The structure of biofilm grown in a drinking water distribution system with heat exchange analyzed with scanning electron microscopy and energy-dispersive X-ray spectroscopy.

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Abstract

Due to climate change, our society must become more climate resilient, and this is also the case for drinking water distribution systems. Climate change implicates that temperatures of the atmosphere are getting higher. This may lead to that the drinking water may become warmer.

One of the consequences of climate change is that alternative ways of using and making energy has to be developed. One of the options is to use drinking water as cooling water. This will however require careful investigation. In this study the effect of higher water temperatures is examined for the purpose of using drinking water as cooling water, and enquires are made as to what effect this has on the structure of the biofilm inside drinking water distribution systems.

A model system, with three setups using PVC pipes, was constructed. The first set up was a reference system with drinking water from the Dutch drinking water system, second set up was a reference system with heat exchange equipment where the water from the Dutch drinking water system goes through the equipment but no heat exchange occurs, the last set up was a thermal exchange system in which the drinking water is heated to a constant 25 degrees with the help of heat exchange. From each system biofilm samples were taken after 1,2,4,12,20 and 32 weeks. These samples were then analysed using scanning electron microscopy and energy-dispersive X-ray spectroscopy.

Some visual trends were seen that were caused by age, and between the system with different temperatures with help of the scanning electron microscopy imaging. However, element analysis with the energy-dispersive X-ray spectroscopy showed no such trends. Whether this was due to no trends being present, or to the limitation of the technique, is unclear.

From the result found in this research it is hard to make any conclusion of the effect of higher water temperatures on the structure biofilm in drinking water distribution systems, and further methods should therefore be tried

Contents

1 Introduction

In drinking water distribution systems (DWDS) microbial communities can be found. It can even be claimed that many of the problems of our DWDS are microbiological, such as nitrification, microbial mediated corrosion and the presence of biofilm [3]. These microbial communities for the most part do not flow around in the water, even though some certainly do. Most of them (95%) are to be found as a slime layer on the pipe surface, which is known as the biofilm [12, 1].

This biofilm is a small micro ecology that consists of bacteria; up to 90% of the dry weight of biofilm is Bacterial Extracellular Polysaccharides (EPS). Also fungi, viruses and protozoa can be present in the biofilm[3]. The EPS synthesized by microbial cells vary significantly in their composition and hence in their chemical and physical properties. However, the EPS is essential for the biofilm and can provide many diverse benefits to the cells in it. It can give structure to biofilm, allowing stratification of the bacterial community and establishing gradients of nutrients and waste products.

Another important role is that EPS can act as molecular glue, allowing the bacterial cells to adhere to each other as well as to surfaces. This adhesion property is crucial in the formation of the biofilm, as it is formed when bacteria adhere to the pipe wall (primary adhesion), and then start to produce the EPS [10]. They then begin creating a matrix to which other microbes can become attached, causing the biofilm to grow. The matrix helps bacteria to attach and increases bacterial growth (secondary adhesion) [6]. The growth of EPS has been shown to lead to an increased microbial count in tap water compared to the amount in the treatment plant. This increase of bacteria in tap water can occur when the biofilm becomes thick and releases bacteria into the drinking water. This process of biofilm formation is shown in Figure 1.

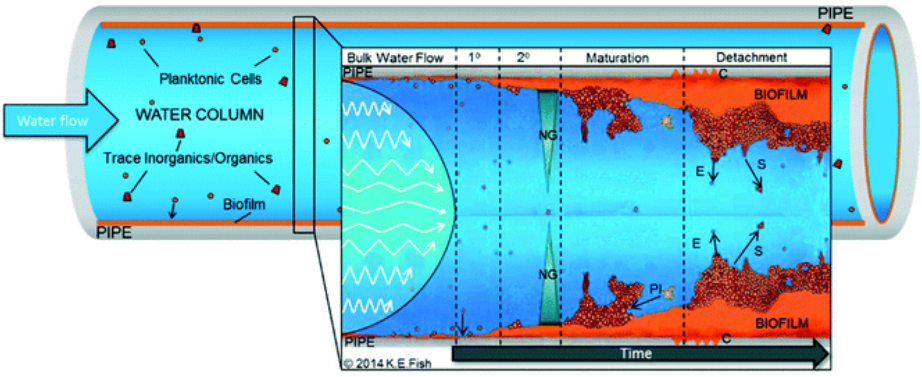


Figure 1: Biofilm development within DWDS. 1: primary adhesion, 2: secondary adhesion, NG: nutrient gradient concentrations within the biofilm, PI: protozoan interactions, C: corrosion of the pipe surface, E: erosion, S: sloughing[8].

