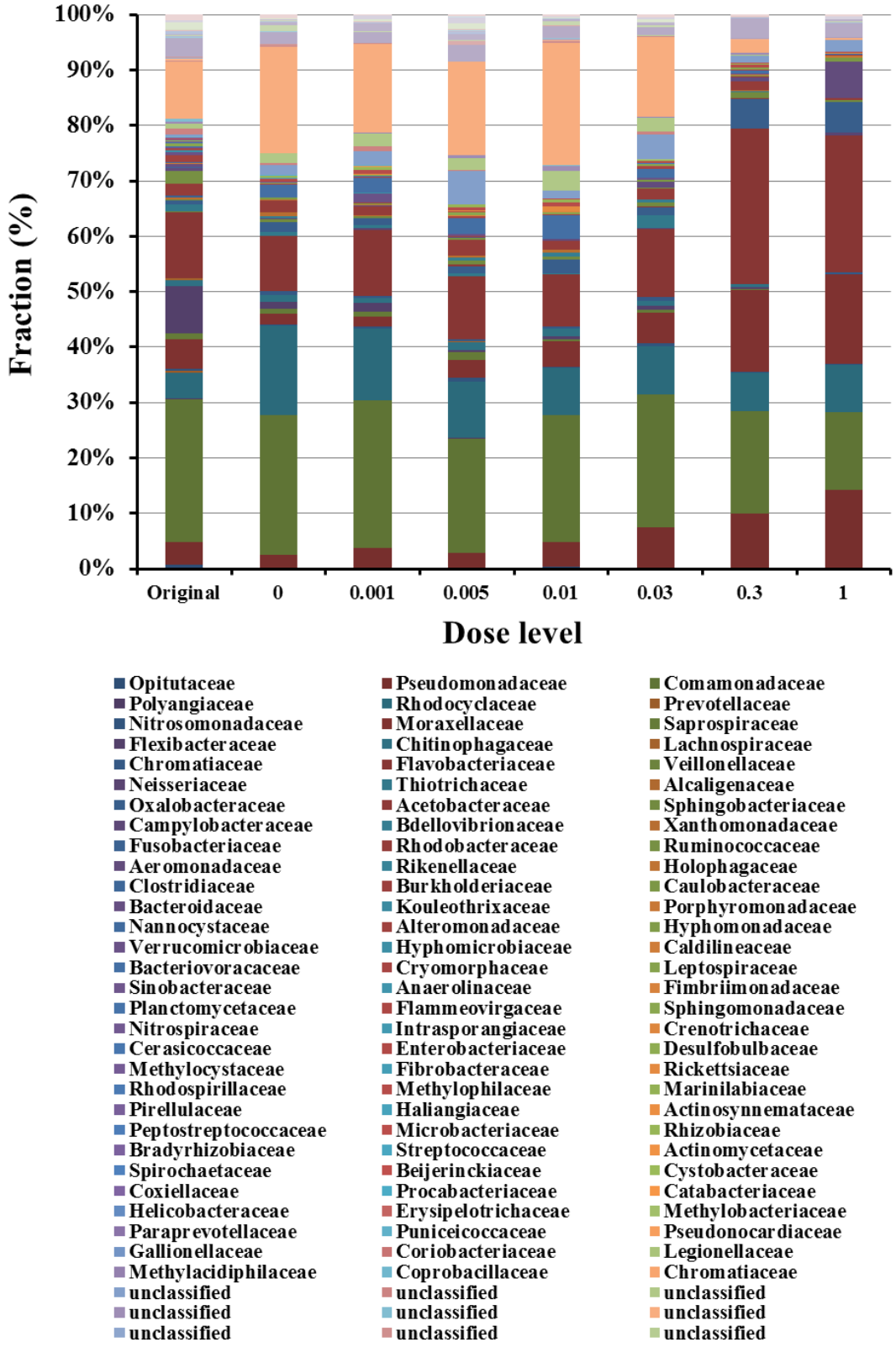

Fig. 6.11 Family level bacterial population changes in Exp. 16 (2).

6.3.2.2 The effect of extract on the bacterial population changes at the OTU level

Figure 6.12 shows the fluctuations of OTUs in the form of heatmap coupled with phylogenetic and taxonomic assignment information.

As showed in Fig. 6.12, the propagation and recession of the OTUs with the different concentrations of extracts were clearly observed. For example, OTU1686, OTU5659, OTU4120, OTU2882, OTU110, OTU5655, OTU2722 and OTU1229 increased due to addition of the extracts. On the other hand, OTU4163, OTU2850, OTU1676, OTU3078, OTU2196, OTU1332 and OTU1207 decreased due to addition of the extracts. The changes of these OTUs suggested they could be stimulated or purged with the increase of extracts.

