Master Thesis
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Assessing the role of the drinking water distribution system on the bacterial community in public buildings

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Abstract

Safe drinking water is important and the water quality is related to the microbial community. In this study, two public buildings that receive drinking water at different locations in a distribution system were selected as sampling points. The drinking water samples in the buildings were sampled every week to investigate the impact of the distribution system on the microbial community. The sampling was carried out for two months, except a week of public holidays in the middle of it, which allowed us to assess the impact of water stagnation on the bacteria in the water.

The research methodology is to analyze the cells through flow cytometry, and preserve the DNA in the water through filtration for further research. The traditional tests for microbes, chemicals and metal analyses in the standard water quality tests were also submitted to Eurofins for testing.

It is found that the drinking water distribution system has a significant impact on the microbial community, and it was detected that more intact cells in building further from the DWTP was 240% higher than the one closer to the DWTP on average. The stagnation of water led to an increase in the number of total cells and percentage of the high nucleic acid (HNA) bacteria content in the water. In particular, the TCC was 120% and 220% higher after stagnation in school 1 and school 2, respectively. In addition, higher the temperature results in a higher cell content in the distribution samples. The distribution samples had lower cell count and fewer bacterial types compared to other samples.

0.1 Popular Science Summary

Drinking water is a daily necessity. Could you taste differences when you are drinking tap water from different places? Do you have any idea why the water taste varies a lot? Microbes might be part of the answer!

In this study, we focused on the impact of the distribution system on microbial communities. Two public buildings were chosen to be studied, one of them is closer to the drinking water distribution system and the other is further away. During March and April in 2021, we went to the buildings to take water samples from different taps and took the water back to Lund University for analysis. One week of stagnation was introduced in the study due to the Easter holiday.

Flow cytometry was the key technique in this project. It's like counting sheep, but instead of you it's the machine and instead of sheep they are cells, also it's faster. Microbiology, chemical and metal analysis were applied as well. It was found that the distribution system has an influence on the microbial communities in the water in the building. The intact cells were present more in the building further down in the distribution system, which was a result of water age differences and pipes. The stagnation effect was investigated based on the cell count increase after the holiday. Moreover, since the weather was getting warmer, the water temperature was climbing during the two months, thus, more cells were detected in the water.

This project could be a useful reference for the government when it's needed to establish important public buildings like hospitals and schools where clean and safe drinking water is fundamental.

0.2 Acknowledgements

I would like to thank the following people, without whom I would not have been able to complete this research, and without whom I would not have made it through my master's degree.

The water group at Applied Microbiology and Water Resources Engineering at Lund University, especially to my supervisor Catherine J. Paul, whose insight and knowledge into the subject matter steered me through this research. And special thanks to my PhD supervisor Mikael Danielson, and my industry supervisor Sandy Chan allowed my studies to go the extra mile. In addition, a thank you to my examiner Kenneth M. Persson.

I would also like to thank everyone at Applied Microbiology for all the help in the lab. And I thank Anna Barup from Byskolan Södra Sandby, as well as Charlotte Wedelsbäck from Stehagskolan for cooperation for sampling, especially during this pandemic.

Contents

0.3 Abbreviations

DWDS: Drinking water distribution system

DWTP: Drinking water treatment plant

DMSO: Dimethyl sulfoxide

FCM: Flow cytometry

HNA: High nucleic acid

LNA: Low nucleic acid

TCC: Total cell count

ICC: Intact cell count

SG: SYBR Green I

PI: Propidium iodide

1 Introduction

1.2 Purpose of the study

The purpose of this study was to investigate the locations of two public buildings, which are both primary schools in the same distribution system of a drinking water treatment plant. The effect of the distribution system and the building pipe system on the microbial community in the buildings was studied. Microbial water quality is defined here as the total bacterial flora in the water and the presence of any indicator bacteria. This project is a joint collaboration with the Swedish Defense Research Agency (FOI) and Lund University. It's collaborated together with water companies that produce and deliver the water to public buildings, which are Sydvatten AB and VASYD. The study is funded by MSB (The Swedish Civil Contingencies Agency, Myndigheten för Samhällsskydd och Beredskap).

1.3 Objectives of the work

These tasks will be compiled to solve research questions:

- ◊ Does the location in the distribution system affect the microbial water quality in the building?
- ◊ How does the location in the distribution system affect the microbial water quality in the building?
- ◊ How does the stagnation affect the microbial water quality?
- ◊ Will the impact be different depending on the season and temperature change?
- ◊ What are the differences among the taps within each building due to the usage pattern?

2 Theory

2.1 Scientific Background

2.1.1 Drinking water quality

Water is key to our civilization and safe drinking water is crucial to human health and development. In ancient times, the Greeks and Romans used sedimentation tanks, filters, and boiling modus to deal with the poor water quality (IWA, 2021). Safe drinking water is crucial to human health and development. In 2019, the World Health Organization (WHO) published that 82,900 people die from diarrhea each year as a result of unsafe drinking water, sanitation and hand hygiene (WHO, 2019). More specifically, the source of diarrhea is due to the presence of microorganisms in drinking water. This has become even more important in connection with the coronavirus pandemic, as clean water and sanitation is the first and most crucial step to protect people from the virus. The United Nations reported in 2020 that the proportion of the global population using safe drinking water has increased from 61% in 2000 to 71% in 2017, whereas globally, 2.2 billion people still lack safe and managed drinking water, and 785 million people do not have basic drinking water (UN, 2020).

As the drinking water quality testing has become an elementary convention, the World Health Organization, individual countries and territories have established evaluation standards for various indicators. For instance, the guidelines for drinking water quality from the WHO sets the limit of chlorine at 5 mg/L. Besides, the restriction for chloride has not set yet, however, the concentration in excess of 250 mg/L is likely to be detected by taste. Furthermore, the number of coliform bacteria has not been set specifically, although zero is of course, preferable. Particularly, in Sweden, more than 10 $cfu/$

100 mL will be considered as not safe for drinking water at the user (Livsmedelsverket, 2020) and *Escherichia coli* must not be detectable in any 100 mL of drinking water sample. The number of *Enterococci* and the total heterotrophic bacteria should be as low as possible, which have little value to epitomize the presence of the pathogen, nevertheless, they can be regarded as indicators of water treatment and disinfection, as well as for evaluating the cleanliness of the distribution system. (WHO, 2018; WHO, 2011)

2.1.2 Distribution system

Already in ancient times, people always placed settlements based on the location of rivers and lakes. Groundwater became the source of drinking water for people when there was no river or lakes, which was pumped up through dug wells. Owing to the necessity of transporting water, the distribution system for drinking water was gradually formed. Nowadays, the production and delivery of drinking water have become a mature industry.

The drinking water quality can be altered due to changes in microbial communities in the distribution system (Block, 1992). The biofilm formed by the microbial communities in the DWDS exists throughout the pipeline. It directly affects the corrosion of the pipeline and the aesthetics of the water (Douterelo, et al., 2007; Fish, et al., 2017). In addition, the stagnation of drinking water in the pipeline brings about changes in water quality (Zlatanović, et al., 2017).

The drinking water studied in this project is produced by Sydvatten AB, which for this drinking water uses Bolmen Lake water as the source. The drinking water treatment plant Ringsjöverket receives the source water by 82 km tunnel and supplemented by a small portion

of groundwater. The water is treated by ferric chloride flocculation, sedimentation, fast sand filtration, slow sand filtration and hypochlorite disinfection, in the end, the DWTP produces 1300 L/sec of drinking water, which is distributed and transported to the recipients (Chan, et al., 2018). The distance obtained by Google Maps between the drinking water treatment plant and school 1 (primary school in Stehag) is 1.2 km, while the distance between the drinking water treatment plant and school 2 (primary school in Södra Sandby) is 23.4 km. The distribution net is more extensive and the drinking water to Södra Sandby passes over the city of Lund.

2.1.3 Flow cytometry

Prompt detection of microbial cells is a task that merits investigation in the microbiology sphere. In 1934, Moldavan (Moldavan, 1934) was the first person who conceived automatic cell detection; In 1936, Caspersson et al. (Signer, et al., 1938) proposed a monochromatic UV microscope; The first fluorescent antibody label was exploited and applied by Coons in 1941 (Childs, 2014). During decades of development, flow cytometry has been improved to become an irreplaceable important tool in the field of analytical cytology. Therefore, flow cytometry has applied in multiple disciplines as immunology and molecular biology, etc. (McKinnon, 2018), due to its rapid, accurate and easy to be operated features. Particularly, FCM has become a general assessment modus for the bacterial community in drinking water treatment (Hammes & Egli, 2010).

Flow cytometry (FCM) is a technique that analyses individual particles or cells by suspending them in a buffer while passing single or multiple lasers (McKinnon, 2018). The flow cytometer is mainly

composed of four parts: fluidic system, optical system, electronic system and analysis system. As shown in Figure 2. 1, the suspended samples are pumped by gas pressure and then enter the flow chamber. The sheath fluid is ejected under high pressure into the fluid system at an angle with the detected cells, thus the sheath fluid can envelop the cells at a high speed and become a circular stream. The tested cells are separated into a single row to pass the flow cytometer. Usually, the laser is used as the excited light source and the beam of laser illuminates the cell stream vertically. The forward scatter (FSC) is used to reflect the size of the cell volume and the signal intensity of the side scatter (SSC) can reflect the complexity of the particles. The computer collects various measured signals and converts them to display, as well as analyze the results.