As biofilm is a significant component of the microbial part of DWDS and a source of increased microbial counts, including pathogens. It is essential to keep it in check within DWDS. It can increase the number of pathogens inside the DWDS and therefore even though drinking water emerges with good microbial counts from the treatment plant. It can also attain high concentrations of pathogens at the endpoint. Therefore, it is crucial to research biofilm, its structure, how it grows and how different variables affect its growth and structure[12].

Its relation to temperature is especially significant, particularly concerning the forthcoming years, due to Global warming. It has already been shown that during the summer season in the Netherlands the growth of microbes increases compared to the winter months, clearly indicating a relation between biofilm growth and temperature[19]. The earth will on average become at least two degrees warmer, and because of the increase in air temperatures the temperatures of drinking water will also increase. The KWR anticipate that water temperatures will rise above 20-25°C and the growth of most (opportunistic) pathogens will thus be enhanced [19]. Therefore the question arises as to what effect this will have on the micro-fauna of the water. Will additional measures be necessary in order to ensure minimal biofilm growth and thus safeguard drinking water? The first step in answering this is to discover what consequences increased temperature will have on the biofilm and its structure and this was the focus of the research described in this thesis.

Apart from effect of increased temperatures, this study will also consider the impact of using drinking water as cooling water, as depicted in Figure 2. This process will reduce the need for cooling equipment, thereby reducing the energy required for cooling. This in turn will contribute to lowering CO_2 emissions into the atmosphere, and could become one of many other methods for mitigating the effect of climate change. Adding a heat exchange in the drinking water system will be placing additional equipment in the system, which next to increase the water temperature, may also affect the water quality on its own.

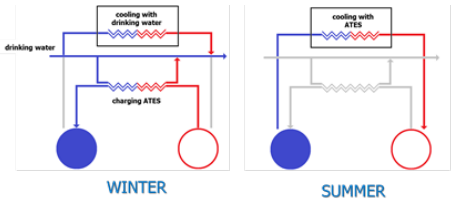


Figure 2: Overview of how to use drinking water as cooling water. [18]

This study is a part of a wider research initiative by J. I. Ahmad at the TU Delft, and the system used to sample the biofilm is her system. This system will hopefully contribute to our knowledge of biofilm by studying its structure when grown at different temperatures both with and without heat exchange equipment. It is to be hoped that further understanding will be gained concerning the consequences of temperature and heat exchange equipment on biofilm growth

and its structure. The structure of the biofilm will be investigated with the help of Scanning Electron Microscopy (SEM) imaging and Energy-Dispersive X−Ray Spectroscopy (EDS). The goal is a greater understanding of the biofilm structure of DWDS and the effect of different water temperatures with and without heat exchange equipment.

2 Methodology

2.1 System

The system that this study is based on consists of three different setups. One reference setup uses drinking water at a temperature equal to the temperature in the DWDS (REF).A second system with thermal energy recovery in which the drinking water is heated to a constant of 25 degrees Celsius with the help of heat exchange (ATER). The third system goes through the heat exchange system but retains the temperature from of DWDS (AHE). In REF and AHE the temperature will vary with the season. An overview of the system can be found in Figure 3.

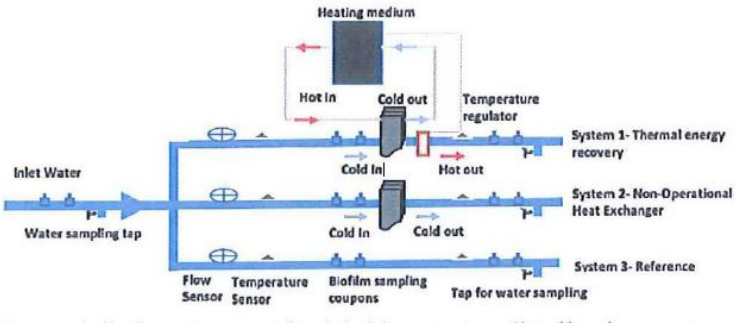


Figure 3: Overview of the system where the samples are taken from. [18]

The pipes of the system are made of PVC, polyvinyl chloride. This is a polymer with the chemical formula $(C_2H_3Cl)_n$. PVC piping is a thermoplastic material consisting of PVC resin compounded with varying traces of stabilizers, lubricants, fillers, pigments, plasticisers and processing aids [20]. PVC is increasingly used in drinking water pipes, especially in the Netherlands. Studies have shown that it has less bacterial abundance or diversity compared to metals or cement[8].