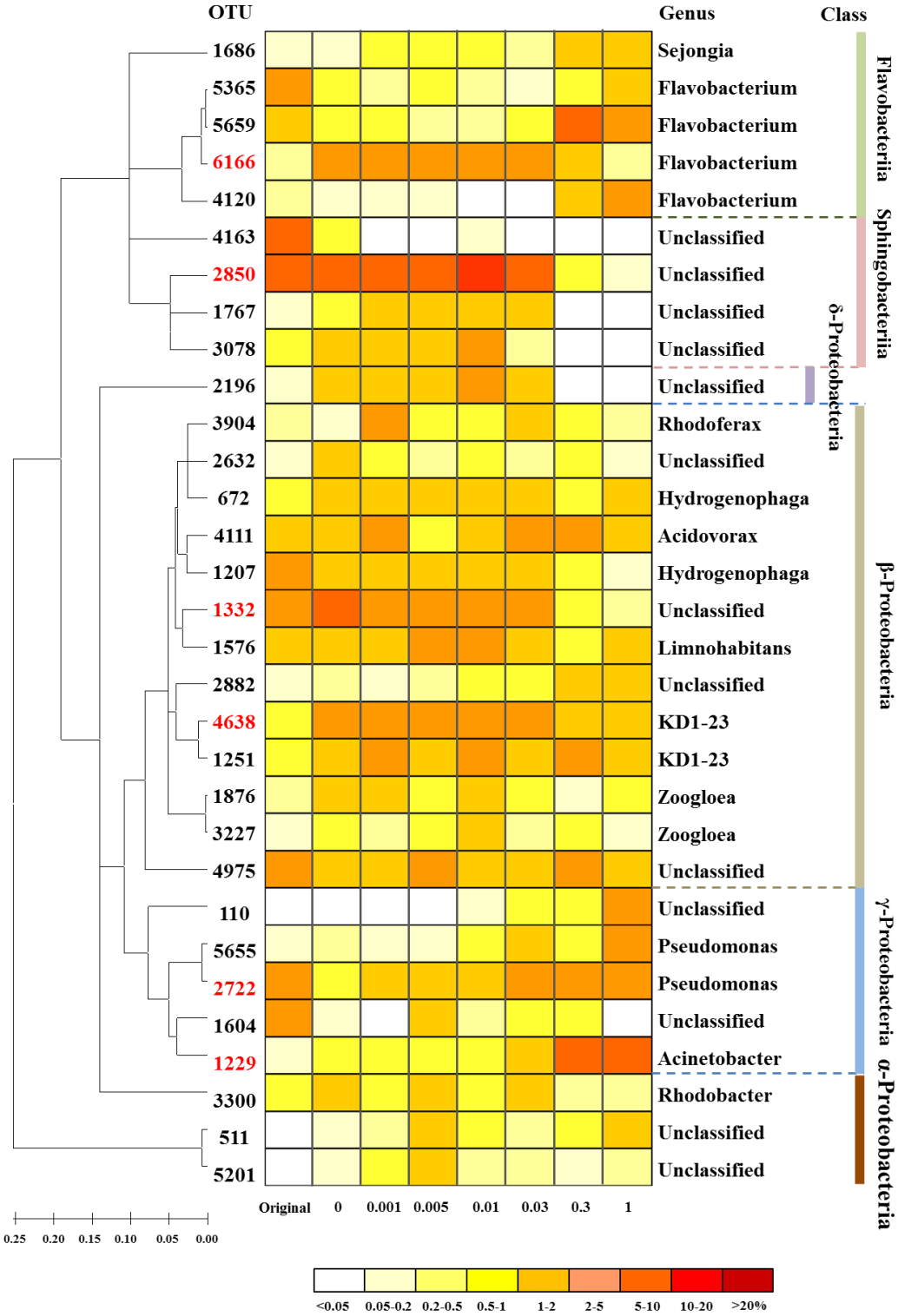

Fig. 6.12 Heatmap representation of the effects of extract addition on bacterial population at different dose levels. The OTUs with average fractions higher than 0.5% are shown. The OTU numbers of the OTUs with the average fraction higher 2% are shown in red color.

Following 6 OTUs had average fractions higher than 2%, and their OUT numbers are shown in red in Fig. 6.12: OTU6166, OTU2850, OTU1332, OTU4638, OTU2722, and OTU1229. The relationships between the fraction values of these OTUs with different levels of extract addition were as shown in Fig. 6.13, where Fig.6.13 (a) on liner and Fig.6.13 (b) on logarithmic horizontal axes. As Fig. 6.13 (b) shows, all the fractions of OTUs changed greatly when dose level was higher than 0.3 ×, especially OTU2850, OTU1332 and OTU1229. But when the dose level was smaller than 0.3 ×, their change were small, as can be seen on OTU2850, OTU1332, OTU4638 and OTU1229. These results suggested the dose-response changes followed a sigmoid curve. OTU6166 also showed similar pattern.

