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Bacterial filtration efficiencies and the bacterial communities' proportions differences between sand size and depths in biosand filters

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ABSTRACT

The biosand filter (BSF) is a household sand filter used with pause periods in between uses, but it is not clear if larger sand particles can be used to increase the flow rate while maintaining the same bacterial filtration rate. Smaller scale BSFs were prepared to first measure if larger sand particles can filter water effectively. It was found that after 2 months of ripening BSFs, there was no significant difference in bacterial filtration rate between BSFs of a sand diameter less than 0.7 mm and less than 1.4 mm after 24 h of a pause period. Bacteria were identified by taking 16S rRNA in three layers of the sand in each BSF. *Lelliottia sp.*, *Enterobacter aerogenes*, *Kluyvera sp.*, *Buttiauxellanoackiae*, and *Pantoea sp.* were identified. By examining the band intensities in denaturing gel gradient electrophoresis, it was found that the microbial communities' proportions differ between the levels of the sand and between the two BSFs of differing sand diameters, indicating differing filtration method priorities between the BSFs of different sand sizes. These findings indicate that, in the case of a 24 h pause period, a BSF with sand sizes 1.4 mm or less is just as viable as the current BSFs, while allowing for easier construction.

Keywords: Biosand filter; BSF; Schmutzdecke; *Lelliottia sp.*; *Enterobacter aerogenes*; *Kluyvera sp.*; *Buttiauxellanoackiae*; *Pantoea sp*

1. Introduction

According to a 2017 progress report on drinking water, sanitation and hygiene released by the World Health Organization, 7 out of 10 people used safely managed drinking water services in 2015, with Sub-Saharan Africa and Oceana having the least access to clean drinking water. Sickness and death due to pathogens in the water are responsible for some 1.7 million deaths a year worldwide [1], and contaminated water is one of the most common causes of sickness and death. It is reported that more than 1.1 billion people do not have access to safe water [2]. Developing countries require water treatment systems, but many water purification machineries are expensive, and thus an alternative solution using "appropriate technology" is attracting attention.

Biosand filters (BSFs) are one of the most widely used water purification methods in developing countries. The 2012 Annual Report of the Centre for Affordable Water and Sanitation Technology (CAWST) presented statistical data indicating that 5,981,000 people are impacted by the water sanitation project encompassing biosand filters (BSF). Furthermore, over 200,000 BSFs have been installed globally. Additionally, 12,346 institutions are participating in supplying BSFs, and 37 countries are taking advantage of the BSF system.

A typical biosand filter is composed of easily obtainable materials, such as sand and pebbles in a plastic or a metal container. It exhibits a high purification efficiency, removing 93–99% of fecal coliform bacteria, 99.9% of protozoa, up to 100% of helminths, and 70–99% of viruses [3,4,5]. Pathogens and dirt in the filter are filtered via mechanical trapping, predation, absorption, and natural death inside the

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filter. Some dirt and pathogens are too large to pass through the sand pores, some pathogens die due to lack of nutrients or oxygen inside the filter, and the microbes prey on other microbes inside the layer, especially in the biolayer or schmutzdecke. BSF provides an appropriate condition for the growth of microorganisms, encouraging them to form a biofilm called schmutzdecke [6]. Various microorganisms from the influent water source attach to the sand surface and accumulate to become part of the schmutzdecke. The microorganisms composing the schmutzdecke, in turn, filter other harmful microorganisms contained in influent water. The participation of water source-originated microorganisms in the schmutzdecke further increases the biological purification efficacy [7].

Building BSFs, while inexpensive and easy to make, requires sifts of various sizes and sand grains with a diameter less than 0.7 mm to be built according to the CAWST manual. According to the CAWST biosand filters knowledge base as of May 2018, the 0.7 mm aperture mesh net needed to filter for sand grains is not available in many countries. If larger sand particles can be used to build the BSFs, it may reduce the cost and make it easier to build one while increasing the flow rate. While a study found that a large sand grain size of 0.30 mm is less effective for bacterial removal compared to the smaller sand grain size of 0.15 mm [8], this paper seeks to find if there is a significant difference in bacterial removal between sand sizes of 1.4 mm and 0.70 mm.