The system is monitored with temperature sensors during the experiment. This resulting data is used to chart an overview of the temperatures during the experiments. The temperature is measured at different points for each setup: at the beginning, for ATER and AHE before the heat exchange equipment, immediately following the heat exchange/equipment, and at the end of the system.

2.2 Sampling

The samples are taken from the system after different time periods. The newest pipes are located at the end of the system in order not to influence the system. The samples are taken after 1, 2, 4 12, 20 and 32-week periods. When sampling a segment of pipe, see Figure 4, is taken out of the system and replaced with a new one. Afterwards it is emptied of water and closed off to keep the inside

dark, and stored in a fridge (4°C) in order to limit any further bacterial growth on the biofilm in the pipes after sampling.

Figure 4: Segment of a pipe used in the experiment. With valves that can close and open at each end.

Immediately before the measurements of the samples commence, they are removed from the fridge and cut into smaller pieces more convenient to analyse. This is achieved with the help of a PVC cutter to cut the pipe in smaller segments, then the sides of the pipe are shaved making the wall then enough to cut the side into smaller pieces with a Stanley knife. The cutting is done as carefully as possible to limit damage on the biofilm, making small pieces approximately 2x2 cm from the PVC pipes, as seen in Figure 5.

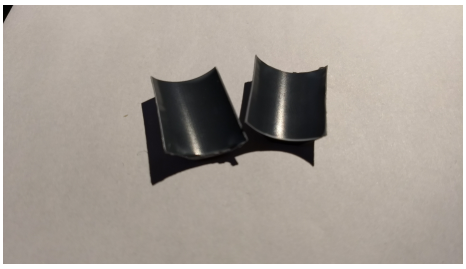
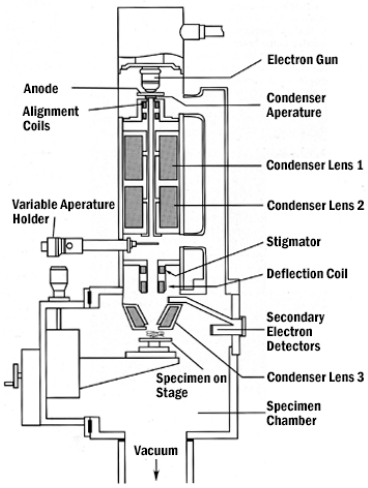
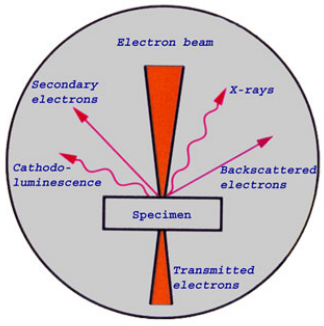


Figure 5: Samples of pipe wall used for SEM and EDS

2.3 SEM and EDS



(a) Overview of the SEM. [16]



(b) Representing the effect of the electron gun on the sample.[16]

Figure 6

The SEM images takes images using a beam of electrons, the image is formed by the detection of freed electrons from the specimen that is loosened because it is being scanned using very high energy electron gun (the secondary electrons). An overview of a general SEM can be found in Figure 6; here you can see the electron gun and the secondary electron detector[16, 17].

As the samples of biofilm constitute biological material they are not dense enough to free sufficient numbers of electrons to form a clear image. To enhance the image a gold layer is incorporated into the sample before imaging using a gold sputter. This more readily easily frees the electrons[11, 16]. Different studies have also used SEM before successfully[1, 2, 4, 9, 11, 14].

The SEM microscope can also be used for EDS measurements. The EDS looks at the X−ray signal rather than the secondary electrons; see Figure 6. This is possible because when the electron beam hits the atoms, holes are created in its shell and the electron will jump to restore balance and send out a photon in the X−ray spectrum. Each atom, having different energy values within its shell, gives out different signals. The EDS detector can detect these signals and the type of atoms present in the material can thus be determined and a calculation of their quantities can be made.

In this study a JSM−840A was used, and for each age and setup of biofilm at least one SEM image each of 10000x, 5000x and 1000x magnification was made. The images were used to examine the biofilm surface. To see whether it was a smooth surface, or whether any particles and discontinuities could be detected.