However, OTU 2722 increased their fractions up to dose level of 0.3 ×, and higher dose resulted in its decrease. Its response was not simple sigmoid pattern. Similarly, OTU5659 showed similar response with OTU2722 but showed a much clear peak at the dose level of 0.3 ×. OTU5659 is expected to give 276 bp fragment (Table 6.3), which is close to 280 bp band showed in Fig. 5.14 which also showed the peak at the dose level of 0.3 ×. Therefore, OTU5659 in genus *Flavobacterium* responded to the extract with a maximum response at 0.3 ×.

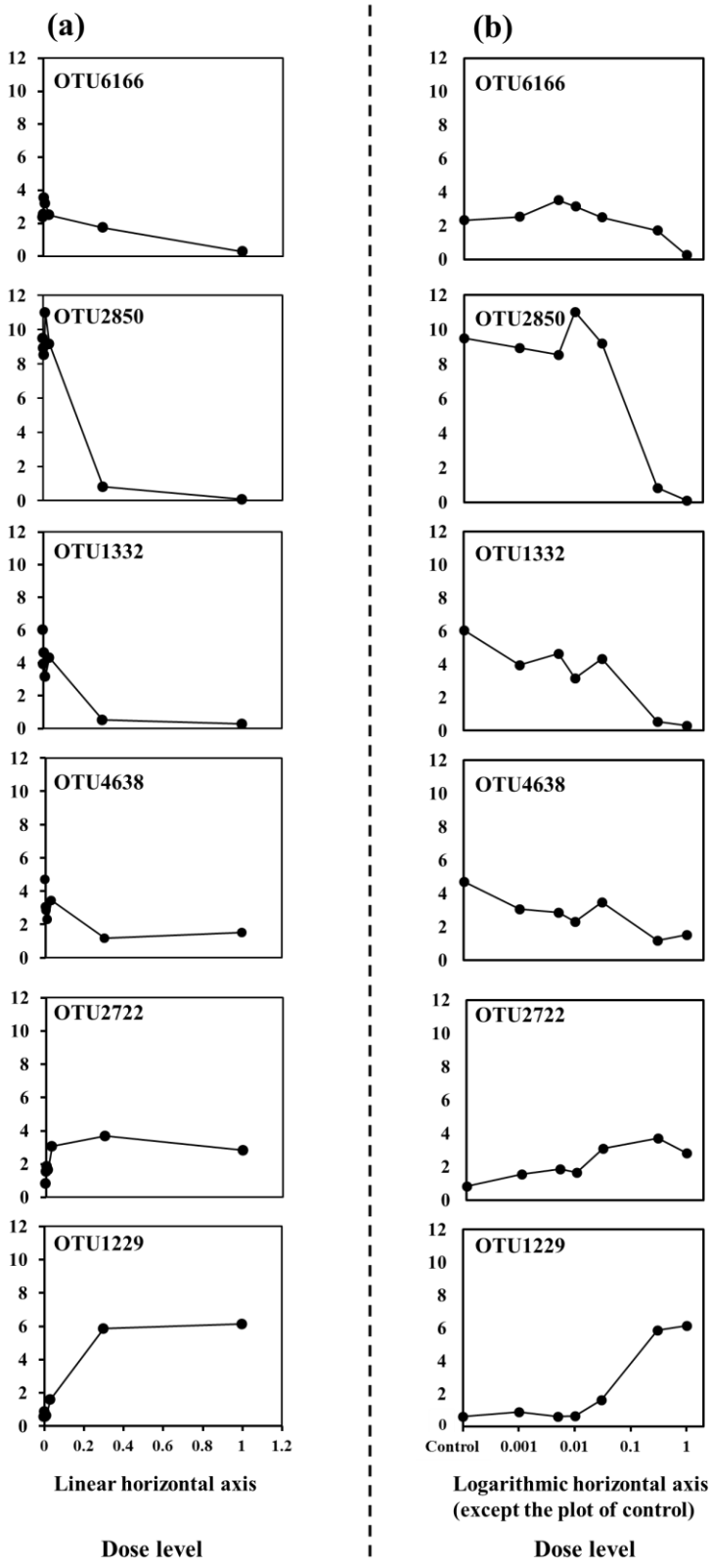

Fig. 6.13 Dynamics of major OTUs (average fractions higher than 2%) with different dose levels.

6.4 Discussion

6.4.1 Bacterial groups affected by crude extracts

Bacterial groups affected by the addition of crude extracts were clarified in this chapter. When the results were observed at higher hierarchical levels, Phylum Proteobacteria, especially order Pseudomonadales in class Gammaproteobacteria, became more by the crude extract addition. On the other hand, phylum Bacteroidetes was reduced. Especially whole class Sphingobacteria significantly decreased and almost disappeared.