Because the schmutzdecke clogs and prevents water flow through the filter as it develops, it should be cleaned or discarded on a regular basis to maintain the BSF. Generally, people using BSFs dump or pour the waste of the schmutzdecke in nearby water sources, such as ditches, lakes, or rivers, for their convenience and without much awareness. This practice has continued because there have been no solid guidelines for the after-treatment of schmutzdecke deposits. No one has questioned the traditional method of discarding schmutzdecke, and the whole BSF filter may result in nearby biological pollution. The main focus of BSF research has been purification and efficiency rather than the potential risks it poses.

Due to different ways that pathogens are filtered, it is feasible that the microbial community would differ depending on the depth of the sand layer in the filter. It is not yet known whether discarding sand in BSFs is safe, and discarding the schmutzdecke may pose a risk of contaminating its surrounding [9]. Examining the potential negative side of schmutzdecke, which has thus far been neglected, this paper aims to perform a risk assessment of schmutzdecke by evaluating opportunistic pathogens.

2. Materials and methods

2.1. Sample collection from Chunmaji Lake

Samples were collected from Chunmaji Lake (latitude: 36.100021/longitude: 129.394265) in Pohang, South Korea. Water was always collected from the same part of the lake, and a volume equivalent to the sand pore volume was poured into the BSFs every 24–48 h as instructed in the 2012 Biosand Filter Construction Manual written by CAWST. Changes in the weather, environment, and temperature were ignored to simulate a real-world scenario.

2.2. Construction of a small-scale BSF

BSFs were constructed according to the manual offered by CAWST. A cylindrical container composed of polyethylene phthalate was used as the casing, and 3 holes were punctured on its side. The holes were blocked with parafilm, which were removed when sand samples were

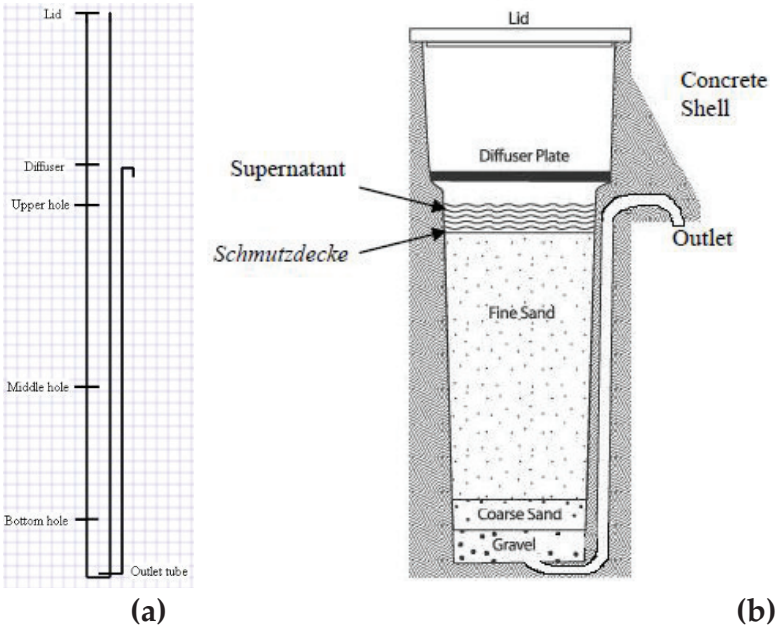


Fig. 1. Biosand filter. (a) Diagram of miniature BSFs. Each grid represents 2 cm; (b) BSF diagram was taken from CAWST *Household Water Treatment and Safe Storage Fact Sheet* released in 2009.

retrieved through the holes. The biosand filter construction manual was adhered to, except for the horizontal length of the container, therefore making the BSFs smaller than the original versions of the BSFs. The diameter of the cylindrical BSFs was 4 cm each, allowing for easy access to different layers of sand in the filter. Two types of silica sand were purchased from the total farm industry (South Korea, Junju), unit 5 sand of 0.7~1.4 mm diameter and unit 6 sand of 0.35~0.7 mm diameter. Sand purchased were uniform in size and shape; one BSF was filled with unit 5 sand, and another BSF was filled with unit 6 sand. Suitably sized pebbles were collected around the source water and washed. Sand and pebbles were autoclaved before being put in the BSFs. Standing water height of 5 cm above the sand surface was maintained throughout the experiment. Sufficient amounts of the schmutzdecke in the top layer of BSFs were developed 8 weeks after the Chunmaji Lake water source was regularly poured into them.