With the EDS in the SEM of each sample images of 5000x or 1000x magnification where made. The EDS gives an overview of which atoms were seen to be present on the surface of the biofilm and of the PVC. The EDS was used to examine the composition of the different biofilms. Graphs were made on which the different elements of the sample can be found, as well as the relative composition in weight and atom percentages.

Initially the PVC was analysed in order to obtain clean PVC samples. Not just the surface of the PVC was analysed but also the inside. This was achieved by cooling the PVC with liquid nitrogen in order to make the PVC brittle. When the PVC cooled it was then broken, and both SEM and EDS was performed inside the break to determine the clean composition of the PVC. It was important to ensure that the elements found on the surface were from the PVC and not from external contaminants. Two EDS from within the PVC were taken, and two from the surface. The result from the EDS was averaged in order to find the average composition of the PVC, as well as how the material was structured. With this a baseline was made for the rest of the samples.

The biofilm was analysed from the surface and two EDS were taken from each sample, with an exemption for the 20-week sample of the reference system where only one EDS was done. This was due to some problems with the SEM machines on that day.

The graphs were used to give some idea of where different elements are to be found. This is especially useful for elements other than carbon and chlorine, as they are the main components of PVC and it is generally expected that they

are to be found everywhere in the sample. However, if particles of metals, or calcium or silicon are detected in the sample, then the EDS can indicate where these are located.

The atom percentage is used to discover whether there is any significant difference between the different biofilm setups, but also between the biofilm and the PVC. To achieve this, two parameters were chosen: the calcium present and the ratio between the carbon and chloride atom percentage. The second is particularly interesting because PVC is made of chains of carbon and chloride and biofilm is organic material that consists mainly of carbon chains. The amount of carbon to chloride is thought to increase when the carbon from the biological material is added and not just the carbon from the PVC and therefore was used to estimate the amount of biofilm.

Concerning the calcium atom percentage and the ratio between the carbon/chlorine atom percentage ratio, the correlations between the age of the biofilm were examined, as well as the parameters, by calculating the correlation factor. The correlation factor (p) is calculated with the help of the variance (Var) and covariance of two variables X and Y:

$$P(X,Y) = \frac{Cov(X,Y)}{\sqrt{Var(X) * Var(Y)}}$$

If the correlation factor is positive this means that X becomes larger with a larger Y or smaller with a smaller Y; if it is negative then X becomes smaller with a larger Y or larger with a smaller Y (inverse relationship). Covariance around 0 means that there is no correlation between X and Y. Larger correlation factor values indicate a stronger correlation and correlation is assumed when values are higher than 0.5.

When examining the difference between the average values for the two indicators for the different systems, as well as the differences between the samples with and without biofilm, a student T-test with two tails for two samples with unequal variances was performed. A students t-test assesses whether the hypothesis that the mean of X equals the mean of Y is correct, or whether there is any significant difference between the two averages. This is achieved by calculating the non-pooled student mean difference:

$$Td = \frac{Xn - Yn}{Sd}$$

Xn is the average of sample X, and Yn is the average of sample Y. Ans Sd is the non-pooled variance:

$$Sd^2 = \frac{Sx^2}{2} + \frac{Sy^2}{2}$$

Sx is the sample variance of x and Sy is the sample variance of y. This is then compared with the critical values of the t distribution. A significance value of 0.05 value is chosen this means that the students mean difference should be 95% likely to be different before it can be said that there is a significant difference

between the two data sets. The P-value related to the T test can be found in tables or calculated with help of an statistic program. If this is not the case then it is not possible to claim that the averages could not be equal [7].

The statistical analysis was performed with the help of the built-in data analysis of Microsoft Excel.

3 Results

3.1 Temperature measurements

The temperature of the system was measured during the time of the experiment. The data runs from the 16^{th} of January until the 2^{nd} of November. Unfortunately, there are some gaps in the data due to scheduled restarts of the computer in which the measurements stopped for a period. There are also some peaks in the measurement because the system was disturbed when the samples were taken. This required some time to return to a stable temperature. The system temperature graph can be found in Figure 7.