On the other hand, OTU5659 and OTU6166 both belonging to genus *Flavobacterium* in phylum Bacteroidetes showed totally opposite response to the extract addition: OTU5659 increased while OTU6166 decreased at higher dose levels (Fig. 6.12).

That is, about phylum Bacteroidetes, while OTUs in Sphingobacteria were negatively affected, in class Flavobacteria, responses were apparently different even when OTUs belonged to the same genus *Flavobacterium*.

Opposite responses of OTUs in the same genus were also observed in genus *Hydrogenophaga* in class Betaproteobacteria: while OTU672 was not affected by the addition of extract, OTU1207 decreased (Fig. 6.12).

These outcomes cannot immediately be generalized, as the phenomenon is thought to be very complex: extracts from source activated sludge contains different interaction chemicals at different concentration levels, and recipient bacterial population in activated sludge is different. More extensive study is needed to clarify the whole picture of the effects of chemicals on bacterial population.

6.4.2 Comparison of RFLP results with pyrosequencing.

As far as the author reviewed, this may be the first one to compare the results of RFLP and pyrosequencing. As shown in Fig. 6.7, the fragment 91 bp was confirmed to be from OTU2850 (unclassified Sphingobacteriales) and OTU5659 (genus *Flavobacterium*), though they belong to different classes under phylum Bacteroidetes. The fragment 206 bp was confirmed to represent OTU 1229 (genu *Acinetobacter*) and OTU 110

(unclassified Aeromonadaceae), although they both belong to the same class Gammaproteobacteria. The two methods, pyrosequencing and RFLP, gave consistent results.

6.5 Conclusions

In this section, the samples obtained in Exp. 16 (1) and Exp. 16 (2) were analyzed by pyrosequencing to clarify bacterial species affected by the addition of the extract E137.

1. Bacterial species that are affected by the crude extracts were clarified. Whole class Sphingobacteria was inhibited by the addition of extract. On the other hand, at order level, growth of Pseudomonadales, Aeromonadales and Flavobacteriales were promoted by the addition of crude extracts. In the same genus of Flavobacterium, two OTUs (OTU6166 and OTU 4120) were oppositely affected by the extracts. Based on the analysis of the major OTUs, OTU1229 (genu *Acinetobacter*) and OTU2850 (unclassified Sphingobacteriales) were the most affected by the addition of extracts: OTU1229 was promoted and OTU2850 was decreased, respectively. (Fig. 6.2, Fig. 6.5, Fig. 6.9 and Fig. 6.12)

2. Even in the same genus, the response of OTUs were not consistent. One example is OTU6166 and OTU4120 in genus *Flavobacterium* as mentioned above, and another was found in genus *Hydrogenophaga*. (Fig. 6.5 and Fig. 6.13)

3. The effect of crude extracts on OTUs basically followed a sigmoid curve with a threshold value. Some of the OTUs showed the maximum response in the middle range dose: the higher or lower dose resulted in smaller response. (Fig. 6.14)

4. The sizes of the restriction fragments calculated for the most affected OTUs by extract addition agreed well with the results of RT-PCR/RFLP. (Fig. 6.7)

Chapter 7 Conclusions

In the present study, activated sludge bacterial population changes caused by the addition of crude extract from activated sludge were investigated. In Chapter 1, three specific objectives as follows were raised.

1. To clarify the response time to cause changes in bacterial population;
2. To clarify the effects of the concentrations of the extract on bacterial population change;
3. To identify bacterial species affected by the addition of the extract.

Here are conclusions corresponding to each objective.

Conclusion for objective 1: The addition of crude extract from activated sludge caused significant bacterial population change in activated sludge only within a couple of hours. (5.3.3)

Conclusion for objective 2: The dose-response relationship basically follow a sigmoid curve when logarithm of dosed crude extract and the relative band intensities from RT-PCR/RFLP analyses were plotted (5.3.4). Some of the bands and their corresponding OTUs showed the maximum response in the middle range dose: the higher or lower dose resulted in smaller response (5.3.4 and 6.3.2).

Conclusion for objective 3: The bacterial species affected by the crude extracts were clarified by pyrosequencing. The growth of bacteria in order Pseudomonadales, Aeromonadales and Flavobacteriales were basically promoted by the addition of crude extracts. But in Flavobacteriales, two OTUs (OTU6166 and OTU4120) in the same genus *Flavobacterium* were oppositely affected by the extracts. On the other hand, class Sphingobacteria was totally inhibited by the extracts. But this outcome stands only for the present study, and more study would be needed to gain a general picture. (Chapter 6)

Further, following achievements were obtained.

In relation to the conclusion for the first objective, following outcomes were achieved.