The combination between FCM and fluorescent stains has been used as a technique to monitor drinking water microbiology (Santos, et al., 2019; Dlusskaya, et al., 2019). The SYBR Green I and propidium iodide were used in this project, and the approach followed the previous study (Chan, et al., 2018). The SYBR Green I (SG) is a type of nucleic acid stain, which can be excited by blue light ($\lambda_{max} = 497\ nm$) and emit green light ($\lambda_{max} = 520\ nm$). The SG binds to double-stranded DNA preferentially, thus can be used to analyses total cell count (Lebaron, et al., 1998). Propidium iodide (PI) is a fluorescent intercalator, it absorbs blue-green light ($\lambda_{max} = 493\ nm$) and emits red light ($\lambda_{max} = 636\ nm$). PI crosses damaged bacterial membranes, but it is unable to enter the membranes with full integrity. Hence, it is a way to distinguish intact cells and damaged cells, or live and dead cells (Rosenberg, et al., 2019).

It can be observed that distinct signal intensity differences between SSC signals and fluorescence. As it in Figure 2. 2, the bacterial community can be divided into two groups consequently as HNA

(high nucleic acid content) and LNA (low nucleic acid content) (Santos, et al., 2019). This microbiological characterization has become a standard method for identifying microbial community "fingerprints" (Berney, et al., 2008; Roy, et al., 2012).

The previous studies revealed the application of using FCM to analyze water. The existences of high DNA content HDNA (HNA) and low DNA content LDNA (LNA) were demonstrated by Gasol, which were detected with SYBR Green I and FCM (Gasol, et al., 1999). The FCM was used to assess the bacteria viability in freshwater, it's found that SYBR Green- PI stains were suitable for this measurement (Berney, et al., 2007). A method of combining the fluorescent stains and FCM was proposed to detect microorganisms in water samples (Hammes & Egli, 2005). The SYBR Green I together with FCM were used to analyses the total cell concentration in drinking water samples during water treatment and distribution (Hammes, et al., 2008). It's shown that the combination of FCM and fluorescent stains is a rapid way to assess the microbial viability in drinking water (Berney, et al., 2008). Prest et al. promoted a method for characterizing the water samples with the combination of fluorescence fingerprint and bacterial cell concentration (Prest, et al., 2013).

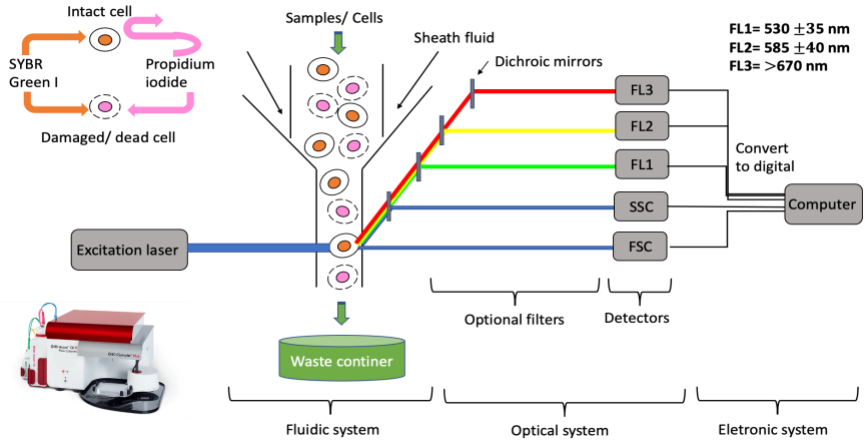
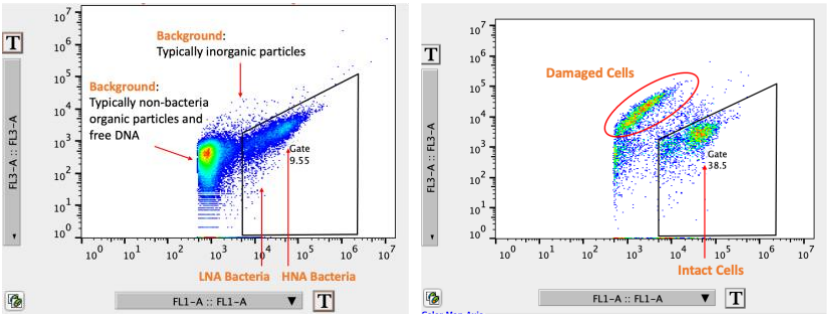


Figure 2. 1 Schematic illustration of flow cytometry: cells flow into the flow cytometer individually while suspended, and then passes through laser vertical irradiation. Multiple fluorescence detectors are in the optical system. SG stains both intact and damaged cells, PI stains only damaged cells. (FL, fluorescence; SSC, side scatter; FSC, forward scatter)



(a). The scatter plot with only SG: the background, LNA and HNA bacteria areas were labelled above.

(b). The scatter plot with SGPI: inside the gate were intact cells, and damaged cells were circled outside of the gate.

Figure 2. 2 The illustration of cells analysis template.

2.2 Approach and Methodology

All water samples were collected from two buildings. One building is located near the drinking water treatment plant Ringsjöverket, and the other building is located downstream of the drinking water treatment plant. The water samples were stored on ice for transportation and analyzed at Applied Microbiology at Kemicentrum in Lund University.

The purpose is addressed by sampling tap water from March to April (a total of 8 weeks) 2021. Samples were taken from three locations in each of two public buildings (primary schools) each week, except for the Easter break. The most commonly used tap in the building was sampled twice, the first collection was to take the water in the building, and the water was taken for the second collection was used as the distribution sample (the water in the distribution pipes), which needed to flush the tap for 15- 20 minutes after the first collection. Since how the distribution system affects the water quality was one of the objectives. There are a total of 68 samples for the entire project. 2.5 L (1 L for filtration for DNA sequencing, 1 L for microbiology test, 250 mL for chemicals test, 50 mL for metals test, and 10 mL for flow cytometer) were sampled for each tap. One extra sampling was carried out the first week after the break to capture the possible stagnation in both recipients. The water samples were filtered onto a 0.22 μm filter and stored at -20 ℃ for DNA sequencing in future projects.

The statistical analysis will be applied to all data to describe the relationship between variables such as "total cell count", "intact cell count" and "distance from water treatment plant", etc. The microorganisms will be collected by filtration and archived for DNA sequencing.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals and Reagents

The following chemicals and reagents were used in the study:

- ◊ Pentahydrate sodium thiosulphate (Stock solution is 20 g/L sodium thiosulphate)
- ◊ DMSO: Dimethyl sulfoxide (Sigma- Aldrich, D8418-100 mL)
- ◊ SYBR™ Green I nucleic acid gel stain (Thermo Fischer Scientific, 1000× concentrate in DMSO)
- ◊ PI: Propidium iodide (Sigma- Aldrich, P4864-10 mL, 1 $mg/\ mL$)
- ◊ Sodium hypochlorite solution (6-14% active chlorine)
- ◊ Sheath solution (BD Accuri™ Bacteriostatic Concentrate Solution mixed with MQ water)
- ◊ BD Accuri™ cleaning solution concentrate
- ◊ Sodium hypochlorite solution (6-14% active chlorine)
- ◊ 8 peak and 6 peak beads for FCM quality check
- ◊ Drinking water samples
- ◊ Ethanol (99.5%)
- ◊ MilliQ water

3.1.2 Equipments

The following equipments were used in the study:

- ◊ Glass bottles (2 L, 500 mL)
- ◊ Falcon tubes (15 mL)
- ◊ Microbiology test bottles (500 mL, from Eurofins)

- ◊ Chemical test bottles (250 mL, from Eurofins)
- ◊ Metal test tubes (50 mL, from Eurofins)
- ◊ Cooling bags
- ◊ Ice bags
- ◊ Temperature meter
- ◊ Filtering glass equipment (a vacuum suction for an e-piston that catches eluate, a filter holder, a funnel where water is poured and a clamp)
- ◊ Isopore membrane filter (0.2 μm pore size, hydrophilic polycarbonate membrane, 47 mm diameter, non-sterile)
- ◊ Sterile petri dish
- ◊ BD Accuri C6 flow cytometer
- ◊ Vortex mixer
- ◊ Eppendorf tubes (2 mL)
- ◊ Pipettes

3.2 Methods

3.2.1 Sampling

The sampling was carried out every week in March and April 2021, except for the first week in April which was the Easter break. The plan was to sample in school 1 every Tuesday and school 2 every Thursday as early as possible, thus the morning water samples could be caught before human activities. 8:30 am was the standard time for sampling. One extra sampling in school 2 was operated the first Tuesday after the Easter break since the stagnation of water would be observed this way. The sampling positions were shown in Figure 3. 1. The sampling locations' name are listed below:

School 1: Stehagskolan (close to the drinking water treatment plant)

- ◊ 1A: Children's bathroom
- ◊ 1B: Classroom (practical studies)
- ◊ 1C: Teacher's kitchen
- ◊ 1D: Distribution sample (same tap as 1C)

School 2: Byskolan Södra Sandby (further down in the distribution system)

- ◊ 2A: North classroom
- ◊ 2B: Storage room
- ◊ 2C: South classroom
- ◊ 2D: Distribution sample (same tap as 2A)

3.2.1.1 Sampling preparation

1) Four 2 L glass bottles were prepared each time. An extra 500 mL glass bottle was needed in school 1 since the sink in the children's bathroom was too small to fit in the 2 L glass bottle, so the 500 mL bottle was used for pouring over.
2) Bleach solution: Approximately 200 mL sodium hypochlorite solution and 1800 mL MilliQ water (about 10% free chlorine) were poured into one of the 2 L bottles, and then separated it into all the glass bottles.
3) The bottles were shook completely and let them stand still for 5 minutes, and then repeated this step.
4) The bleach solution was poured out and rinsed all the bottles adequately 5 times with MilliQ water until the chlorine smell was gone.
5) The bottles were labelled with sampling date and location names.
6) Four 15 mL falcon tubes were prepared and 10 µL of 20 g/L sodium thiosulphate was added into each tube.
7) Glass bottles, falcon tubes, cooler bags and temperature meter were packed in the cooling bags.
8) Eurofins bottles: Two microbiology sampling bottles, one chemical sampling bottle and one metal sampling tube were needed in each location. They were labelled with sampling date and location names and packed in one box.

3.2.1.2 Sampling procedure

1) Medium (normal) flow of tap water was used in this project. This was important since the closer to the reality, the better.
2) First, hands were sprayed with hygiene alcohol and measures to avoid touching the mouth of the bottles taken.

3) The 2 L glass bottle was filled with the very first water from the tap, as full as possible.
4) Thereafter, the chemical bottle was filled completely, up to the mouth. That was more than 250 mL.
5) Finally, the metal tube was filled completely, maximum up to the mouth.
6) The temperature was measured before turning off the tap for about one minute until the number was stable.
7) The water was poured from the 2 L bottle into the microbiology test bottles. The microbiology bottle was filled up to the mark 500 mL (which was about 90% of the entire volume of the bottle. It was important that the bottle was not completely filled).
8) The water was poured from the 2 L bottle into falcon tube to about 10 mL.
   The tube contained 10 μL of 20 g/L sodium thiosulphate which was important to keep inside.
9) When all 5 types of bottles were full, caps were closed properly and the bottles placed in a cooler bag before transporting them to the laboratory.
10) For taking the distribution system sample, one of the taps needed to be running for 15- 20 minutes before sampling. For this sample, the temperature was measured before taking water, which was to make sure the temperature was stable, since a stable temperature indicated the fresh water from the main pipes where it had not been affected by building temperature.

During this pandemic, the sampling work was not easy to carry out according to the restrictions in schools. In school 1, the samples were taken by me, while in school 2 the 2 L bottle, chemical test bottles and

metal test bottle were filled up by the science teacher in the school, who was sufficiently communicated with us in advance, and myself was in charge of pouring over the water from the 2 *L* bottles.

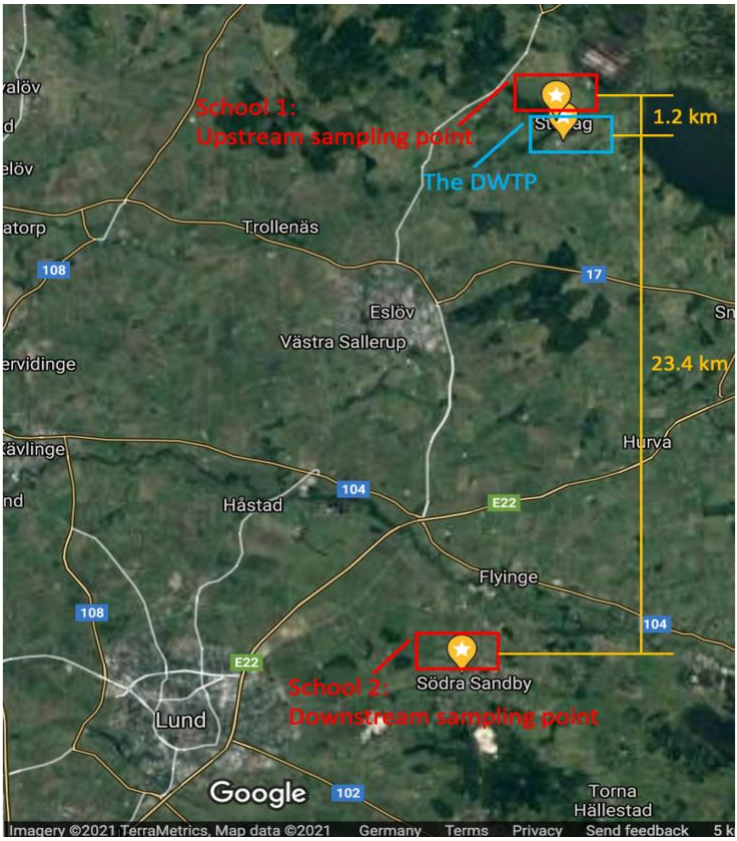


Figure 3. 1 The map of the DWTP and two sampling places. School 1 is the primary school in Stehag, and School 2 is the primary school in Södra Sandby. The distance is the linear distance. The actual pipes pass over the city of Lund. (Map generated from Google Map)

3.2.2 Flow cytometry

All reagents must be checked before starting the Accuri C6: the FACS (the basic flow cytometry staining buffer) clean solution, sheath solution and decontamination solution should be enough for the upcoming procedures. If the waste tank was almost full, the water liquid had to be poured out and autoclaved afterwards.

3.2.2.1 Quality check

The quality check was fundamental before the formal FCM procedure could start. According to the handbook, MQ, clean solution, decontamination solution, MQ, 8 peak beads, 6 peak beads and MQ should be placed properly from A1 to A6. Thereafter the quality check template was run. This step took about 10-15 minutes if the beads were mixed well. Statistics was checked after the running was done. If the required fluorescence detectors failed the quality check, the new reagents were suggested to make to solve this problem.

3.2.2.2 Preparation of the samples

SG stock solution was 1000 × concentrate, and the 100 × concentrate solution was made by adding 495 μ***L*** DMSO into 5 μ***L*** SYBR Green, and the PI solution was made by adding 105 μ***L*** SG solution (100×) into 21 μ***L*** Propidium iodide. The solutions were vortexed right after the previous step and had to be covered by tin foil, since they are sensitive to light.

3.2.2.3 Water sample standard procedure

As can be seen in Figure 3. 2, 24 Eppendorf tubes were used for 4 duplicate samples, “S” was referred to 495 μ*L* water sample plus 5 μ*L* SG solution, and “P” was referred to 494 μ*L* water sample plus 6 μ*L*

PI solution. In the MQ, there was 500 µL MilliQ water. Since there were four different samples in each sampling, sample "1- 4" were named here to represent positions "1A- 1D", or positions "2A- 2D".

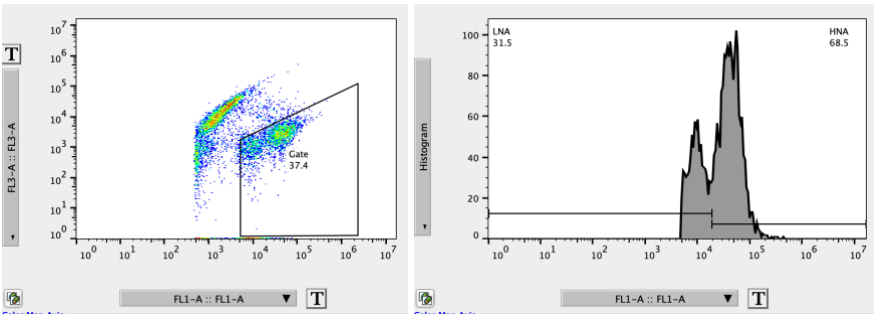
After the mixing of samples were performed, they were vortexed and incubated in 37 ℃ incubator for 15 minutes.

The sample names were written down on the computer of FCM and the settings were changed for all the samples as below:

- Run limits: 50 µL, tick the box on the left
- Fluidics: Medium (flow rate: 35 µ$L/\ min$; core size: 16 µm)
- Set threshold: FL1-H, less than 500
- Agitate plate: None, every 1 well

After 15 minutes of incubation, all samples were vortexed again, the lids were cut and placed correctly in the FCM rack. Then, auto run display was opened.

Figure 3. 2 The structure of duplicate samples for flow cytometry (S1, sample 1 with SYBR Green; P1, sample 1 with Propidium iodide; MQ, MilliQ water, etc.)



(a). The scatter plot: the gate need to include the target cells and exclude the background and noise.

(b). The histogram plot: LNA and HNA will be automatically performed, the gate was adjusted to fit all the samples.

Figure 3. 3 The gate setting strategy. Same gating was applied to all the samples.