2.3. Bacteria colony forming unit (CFU) enumeration of water source and filtered water

After 2 months of BSF usage and allowing them to ripen, 100 µL of source water and filtered water from two BSFs were plated on Plate Count Agar (PCA) to measure the CFUs of the influent and effluent water. The plates were incubated in a 37°C incubator for 24 h and the colonies were counted.

2.4. DNA isolation

Using an i-genomic Soil DNA Extraction Mini Kit (iNtRON Biotechnology, Inc. South Korea), 100 mg of sand was isolated through the three holes in BSFs. Each isolated sample was added to a 1.5 ml sample grinder tube, vortexed in lysis buffer and inhibitor remover and vortexed for 10 min at 13,000 g. After collecting the supernatant into a new tube, precipitation solution was added and then centrifuged at 13,000 rpm for 3 min. Then, 300 µL of supernatant was transferred into a new 1.5 ml tube. Then, 300 µL of elution buffer was added, followed by 300 µL of 100% EtOH, and the tube was inverted several times. Lastly, 800 µL of the mixture was isolated into a spin column inside a collection tube and was centrifuged at 13,000 rpm for 1 min. Flow-through was discarded, and after adding the remaining mixture into the spin column, the tubes were centrifuged at 13,000 rpm for 1 min. Then, the washing step and elution steps were followed. Then, the binding step, washing step, and elution steps were followed to isolate DNA, and the samples were kept at –20°C.

2.5. Polymerase chain reaction amplification

Extracted DNA was amplified through polymerase chain reaction (PCR) with a Veriti R TM 96-well Thermal Cycler (Applied Biosystems, Marsiling, Singapore), using the 16S rRNA universal primer pair of 341F (5′-CCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′). The DNA template (1.0 µL) was mixed with 5 µL of 10× Buffer, 4 µL of 10 mM dNTP, 1.0 µL of forward primer (10 pmol), 1.0 µL of reverse primer (10 pmol), 0.25 µL of Ex-Taq (0.25 µL), and 37.75 µL of DDW. The total volume was 50 µL. A kit from Takara Bio USA, formerly known as Clontech, was used.

The resulting product was used as a template following PCR to add the GC clamp. The GC clamp was ligated at the 5′ end of the 341F primer (5′-CGCCCGCCGCGCGGGCGGGCGGGGCGGGGGC-CGGGGGGACTCCTACGGGAGGCAGCAG-3′). The new DNA template (2.0 µL) was mixed with 5 µL of 10× Buffer, 4 µL of 10 mM dNTP, 1.0 µL of forward primer (10 pmol), 1.0 µL of reverse primer (10 pmol), 0.25 µL of Ex-Taq (0.25 µL) and 36.75 µL of DDW.

2.6. DNA Gel electrophoresis

Twenty-five milliliters of 1X Tris base, acetic acid and EDTA (TAE) solution was mixed with 0.5 g of agarose (Promega, Spain) and ShinyStar gel stain (NanoHelix, South Korea), then was cast in a tray with wells to make a gel for electrophoresis. DNA gel electrophoresis was run for 25 min at 120 V and examined under UV light to confirm successful PCR. Gel electrophoresis was run for both 341F PCR and GC341F PCR.

2.7. Denaturing gradient gel electrophoresis (DGGE)

Six percent acrylamide gel was prepared with a 0–70% denaturing gradient. Formamide and urea were used for the gradient. After letting the liquid solution set, the gel was immersed in 60°C 1X Tris base, acetic acid and EDTA (TAE) solution. After normalizing the DNA amount based on the gel band intensities measured by the ImageJ program, 30 µL of each PCR sample mixed with 6 µL of 6X staining dye was pipetted into wells in the gel. The voltage was set to 104 V, and amperes to 500 mA, and the gel electrophoresis was run for 960 min. The DCodeTM Universal Mutation Detection System (Bio-Rad Laboratories, Japan) kit was used. The gel was then isolated and put in a light-blocking tray with 250 ml of 1X TAE buffer with 25 µL of DNA dye solution added. After 15 min, the gel was viewed under 302 nm UV light, photographs were taken, and the gel was then moved to a UV tray to excise the visible DNA bands.