- Population change caused by the addition of crude extract can be observed even

when increase of biomass is only 20% to 30%. (5.3.1, 5.3.3)

- Crude extract not only stimulated the growth of selected species but also killed or eliminated other bacterial species, as some of the bands or OTUs were intensified but others disappeared as incubation proceeded. (5.3.1, 5.3.3)

In addition, in this study, different activated sludges were used as the source of extraction and as the seed for incubation. In this regard, following outcomes were achieved.

- Different activated sludges have different sensitivities to the same extract (5.3.2).
- Extracts from different activated sludge have different effects to change bacterial population in activated sludge. (5.3.5)

Lastly, there is an additional conclusion from methodological point of view. In the present study, the same samples were analyzed by RT-PCR/RFLP and RT-PCR/pyrosequencing.

- The sizes of the restriction fragments calculated for the major OTUs that were affected by extract addition agreed well with the results of RT-PCR/RFLP. (6.3.1.3, 6.3.2.3)

In this study, crude extracts from activated sludge was demonstrated to show very diverse and very complicated nature to affect bacterial population in activated sludge. Bacterial population was found to change within a couple of hours by the addition of extract. In general, wastewater engineers consider that bacterial population changes in the order of sludge retention time of a couple of days to tens of days. But the outcome here showed bacterial population can significantly change in the order of hours. The author would like to mention followings as the concluding remarks.

- The extent of bacterial interactions mediated by chemicals should be studied in relation with the performance of activated sludge processes such as nutrient removal, and bulking control.
- If more studies are done, different chemicals that mediate bacterial interactions are isolated and identified, and bacterial species that are affected by them are identified, it would become possible to control bacterial population in activated sludge. That will improve the performance of wastewater treatment.Different activated sludges have different sensitivities to the same extract (5.3.2).

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APPENDIX

CONTENTS OF APPENDIX

Appendix 1: Primers used in pyrosequencing

For pyrosequencing analysis of the samples in chapter 6, all the DNA sequence of primers were showed in Table A1.

Table A1 Primers used in pyrosequencing

Forward primers

Dose level	Incubation time	Primer sequences		
		Adapter A region	Barcode	27f primer region
-	0 hr (original sludge)	for all samples, 5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG-3'	5'-CAG AGA GC-3'	for all samples, 5'-AGA GTT TGA TCM TGG CTC AG-3'
1×	2 hr		5'-AGC TGC TG-3'	
1×	4 hr		5'-ATC AGA TC-3'	
1×	10 hr		5'-ATC AGC TG-3'	
1×	16 hr		5'-ATC ATC AG-3'	
1×	20 hr		5'-ATC ATC TC-3'	
1×	24 hr		5'-CAG CAT GC-3'	
0 (control)	2 hr		5'-CAG CTC TC-3'	
0 (control)	4 hr		5'-CAT CTC TG-3'	
0 (control)	10 hr		5'-CAT CTG AG-3'	
0 (control)	16 hr		5'-CAT GAG AG-3'	
0 (control)	20 hr		5'-CAT GAT GC-3'	
0 (control)	24 hr		5'-CAT GCA GC-3'	
0.001×	24 hr		5'-CAG AGA TG-3'	
0.005×	24 hr		5'-CAG AGC AG-3'	
0.01×	24 hr		5'-CAG AGC TC-3'	
0.03×	24 hr		5'-CAG ATC TG-3'	
0.3×	24 hr		5'-CAG ATG AG-3'	
1×	24 hr		5'-CAG CAG AG-3'	

Reverse primer (common to all samples)

Adapter B region	519r primer region
5'-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG -3'	5'-GWA TTA CCG CGG CKG CTG-3'

Appendix 2: The performance of SBR1

As has been described in section 3.2.1, SBR1 has been operated for taking the activated sludge samples for chemical extraction and incubation. The performance of the reactor was showed in Fig A1.