3.2.3 Gate Strategy

The flow cytometry data analysis is based on gating. A gate is a graphical boundary, which can be used to separate the cell population from particles for further analysis (Reynolds, 2020). The markers that can be visualized by fluorescence are the premises of the gating strategy. Specifically, in this project, the function of the gate is to select the cells and exclude the background and noise, even separate the live cells and membrane-damaged cells. As well as the %HNA and %LNA are generated. All the samples were performed with the same gate in this project. Gate strategy example in Figure 3. 3.

It's considered that gate testing is preferable since the boundary of the gate is unconvincing by the sense of sight. The humic acid and the non-pathogenic *E. coli* were used to ensure the HNA and LNA were separated properly, and the dead non-pathogenic *E. coli* was used to check if the membrane-damaged cells were excluded properly. The bacterial spiking was to pick one colony of the non-pathogenic *E. coli* in the microbiology lab at Applied Microbiology and dissolve it into 500 mL MQ as the live bacteria. And the same step was repeated, then put the bacterial solution at 80 ℃ for 80 minutes to inactivate. The FCM procedure was the same as before, therefore, instead of using 495 µL water sample, 20 µL humic acid/ bacterial solution was combined with 475 µL water sample here. Interestingly, after the gating testing, it's found that the gate was too tall so there were 2 different clusters in the SG staining dot plot, thus the gate was adjusted to exclude the background cluster.

3.2.4 Water filtration

The drinking water samples were stored on ice before entering the lab, it was ideal to filter them as soon as possible.

In order to use the clean filtration equipment, the 10% bleach solution (10% sodium hypochlorite, 90% MQ water was made for soaking the funnel and the filter holder for 10- 15 minutes before start. The equipment was rinsed with MQ water until the bleach smell was gone. 99.5% ethanol was used to clean the blench and 4 sterile plastic petri dishes were prepared for storing the filter paper afterwards, which were labelled with sampling dates and location names. The equipment was set up as Figure 3. 4 and the filter paper was put on the filter holder, some MQ water was poured in the funnel, the pressure button was twisted to start and make sure there was no leakage. It was essential to rinse the funnel and the filter holder and the water sample with MQ water before filtering each sample. 1 L of each sample was filtered in this project. All the filter papers were kept in the sealed petri dishes and put in the -20 ℃ freezer.

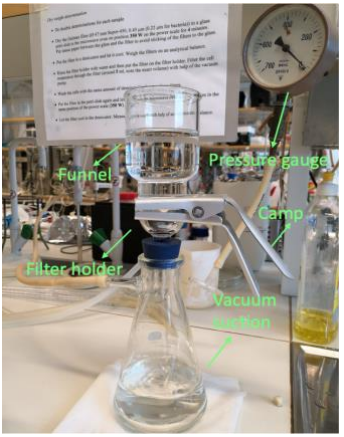


Figure 3. 4 The filtering equipment

3.2.5 Routine microbiology, chemicals and metals analysis

Routine water quality analysis (Table 3. 1) was performed by Eurofins company. For each sample, two microbiology bottles, one of the chemical bottle and one of the metal tube were taken. Thus, there were 12 bottles and 4 tubes packed in the cooler box each time, and they were delivered to Eurofins package dropping place the same day as soon as possible.

Table 3. 1 The Eurofins tests list

Microbiology	**Chemical**	**Metal**	**Others**
Micro fungus	Ammonium nitrogen (NH_4-N)	Manganese Mn (after acidified)	Odor, strength, at 20 ° C
Cultivable microorganisms 22 ° C	Nitrate nitrogen (NO_3-N)	Magnesium Mg (after acidified)	Odor, species, at 20 ° C
Slow-growing bacteria	Alkalinity	Calcium Ca (after acidified)	pH
Intestinal enterococci	Nitrite nitrogen (NO_2-N)	Sodium Na (after acidified)	Taste, strength, at 20 ° C
Coliform bacteria 35 ° C	Fluoride	Potassium K (after acidified)	Taste, species, at 20 ° C
Escherichia coli	Sulphate	Aluminum Al (after acidified)	Conductivity
Mold	Phosphate phosphorus (PO_4-P)	Iron Fe (after acidified)	Total hardness (° dH)
Yeast	Phosphate (PO_4)	Color (410 nm)	Turbidity
Actinomycetes	Nitrite (NO_2)		
Presumptive Clostridium perfringens	Nitrate (NO_3)		
	NO_3/ 50 + NO_2/ 0.5		
	Ammonium		
	Chloride		
	TOC		
	COD-Mn		

4 Results

The sampling was carried out in March and April in 2021, from week 1 to week 4, and then from week 6 to week 9, week 5 in between was the Easter holiday. All samples were analyzed by FCM and compared with other water quality parameters.

The two schools were labelled as school 1 (school in Stehag, closer to the DWTP) and 2 (school in Södra Sandby), each of which had A, B, C and D positions of taps. To simulate the water in the distribution pipes, the most often used taps were chosen to be regarded as distribution samples by flushing the tap for 15- 20 minutes. To be specific, "1D" is the distribution sample from the same tap with "1C" and "2D" is the distribution sample from the same tap as "2A".

In week 6, sampling was performed twice at school 1, since Thursday was the routine sampling day at school 1, but the Tuesday in week 6 was the first day after the holiday. Thus, the first sampling was on Tuesday to catch the stagnation of the building, which was labelled as "Week 6_A" and the second sampling was the routine sampling, which was labelled as "Week 6_B". Besides, the Eurofins data have all been analyzed, although only the cultivatable microorganisms (3 days at 22 ℃) and slow-growing bacteria (7 days) are shown in graphs below since the changes were abundant compared to other parameters.

4.1 Total cell count

In Figure 4. 1, the total cell count of all the samples per mL is displayed via a column chart.

The total cell count ascended in week 6, in particular, the increases were sharp in school 2 except for 2D. Generally, the TCC was between 100,000 $cells/mL$ to 250,000 $cells/mL$. However, it's worth mentioning that TCC in week 6 was 40% and 120% higher than the average of week 1 to 4 in school 1 and 2 respectively. Afterwards, the values were back to the normal range with fluctuations. It can be seen that after using the taps for four weeks (week 6- 9), the TCC of samples still had higher numbers than before (week 1- 4). To be specific, if referred to the raw data of the cell count, the average of the cell counts was larger after Easter, specifically, the average TCC from week 7- 9 compared to values from week 1- 4 was 44% and 32% higher in school 1 and school 2, independently. Besides, except for week 6, the TCC in school 1 was higher than the values in school 2 most of the time for all the taps, except for 2A. Specifically, in school 1, the 1A and 1B had more cell count than 1C and 1D, furthermore, 1D had the lowest TCC in the sampled water. As for school 2, 2D had always the lowest number of TCC when 2A had the highest TCC in the water during the entire sampling period. In particular, the TCC of building samples were 7% and 25% higher than distribution samples in school 1 and school 2 on average.

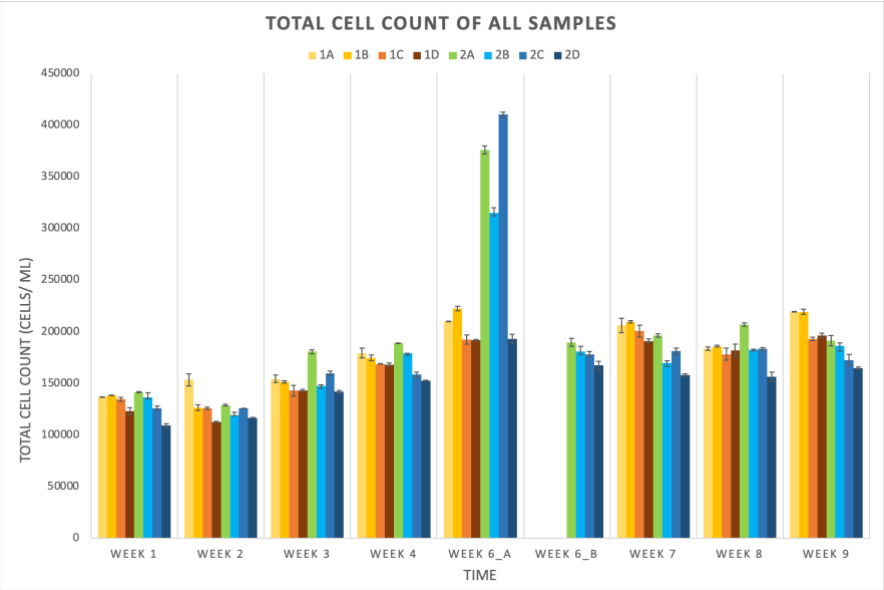


Figure 4. 1 Total cell count of all samples. The error bars were obtained based on the standard deviation of the duplicates. The warm-toned colors were used for positions in school 1, while the cold-toned colors were chosen for positions in school 2.

4.2 Intact cell count

The intact cell count of all samples per mL was applied by a bar chart, and the color of each position was the same as it in the TCC figure. It's needed to clarify that the error bar in form of the value of the standard deviation of 2C in week 2 was absent since the duplicate value was too low to be trusted, so only one value of the duplicates was shown.