Each visible gel band was put in microtubes, and 25 µL of DDW was added to the bands. The tubes were frozen at –20°C and thawed to allow for DNA elution. The samples were sent for sequencing at Macrogen (Seoul, South Korea).

2.8. DNA sequence analysis

Sequences for the 16S rRNA of the isolates were analyzed and identified for the nearest phylogenetic neighbor using the Basic Local Alignment Search (BLAST) tool from NCBI (National Center for Biotechnology Information, USA) [10].

3. Results

3.1. Counting CFU for influent water and effluent water

3.2. Comparing the band intensities of bacterial 16S rRNA DNA

3.3. Constructing the phylogenetic tree of the identified bacteria

Our results show the differing band intensities of bacteria collected. For unit 5 BSF, *Lelliottia sp.* displayed the

weakest band intensity for the top layer of sand, while the middle and bottom layers displayed band intensities. Bands corresponding to *Enterobacter aerogenes* showed similar band intensities across all three layers of sand. Bands of *Kluyvera sp.* showed similar band intensities across the top and middle layers of sand, but the bottom layer displayed the weakest band intensity. The gel band for *Pantoea sp.* was only visible for the bottom layer when cutting the gel. The gel band for *Buttiauxellanoackiae* showed similar intensities at the bottom of the gel.

For unit 6 BSF, the *Lelliottia sp.* band showed the strongest intensity at the middle layer and the weakest at the bottom layer, while the top layer had medium intensity. DNA gel bands of *Enterobacter aerogenes* displayed similar band intensities across the three sand depths. Bands for *Kluyvera sp.* showed the strongest intensity at the middle level, medium strength at the top level, and weakest intensity at the bottom level of sand. *Buttiauxellanoackiae* showed similar band intensities at the bottom of gels, and *Pantoea sp*. displayed similar band intensities at the top and middle layers of sand, but no band was visible at the bottom layer of sand.

4. Discussion

4.1. Microbial filtration rate difference between unit 5 and unit 6 BSFs is not significant

When water was poured into BSFs to measure the sand pore capacity, it was noted that finer sand was collected at the bottom of the BSFs. This was noticeable in both unit 5 and unit 6 BSFs. The rubber tubing connected to the bottom outlet prevented the finer sand particles from leaving the filter.

It took 2 months of regular lake-water filling of the sand pores of the BSFs to develop a bacterial filtration rate that is similar to the average 87.9% bacterial filtration rate of household BSFs in Ethiopia [11]. Chunmaji Lake is a standstill lake located a few kilometers away from the residence and was expected to contain high amounts of bacteria, thus forming a schmutzdecke layer at the top quickly, but it took longer than the expected one month. However, it should be noted that any debris such as sand, branches, or leaves was removed from the water before it was poured into BSFs.

The lack of differences in microbial filtration efficiencies in the two sand filters was contrary to our expectations. It is reasonable to assume that using finer sand particles, as dictated in the CAWST BSF construction manual, would result in more effective filtration than using sand particles of larger diameter. However, the number of bacteria in the filtered water from the two BSFs was enumerated by plating on PCA and counting the colony forming units, which showed that there is no significant filtration rate difference between the two (Fig. 2).

4.2. Amount of bacterial DNA found in the sand of BSFs may differ based on the size of sand particles and depth of the sand

DGGE was run to evaluate the microbial ecology and analyze multiple samples simultaneously to understand the microbial communities at each of the sand layers [12]. Two of the five bacteria identified in DGGE (*Enterobacter aerogenes*, *Buttiauxellanoackiae*) showed similar band strengths