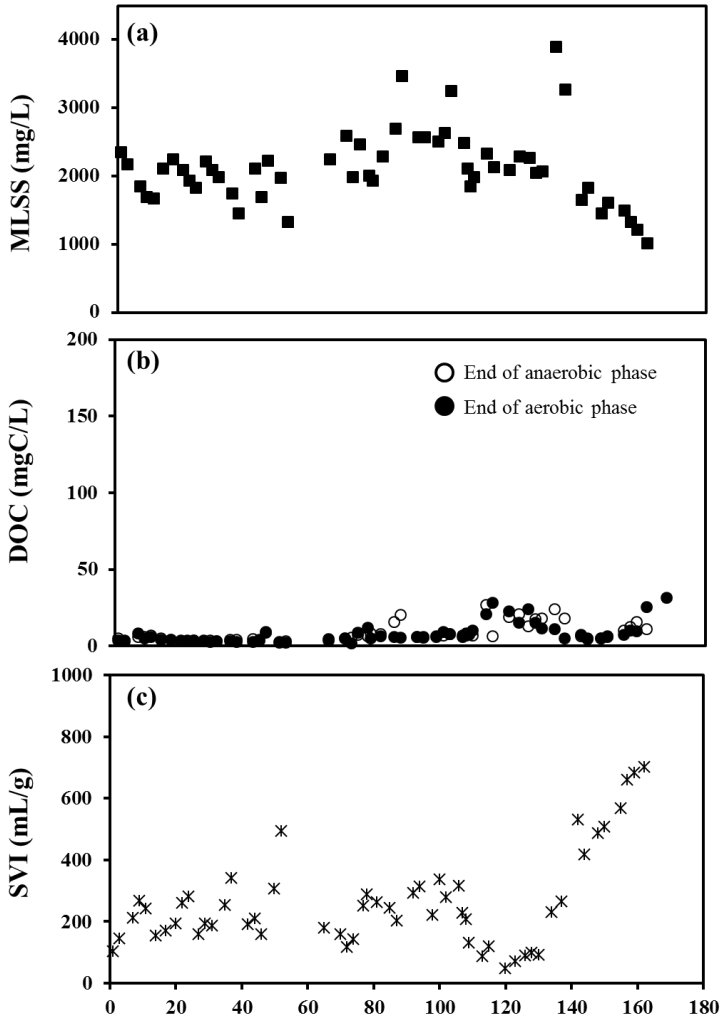

Fig. A1 Performance of the laboratory-scale activated sludge reactor.

Appendix 3: Identification of affected bacteria in Exp. 13

As has been described in section 5.1, the bacterial population changes by addition of extracts have been clearly detected by RT-PCR/RFLP analysis. To further identify the affected bacterial species, the samples were re-analyzed by pyrosequencing and the heatmap of affected bacteria (average fraction higher than 0.5%) was showed in Fig. A2.

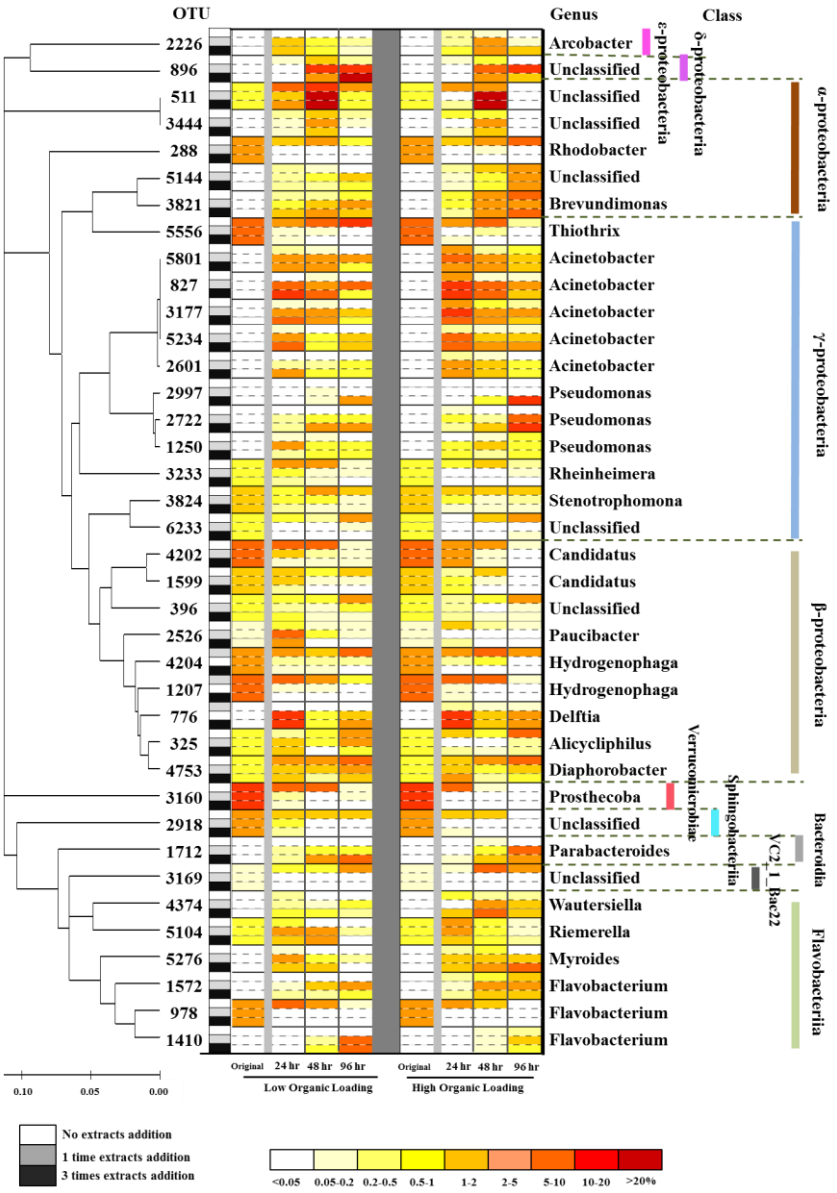

Fig. A2 Partial tree and Heatmap on showing the distribution of OTUs (Average fraction higher than 0.5%) with the different concentration of extract of experiment 13.