ICC had smaller order of magnitude than TCC, which are regularly between 10,000 $cells/mL$ to 100,000 $cells/mL$ (Figure 4. 2). Moreover, there was a dramatic rise at school 2 in week 6, the ICC went up to almost 250,000 $cells/mL$. The ICC in week 6 was 47% and 260% higher than the average of week 1 to 4 in school 1 and school 2, respectively. Hereafter, the average ICC from week 7- 9 compared to it from week 1- 4 was 57% and 33% higher after week 5 in school 1 and 2. It is apparent that school 2 had a higher number of ICC every time, which was 240% higher on average indeed, excluding week 6. The ICC at 2A had the highest value, except for 2C occupied the peak in week 6. Same as the trend found in TCC, the distribution samples always had the lowest ICC. Particularly, the building samples were 40% and 61% higher than the distribution samples on average in school 1 and school 2, respectively.

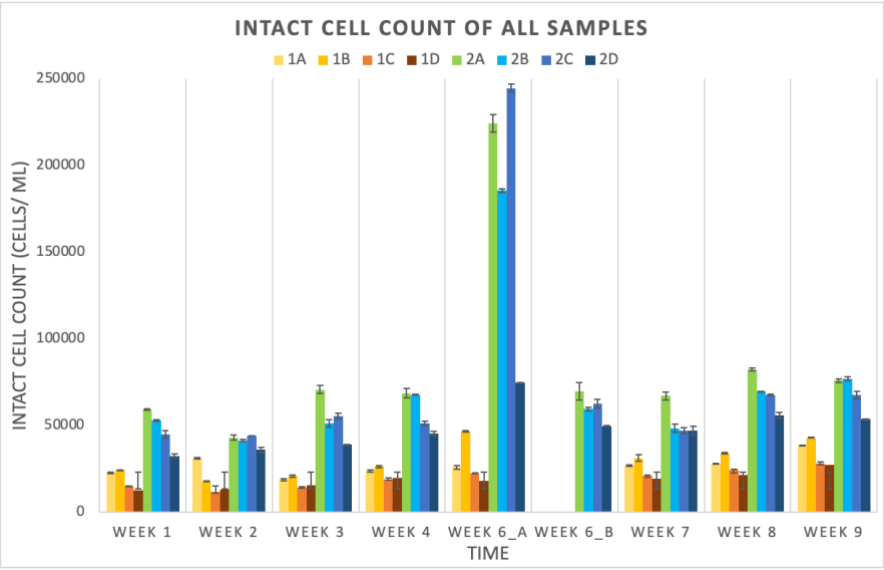


Figure 4. 2 Intact cell count of all samples. The error bars were obtained based on the standard deviation of the duplicates. The warm-toned colors were used for positions in school 1, while the cold-toned colors were chosen for positions in school 2.

4.3 Distribution samples (TCC& ICC)

The distribution samples were analyzed separately based on TCC and ICC combined with sampling temperature of the water.

The cell count fluctuated, in addition, the TCC and ICC of school 2 both had a peak in week 6, as well as the sampling temperature. The ICC of school 1 didn't have a noticeable trend, while the TCC of school 1 increased slightly from week 2 to 6, then descended after. The temperature curve has shown a similar pattern with TCC changes. As is shown in Figure 4. 3, the temperature undulated as the cell count distribution samples. For example, the temperature in school 1 went up in week 6 and kept climbing afterwards, as well as the TCC in school 1. In school 2, the peak in week 6 can be discovered in TCC, ICC and temperature. As is markedly shown in Figure 4. 3, school 2 had higher ICC numbers than school 1, yet the TCC values were close in both schools. Additionally, the numbers of the percentage of ICC (ICC over TCC) were performed, the highest %ICC of school 2 was 39% in week 6 and the highest %ICC of school 1 was 14% in week 9.

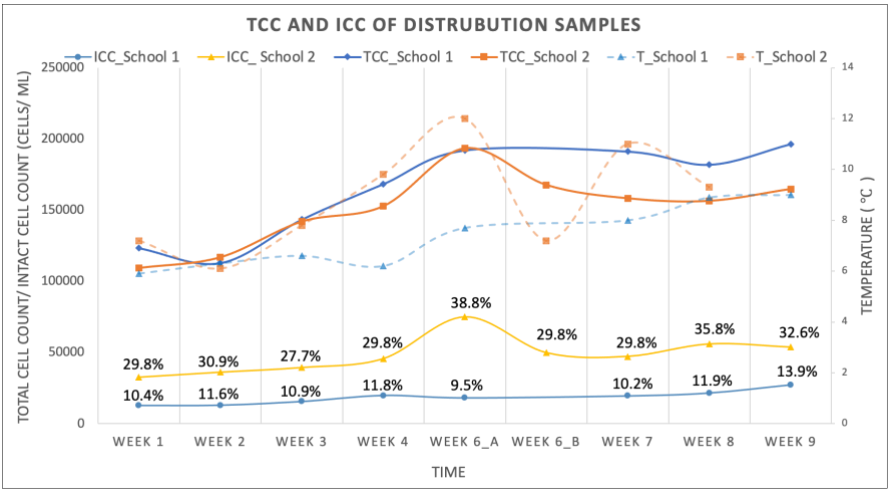


Figure 4. 3 The total cell count and intact cell count of distribution sample. The imaginary lines were the sampling temperature. The percentage above the ICC points were the %ICC (ICC/ TCC). (The light blue, yellow, dark blue and orange curves were ICC of school 1 and school 2, TCC of school 1 and school 2, respectively).

4.4 TCC vs %HNA for TCC

The dot plot (Figure 4. 4) shows the TCC (per mL) vs %HNA for TCC (per mL), for all the samples the color for each position was the same as in previous graphs.

The TCC was within the range of 100,000 to 250,000 $cells/mL$ for most samples, while three dots on the top right were between 300,000 to 450,000 $cells/mL$ (Figure 4. 4). The dots were quite clustered together, nonetheless, some trends can be discovered. The samples in school 1 had higher %HNA for TCC than school 2. If calculated the average of %HNA for TCC (excluding the values in week 6) for each school, the number of school 1 was 5% higher than school 2. The %HNA of TCC were 6% and 5% higher after stagnation in school and school 2, respectively. The three dots on the top right were the samples taken after 7 days of stagnation, of which the TCC and %HNA for TCC were higher in comparison with others.

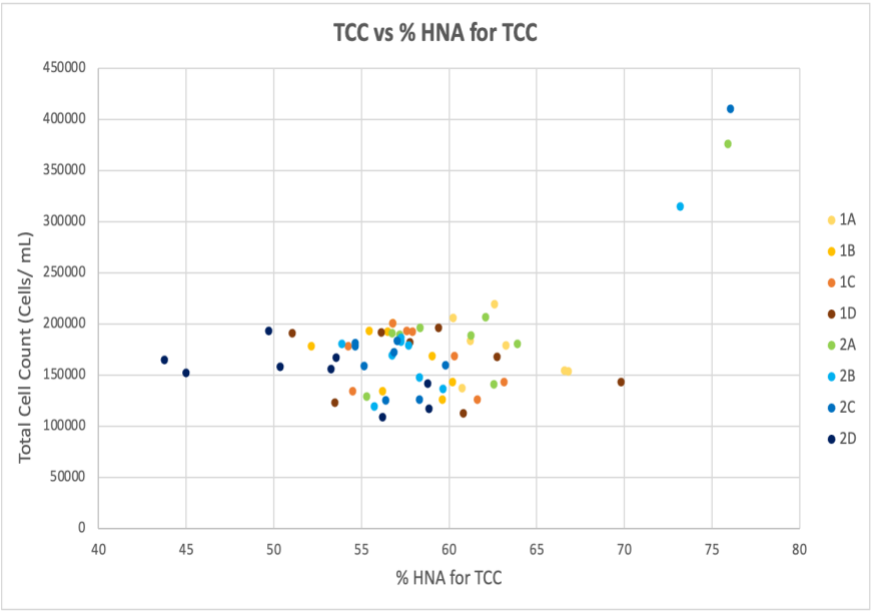


Figure 4. 4 Total cell count vs %HNA for total cell count. The average of the duplicates of all samples were applied.

4.5 ICC vs %HNA for ICC

The dot plot shows the ICC (per mL) vs %HNA for ICC (per mL) , all the samples were contained. The color for each position was the same as in previous graphs.

As is shown in Figure 4. 5, the ICC was within the range of 500 to 10,000 cells/ mL, apart from three dots on the top right. Also, the %HNA for ICC was in the range from 30% to 95%. It's distinctly plotted that the samples in school 2 had higher ICC than those in school 1. Additionally, the samples in school 1 had higher %HNA for ICC by comparison with school 2, which was 25% higher if calculated the average number of all the samples except for samples in week 6. More than half of the samples in school 1 had very high %HNA, which were up to 80% to 90%. The %HNA of ICC were 2% and 21% higher after stagnation in school and school 2, respectively. In both schools, distribution samples had lower ICC as well as %HNA for ICC. The three dots on the top right were the samples taken after 7 days of stagnation, of which the ICC number was significantly high.