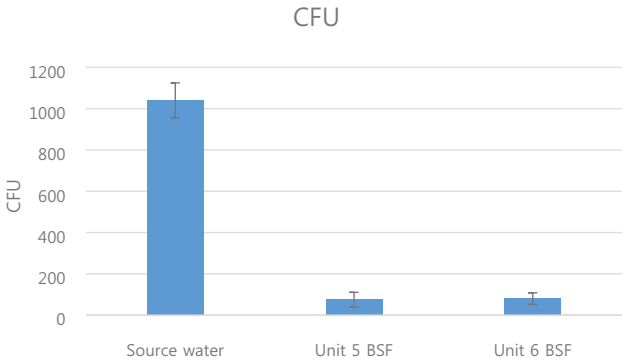


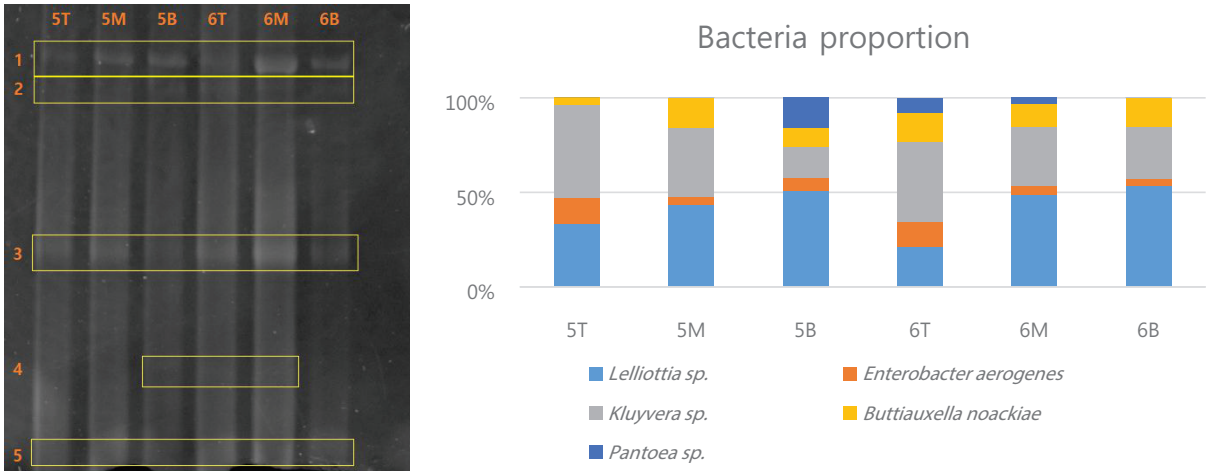
Fig. 2. CFU of source water and effluent water from two BSFs. Y-axis unit is CFU/ml.

across sand depths. This is surprising as the development of schmutzdecke or the biofilm at the top of the filter is what is known to be responsible for the increased filtration rate of water, leading us to expect higher band intensities at the top of the BSF.

Lelliottia belongs to the family of gram-negative enterobacteria and is pathogenic. The cells are straight rods, 0.6–1.0 µm × 1.5–3.0 µm, and motile by peritrichous flagella. They are facultatively anaerobic, and the colonies are unpigmented, round, convex, and smooth [13]. *Enterobacter aerogenes* is a gram-negative, anaerobic facultative, and catalase positive rod-shaped bacterium. The bacterium is approximately 1–3 microns in length and is capable of motility via peritrichous flagella. It is a nosocomial pathogenic bacterium that causes opportunistic infections, including most types of infections [14]. *Kluyvera* is a gram-negative, facultatively anaerobic bacteria with a peritrichous flagellum, sharing the properties of most members of the Enterobacteriaceae family [15]. *Kluyvera*are found in water, soil, and sewage and can cause opportunistic infections in immunocompromised patients [16]. *Buttiauxellanoackiae* is a gram-negative bacterium that forms circular, convex, grayish, and smooth colonies on nutrient agar [17]. *Pantoea*is a genus of gram-negative bacteria of the family Enterobacteriaceae, with a cell size of 1.2 µm by 0.6 µm [18], recently separated from the Enterobacter genus. The cells are motile and cause infections in humans and are also pathogenic to plants [19].

Lelliottia sp. displayed consistently strong band intensity at all sand layers of BSF except for unit 6 BSF's top layer. *Enterobacter aerogenes* showed similar band intensities across all layers of both BSFs. *Kluyvera sp.* displayed the greatest band intensity at the top layer of unit 5 BSF, reduced intensity in the middle layer, and the least intensity in the bottom layer. It showed the same pattern for unit 6 BSF. When cutting the gel on the UV light stand, *Pantoea* was visible in the unit 5 bottom layer and the unit 6 top and middle layers, but not the other layers. All these differences in band intensities suggest that the bacterial population varies at different depths in the sand and differing sand diameters.