Appendix 4: Identification of affected bacteria in Exp. 14

As has been described in section 5.2, the difference of sensitivity of activated sludge from SBR and WWTP was clearly detected by the RT-PCR/RFLP analysis. To further identify the sensitivity of affected bacterial species, the samples were re-analyzed by pyrosequencing and the heatmap of affected bacteria (average fraction higher than 0.5%) was showed in Fig. A3.

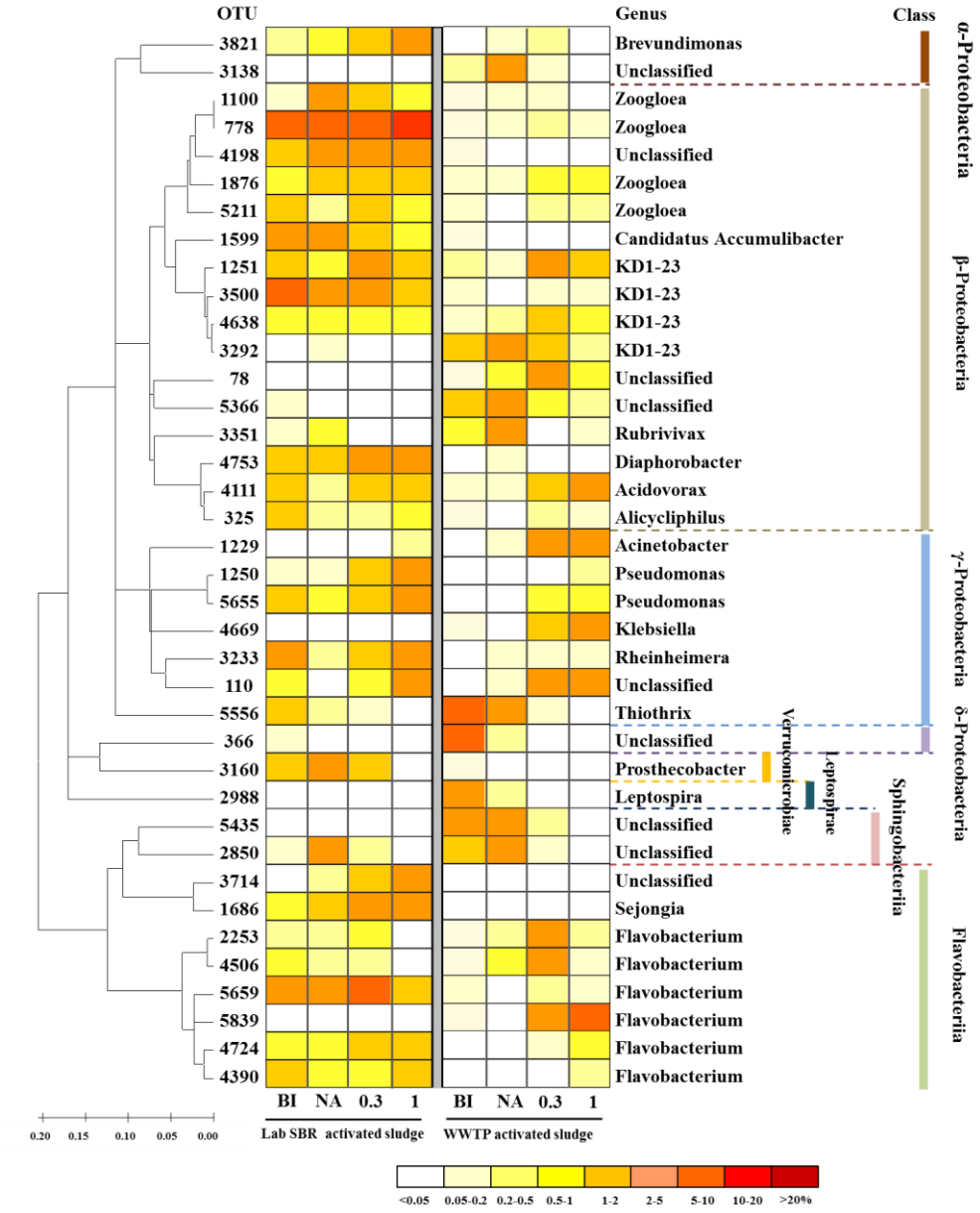

Fig. A3 Partial tree and Heatmap on showing the distribution of OTUs (Average fraction higher than 0.5%) with the different concentration of extract of experiment 14.