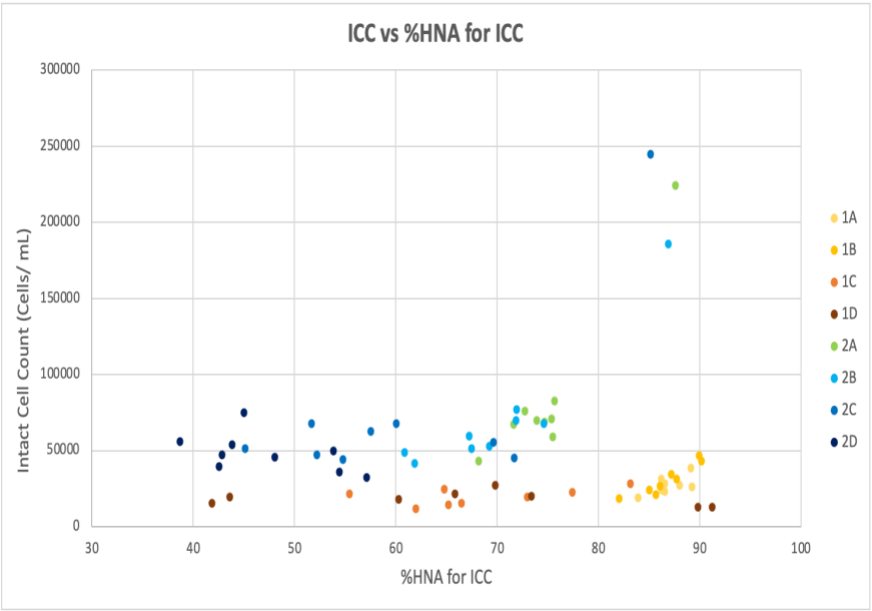


Figure 4. 5 Intact cell count vs %HNA for intact cell count. The average of the duplicates of all samples were applied.

4.6 Fingerprints

The flow cytometry fingerprints of all the samples with SG staining are presented below. It can be seen that similar trends discovered above were also found here.

In Figure 4. 6 and Figure 4. 7, the shapes of the samples from the same tap had similarity, although the samples in week 6 were different and had larger graphic areas, which was more obvious in Figure 4. 7. In addition, as is shown in Figure 4. 6, 1A had the highest percentage of HNA while others had the similar numbers. The fingerprints of the distribution samples in school 1 (1D) had no significant difference compared to others, while the fingerprints of the distribution samples in school 2 (2D) were quite different. The “week 6_1” in Figure 4. 7 was the sampling after the Easter holiday, and it can also be illustrated that the distribution sample had the lowest number of %HNA for TCC. Additionally, the %HNA for TCC was decreasing afterwards.

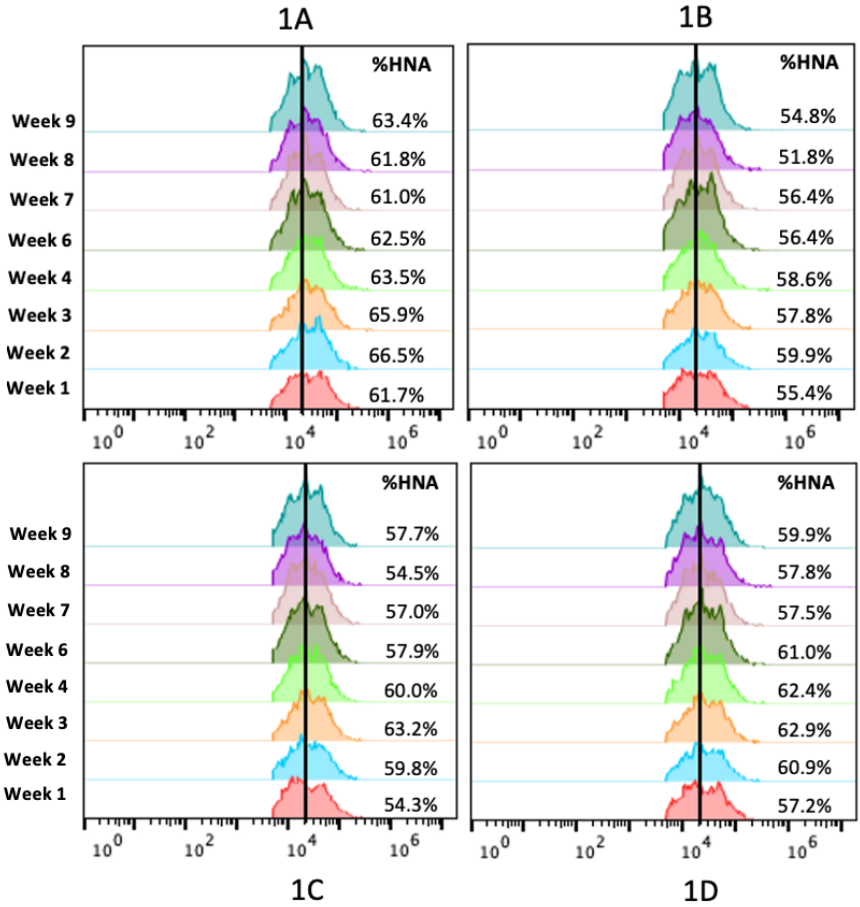


Figure 4. 6 Fingerprints of 1A to 1D with SG staining, the %HNA has been extracted from raw data and written on the plots. The vertical line separated LNA and HNA. (From left to right, they were 1A to 1D, respectively)

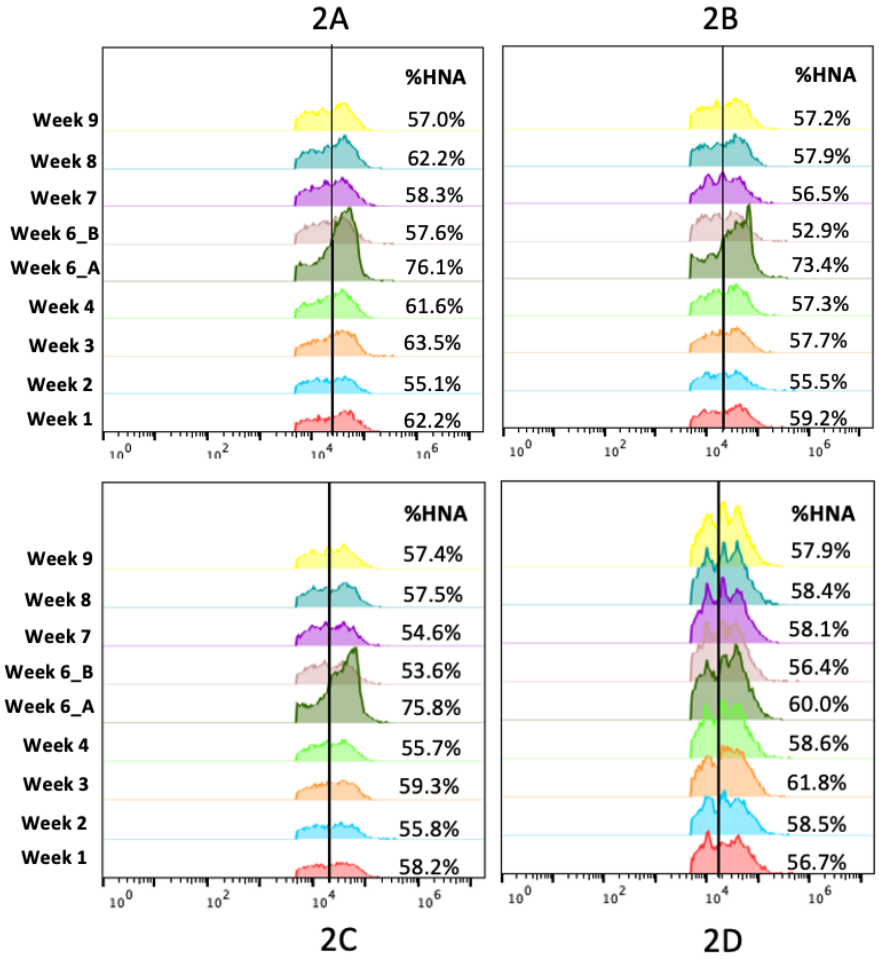


Figure 4. 7 Fingerprints of 2A to 2D with SGPI staining, the %HNA has been extracted from raw data and written on the plots. The vertical line separated LNA and HNA. There were 2 data from week 6 in school 2. (From left to right, they were 2A to 2D, respectively)

4.7 Temperature

The sampling temperatures are shown in bar chart (Figure 4. 8). It can be seen that the water temperature in school 2 was mostly higher than school 1, at least for tap 2A and 2B. Overall, the temperatures in school 1 were close, although the temperature in school 1 sightly increased after stagnation. In most time, except for week 2, the temperature of 2D was lower than 2C after flushing the tap water. And the temperature in school 2 almost stayed at the same level, while 2C and 2D increased at week 6. 2A and 2B had always the highest temperature, 2D had the lowest number comparably. Same as school 1, 2D had lower temperature than 2A after flushing.