When comparing this pattern to the 16S rRNA phylogenetic tree, *Kluyvera* and *Pantoea* show the greatest similarity, as can be shown by the fewest nucleotide substitutions per site, and the highest bootstrap value (Fig. 4). However, the pattern between the two species is different for unit 5 and unit 6 BSFs. For the unit 5 BSF, *Kluyvera sp.* decreases in the



(a) (b)

Fig. 3. DGGE pattern. (a) DGGE labeled wherever a visible band was present. The first number represents the unit of BSF and the following alphabet notes the sand layer: T = Top layer, M = Middle layer, B = Bottom layer. DGGE row 1 corresponds to the *Lelliottia sp.* Row 2 corresponds to *Enterobacter aerogenes*. Row 3 corresponds to *Kluyvera sp.* Row 4 corresponds to *Pantoea sp.* Row 5 corresponds to *Buttiauxellanoackiae.*; (b) Bacterial DNA proportion graphed on a stacked bar graph. DNA measured using the intensity of the DNA bands.

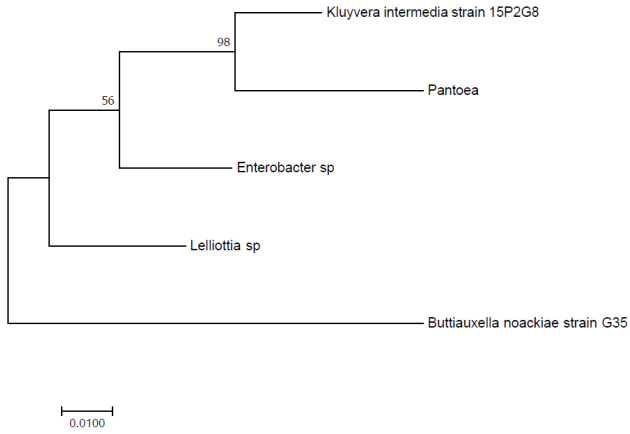


Fig. 4. Phylogenetic tree of the bacteria in BSFs. Phylogenetic tree based on neighbor-joining analysis of 16S rRNA gene for BSF sand layers. The bottom number indicates the length of the branch that represents the amount of genetic change of the number (nucleotide substitutions per site). Numbers at diverging branches are the bootstrap values.

bacterial proportion from the top to the bottom sand layers, while Pantoea is found in the bottom-most layer. This alludes to the competition of the two species. For the unit 6 BSF, however, both Kluyvera sp. and Pantoea decrease in their bacterial proportions from the top sand layer to the bottom. Competition may be a significant factor in determining their proportions in the unit 5 BSF, but it is less significant.

For all the bacteria and both BSFs, the DNA gel band corresponding to the middle depth of sand in the BSF displayed a band intensity higher than or equal to the other depths of sand. This raises the possibility that the majority of the filtration of microbes occurs not in the schmutzdecke or the top layer of the BSF but in the middle level of the BSF.

5. Conclusions

We found that, when using BSFs with a pause period of 24 h or more, there is no difference in the bacterial filtration rate between BSFs built using sand particles that are 0.7~1.4 mm diameter or ones with 0.35~0.7 mm diameter sand. Using larger sand particles for BSF construction not only simplifies building the BSF but also increases the flow rate, addressing one of the major complaints about using BSFs. Using larger sand particles for filtration should be tested more with shorter pause periods. More importantly, countries without proper mesh net to make a BSF filter can use a mosquito mesh net to sift for sand with a diameter of 1.2 mm, allowing for a water filtration rate comparable to the unit 7 BSF.

It has been thought that the BSF's schmutzdecke at the top of the sand layer has the largest role in the filtration system, but the significant bacterial DNA population in the middle layer of sand filter suggests that the middle layer of the BSF may have a larger role in filtration than was previously thought. Additionally, when discarding BSFs, one should be careful to dispose of not only the top layer but also the middle layer, as they possibly contain the opportunistic pathogens they were meant to filter.