Appendix 5: RT-PCR/RFLP results of Exp. 15

In Exp. 15, the extract was obtain on 137th operation day of SBR1 and extracted by ethanol. The incubated activated sludge was from the WWTP activated sludge taken on Feb. 13th and feed with the F/M ratio of 0.5 (kgCOD/kgMLSS) for 24 hours incubation. In this experiment, For investigation of the activated sludge bacterial population changes with the time, the incubated activated sludge was taken on 3hr, 5hr, 8hr, 16hr, 19hr, 21hr and 24hr respectively.

The RT-PCR/RFLP results were showed in Fig. A4, compared with the control, the bands pattern of the sample with the extract addition were changed after 16 hrs incubation, but the how about the changes between 8hr and 16 hr were not clear due to the limitation of the samples taken.

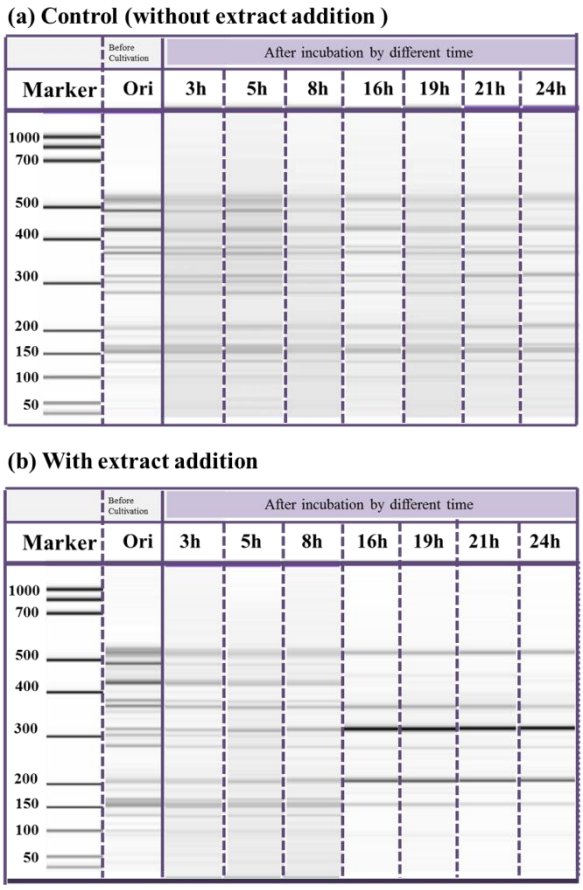

Fig. A4 RT-PCR/RFLP results for the activated sludge (WWTP on Feb. 13th) incubated in microplate with and without extract of E137 with different incubation time.

Appendix 6: Reproducibility of Exp. 16(1) by RT-PCR/RFLP

In Exp. 16 (1), for each condition, two replicates were applied for grasp the bacterial population changes of activated sludge with time during by addition of the extracts. Fig. A5 showed the reproducibility of the RT-PCR/RFLP results.

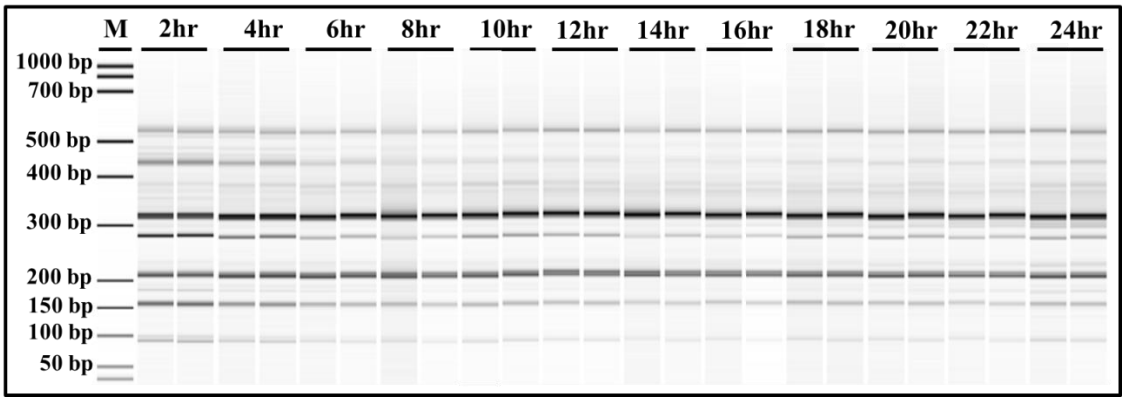

Fig. A5, RT-PCR/RFLP results of the activated sludge bacterial population changes by extracts addition with time during with two repeats.