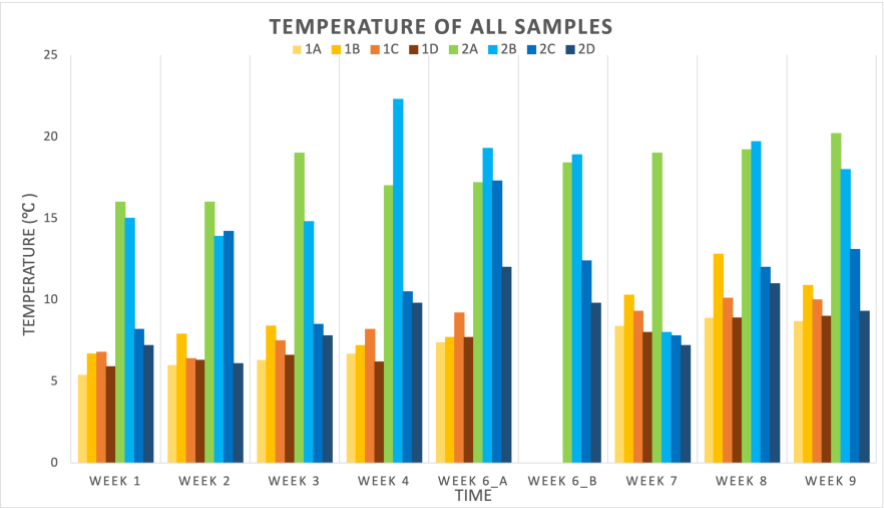


Figure 4. 8 The sampling temperature of all the samples.

4.8 Data from Eurofins

The cultivable microorganisms were cultivated under 22 ℃ for 3 days, and the slow-growing bacteria were cultivated for 7 days at the same temperature. These parameters are parts of the regular drinking water quality check in Sweden. The plots were obtained by using the same color for each position as in previous figures. On week 6, twice of the sampling were carried out at school 2, the first one was supposed to catch the stagnation of the water in the building and the second one was the routine sampling day. Raw data are available in Appendix Table 7. 1 and Table 7. 2.

4.8.1 Microbiology data

4.8.1.1 Cultivable microorganisms (3 Days)

According to Swedish Food Agency, the number of cultivable microorganisms is preferably under 100 $cfu/\ mL$ (Livsmedelsverket, 2020). Except for 1A in week 4, all of the counts of the colonies were less than 100 $cfu/\ mL$ (Figure 4. 9). The dramatic growth was before the Easter holiday (week 5), which was different from FCM data since the crucial changes happened after the Easter holiday. Tap 1A and 2A always had higher numbers than other taps, and the distribution samples had the lowest values.

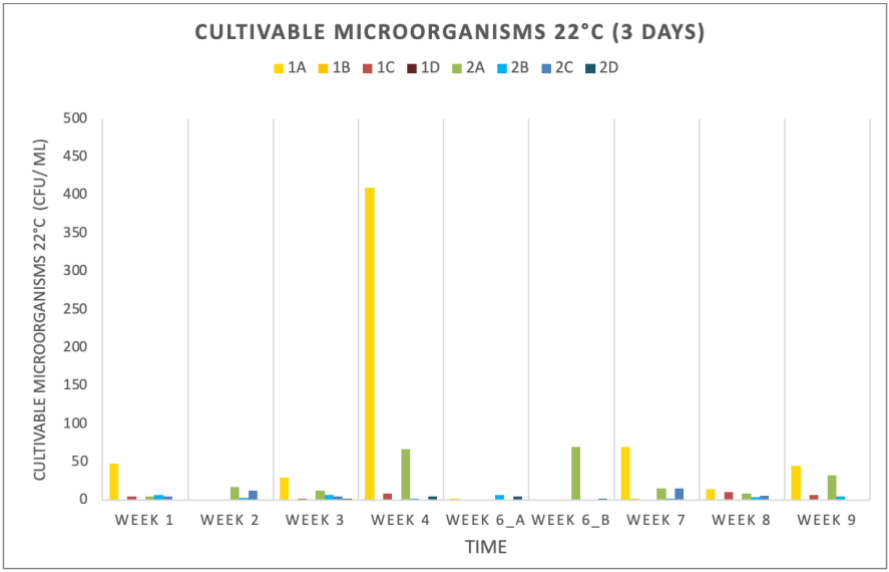


Figure 4. 9 The cultivable microorganisms (3 days of cultivation) in all samples.

4.8.1.2 Slow-growing bacteria (7 Days)

The limited number of the slow-growing bacteria cultivated for 7 days is 5000 $cfu/\ mL$ (Livsmedelsverket, 2020). Apparently, all of the samples were within this range, and most of which were even below 200 $cfu/\ mL$. Same as the 3 days cultivation microorganisms, it's exhibited in Figure 4. 10 that the immense raise was at week 4, particularly at 1A and 2A, which had higher number of colonies than others. After the stagnation, the numbers of slow growing bacteria decreased. In school 2, 2A and 2D were from the same tap and it can be demonstrated that after running the water for 15 to 20 minutes, the bacteria had a clear reduction.

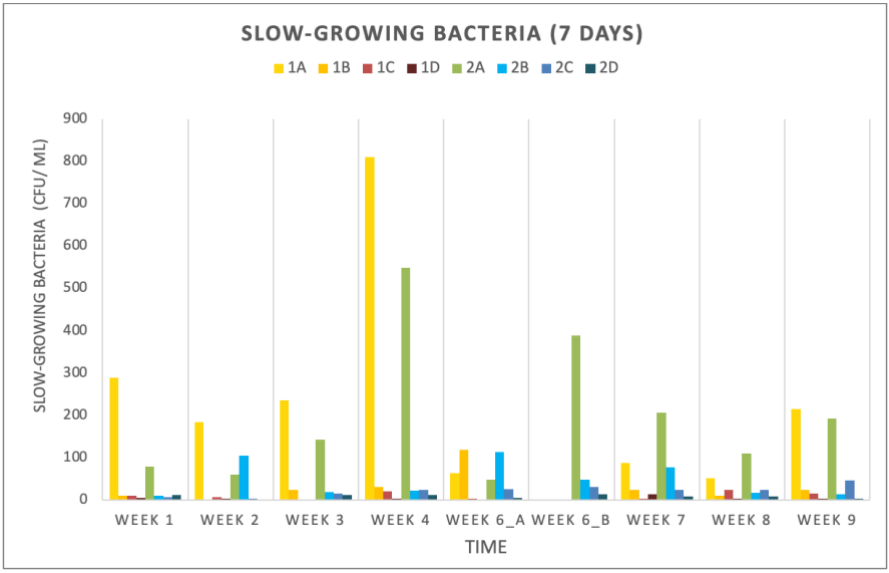


Figure 4. 10 The slow-growing bacteria (7 days of cultivation) in all samples.

4.8.2 Other parameters

Multiple parameters were analyzed based on the standard procedure. Although, there was no obvious rebound among all the samples over time. Moreover, the values of some parameters were lower than the detection limit, thus only a few numbers were extracted below.

4.8.2.1 Chemical data

More than half of the chemical parameters had values lower the detection limit, hence, only six parameters were summed up here. Specifically, the chemical oxygen demand (COD)-Mn was within the range of 1- 2 $mgO2/L$, the nitrate-nitrogen ($NO_3 - N$) was present 0.2- 0.3 mg/L, the alkalinity was around 44- 50 mgHCO_3/L, the sulphate was 5- 7 mg/L, the total organic carbon (TOC) was 2.1- 2.5 mg/L and the chloride was 25- 30 mg/L. The changes among different taps weren't found.

4.8.2.2 Metal data

Four of the metal data were summarized, such as Magnesium (Mg) was 1- 2 mg/L. Calcium (Ca) was 20- 25 mg/L. Sodium (Na) was 9- 13 mg/L and Potassium (K) was 1- 1.2 mg/L. The values of all the samples were too close to conclude any patterns.

4.8.2.3 Others

The pH was 7.5- 8.5, the conductivity was 18- 20 mS/m. The total hardness was $<4\ °dH$, which is regarded as soft water. The turbidity was <0.2 FNU (the higher the number, the higher the risk for gastrointestinal disease). The color (410 nm) was $< 5 - 11\ mgPt/L$. And there was no odour and taste in any of the samples.

5 Discussion

As mentioned above, the sampling was operated four weeks before and after Easter each, therefore, the effect of stagnation was one of the objectives to study in this project. Previous studies (Bédard , et al., 2018; Zlatanović, et al., 2017) have proved that stagnation of the drinking water leads to an increase in cells level. After 6 days of stagnation, the cell counts could increase from $10^3\ cells/mL$ to $7.8 \times 10^5\ cells/mL$ (Ling, et al., 2018).There was a striking increase of TCC and ICC in both schools, particularly in school 2. After the stagnation (week 5), there was a higher percentage of HNA bacteria. It was developing as expected, since, after 7 days of stagnation, the drinking water had been standing in the building, in which situation, the water would get more contact with the biofilm in the building pipe and also got warmer. Then, after the routine usage of the tap water, the cell count decreased as the stagnated water was flushed out. Similar results can be seen from the distribution samples which had fewer cell counts compared to the other taps, which was due to the distribution samples were taken after 20 minutes of flushing before sampling of those samples. An interesting study analyzed *legionellosis* cases after the COVID-19 appeared, since water stagnation in buildings leads to *Legionella* growth (Rhoads & Hammes, 2020). Another study indicated that the reduction of water consumption led to longer stagnation time, and longer stagnation time is weakly related with an increase in *Legionella* (Christian, et al., 2020).