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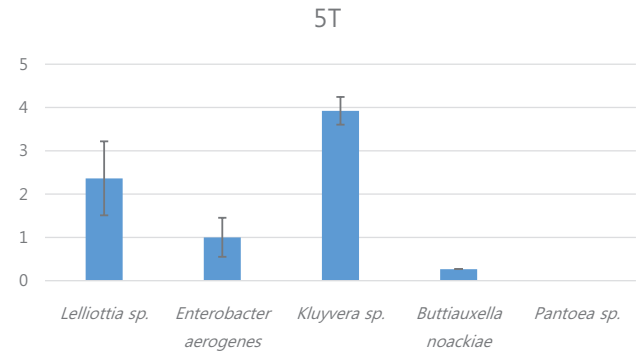
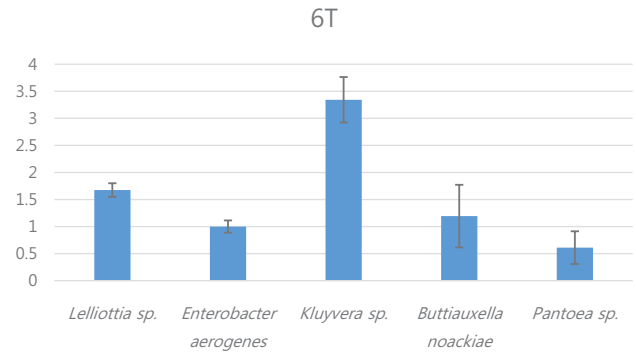
Supplementary data



DGGE DNA band intensities relative to *Enterobacter aerogenes*.



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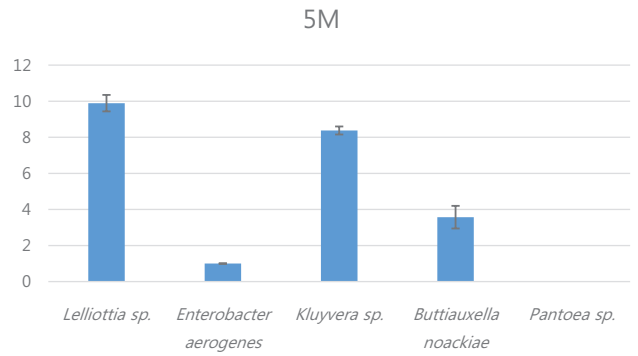

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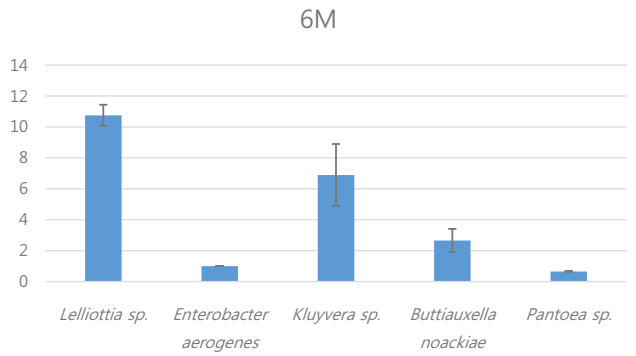
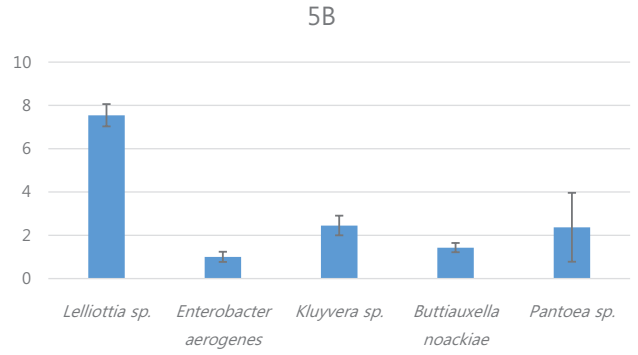
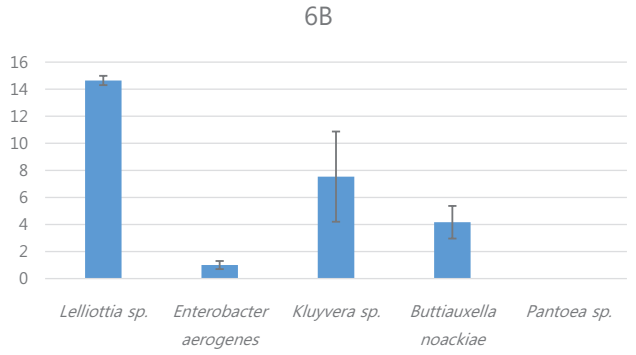


DGGE DNA band intensities relative to *Enterobacter aerogenes*.



DGGE DNA band intensities relative to *Enterobacter aerogenes*.



DGGE DNA band intensities relative to *Enterobacter aerogenes*.