The water temperature was monitored in water sources and DWTP, as it is relevant to chemical and microbial impact in the drinking water (Agudelo-Vera, et al., 2020). A previous study (Agudelo-Vera, et al., 2020) indicated that the soil temperature influences the drinking water

temperature effectively. In this case, the pipe underground could be affected. It's illustrated that the elevated water temperature is correlated with the increased bacteria abundance in DWDS (Zhou, et al., 2017). In this study, the temperature had been measured every time at every tap. It was discovered that the temperature changes matched the cell count patterns, which were the school 2 had higher water temperature and higher intact cell count than school 1, as well as distribution samples in both schools had higher cell counts when the temperature increased. Therefore, stagnation effects couldn't be shown in the temperature among all taps in school 2 since the temperature in school 2 was stable, particularly at tap 2A and 2B. This is interesting since the increase in cells can't be explained by temperature changes in the building samples.

In school 2, there was a higher ICC and lower %HNA of the ICC, as well as lower %HNA for TCC. In addition, the distribution samples had lower cell count and lower %HNA for TCC and ICC. It's interesting to see that more than half of the samples in school 1 had very high %HNA of ICC. As the water passes through the DWDS, the percentage of intact cells in the water increases (Chan, et al., 2019). The same trend was observed in this study since the school 2 is further along in the DWTP which had more intact cells. Hydrodynamics is also an essential factor affecting biofilm. The previous study (Prest, et al., 2016) demonstrated that the increase in hydraulics caused by high water consumption or pipeline flushing will cause the separation of biofilms and the resuspension of sediments. At the same time, it's well-known that the pipe diameter in the DWDS will be larger than the pipe diameter in the building, which causes the hydraulic power to be different in these two situations. Therefore, it is assumed that the water in the distribution system will quickly pass through the pipeline so that the contact with the pipeline biofilm is less. As for the water

in the building, the water contacts the biofilm more and repeatedly suspends the sediment due to the thinner pipes. This explains that the sample of the distribution system contained fewer cell counts and cell types.

To conclude the differences between school 1 and school 2, it can be elaborated that the bulk water (taken from taps) in school 2 had more intact cell count and higher %HNA for ICC. This might be the water age was larger in school 2, the bulk water bacteria regrowth in the DWDS or the biofilm bacteria inhabited on the pipes. The taps in the building showed differences in TCC and ICC, the reasons could be their different locations in the building. Unfortunately, the layouts of the pipes in the buildings haven't been extracted, therefore, it could be interesting to find out in the future. In addition, a previous study illustrated that several factors could affect the bacteria community in DWDS, such as seed bacteria, disinfection methods, nutrient composition, pipe materials and hydraulic conditions, etc. (Liu, et al., 2014).

The comparison between FCM data and microbiology data from Eurofins has demonstrated above. The essential difference was that it can be seen the increase of the cell count after week 5 in FCM but not in microbiology data. Furthermore, there was an increase of 3 days and 7 days cultivated bacteria at the week before week 5, and they were the least in week 6. However, it's inappropriate to compare the microorganisms cultivated in a specific medium at a specific temperature with the whole bacteria community in the samples. Since generally, there are 10^3 to 10^6 cfu/mL in the drinking water (D. Hoefel, 2005; Hammes, et al., 2012). In addition, it is possible that the increased cells in the water from stagnation could use up nutrients supporting the heterotrophic bacteria that would be detected by

culturing. That an increase in the abundance of bacteria in the tap water could have an impact on the heterotrophic counts is intriguing.

Coliform bacteria were found from the last two samplings on 20th April and 27th April 2021 at 1A, which is the children's bathroom in school 1. 8 and 21 cfu/mL coliform bacteria were detected respectively. Since more than 10 cfu/mL coliform bacteria are unsuitable in Sweden, the continuously work such as cleaning and flushing the tap were carried out and several weeks of sampling was going on by VASYD to monitor the water quality after cleaning. It was also interesting to note that school 1 had generally higher numbers of slow growing heterotrophs than school 2, which was further in the DWDS.

The extra disinfectant has been added into the drinking water to irreversibly inactivate the microorganisms. Chlorine, for instance, is commonly used for this (Zhou, et al., 2017). The number differences of the residual disinfectants, such as chlorine differences were not found in chemical analysis data, which was expected to be higher in school 1 since it's closer to the DWTP, even though the concentration of the disinfectants was ideally to maintain in the pipes in order to control the formation of the microorganisms. It's indicated that the particle deposition proves attachment position for bacteria and also reduced the concentration of the disinfectant (Simões & Simões, 2013). Therefore, theoretically, due to the accumulation of particles by DWDS, the residual chlorine in school 2 is considered to be lower.

Another detail to be discussed is that after filtering the water, the filter paper in school 1 was more yellow than it was in school 2, also, the distribution sample was the least yellow (Example available in Appendix). It's considered that there was corrosion of the pipes or more organics in school 1, nevertheless, the total organic carbon data

couldn’t explain this since the numbers among all the samples were close with no significant trend.

This project will continue after this thesis, and include a number of sequencing approaches, including the bacteria communities and the relative abundance of the different taxa can be determined. Thus, the types of bacteria and their relative abundance can be obtained, which would be interesting for deeper analysis. For instance, it can be related to the changes due to the water transporting via the distribution system.

As this project was carried out on a special occasion during the COVID-19 pandemic, the sampling plan included a number of restrictions and exceptions. Several changes can be considered in the further similar study. For instance, the sampling is preferable to be taken on the same day at both schools as early as possible, the pouring step (pour water sample from 2L bottles to others) can be done under a more appropriate atmosphere (instead of in the bathroom, kitchen, even windy outside). Also, about the FCM running, the agitate function was not recommended at TMB, more discussion about the machine should be carried out.

6 Conclusions

- ◊ The stagnation effect was investigated in both schools based on the cell count increase after the holiday, especially in school 2. Since the standing water had more contact with biofilm in the pipe.
- ◊ The water temperature/ season change was relevant to the bacterial communities. Higher the temperature/ warmer the weather results in a higher cell content in the distribution samples.
- ◊ The intact cell count was higher in school 2 compared to school 1. The %HNA was higher in school 1 compared to school 2. The reasons could be the water age differences, the disinfection strategy and pipe materials, etc.
- ◊ The distribution samples had lower cell count and fewer bacterial types compared to other samples.
- ◊ Slow growing bacteria decreased after stagnation, which might be the increased cells used up nutrients supporting the heterotrophic bacteria.
- ◊ Overall, the water quality was good in both schools.

7 Appendix

Figure 7. 1 The example of the filter papers. On the left, they were 1A and 1D from left to right. On the right, it was 2A.

Table 7. 1 The raw data of the cultivable microorganisms 22°C (cfu/ mL)

Cultivable microorganisms 22°C (3 days cultivation) (cfu/ mL)									
Week	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	2021/3/2	**2021/3/9**	**2021/3/16**	**2021/3/23**		**2021/4/6**	**2021/4/13**	**2021/4/20**	**2021/4/27**
1A	48	0	30	410		2	70	15	45
1B	1	0	1	0		1	2	0	1
1C	5	0	2	9	**7-day**	0	0	11	7
1D	1	0	0	1	**stagnation**	0	1	0	0
	2021/3/4	**2021/3/12**	**2021/3/18**	**2021/3/25**		**2021/4/6**	**2021/4/8**	**2021/4/15**	**2021/4/22**	**2021/4/29**
2A	5	18	13	67		0	70	16	9	33
2B	7	3	7	2		7	0	2	4	5
2C	5	13	5	1		0	0	16	6	0
2D	0	1	2	5		5	2	0	1	0

Table 7. 2 The raw data of the slow-growing bacteria (cfu/ mL)

Slow-growing bacteria (7 days cultivation) (cfu/ mL)									
Week	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	2021/3/2	**2021/3/9**	**2021/3/16**	**2021/3/23**		**2021/4/6**	**2021/4/13**	**2021/4/20**	**2021/4/27**
1A	290	184	236	810		65	89	53	215
1B	11	1	25	31		120	24	11	25
1C	11	7	3	21	**7-day**	5	5	24	16
1D	6	4	3	5	**stagnation**	2	15	5	5
	2021/3/4	**2021/3/12**	**2021/3/18**	**2021/3/25**		**2021/4/6**	**2021/4/8**	**2021/4/15**	**2021/4/22**	**2021/4/29**
2A	80	61	143	550		49	390	207	111	194
2B	8	5	16	24		26	31	25	25	47
2C	11	105	20	23		115	49	78	18	15
2D	12	3	13	12		6	14	10	10	5

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