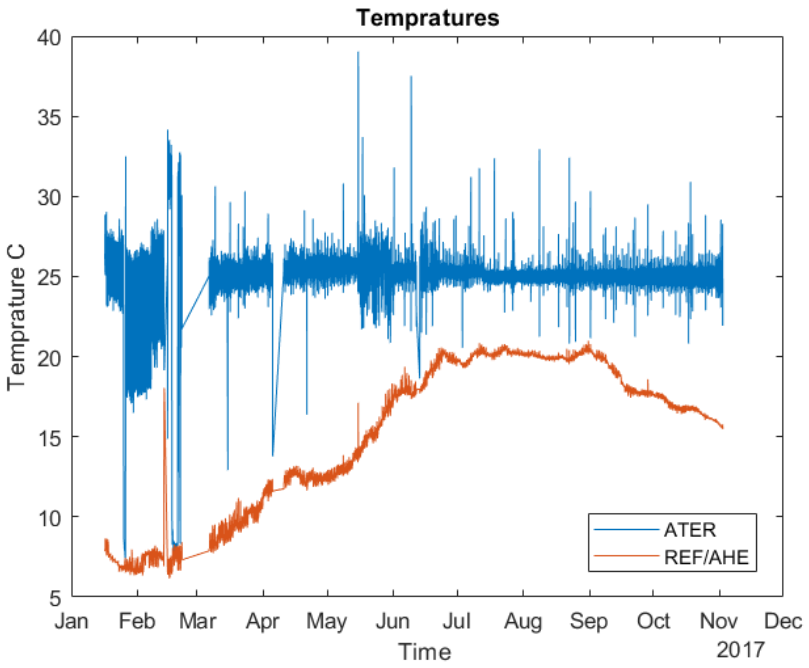


Figure 7: Graph of temperatures. Where the temperature of ATER is kept at 25°C and the temperature of the drinking water, due to the graphs of AHE and REF, is approximately the same, they are represented as one graph in this figure.

3.2 SEM Imaging

Multiple pictures of the biofilm were taken with the SEM in order to examine how the surface appears. The most interesting result is shown below. Within the PVC small particles acting as filler can be seen, as shown in Figure 8. This can also be found on the surface of the PVC, as shown in Figures 9 and 10. It

can also be seen that there is some cratering on the surface of the biofilm and that lines can be found on the surface.

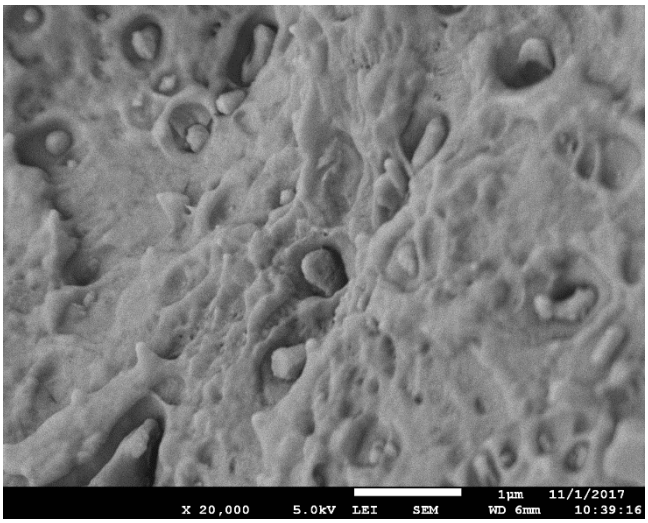


Figure 8: Image of the inside of the PVC taken with 20000x magnification. The bar represents 1 μm.

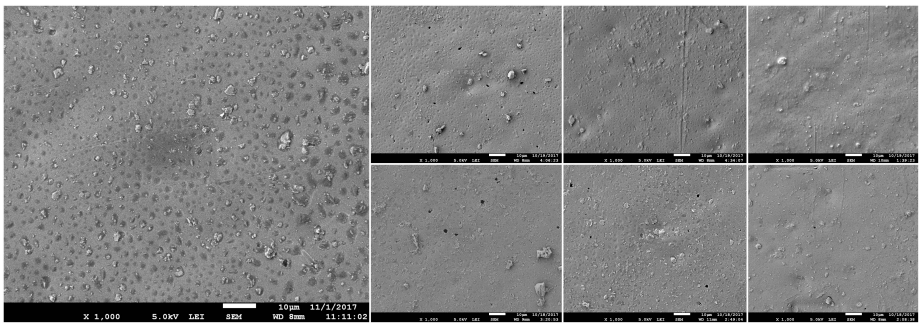


Figure 9: The large picture is the clean PVC. The upper row is the 1-week old biofilm from ATER, REF and AHE respectively. The lower row comprises 32-weeks old biofilms from ATER, REF and AHE respectively. The picture is taken in SEM at 1000x magnification. The bar represents 1 μm.

As seen in Figure 9 and 10, the craters and lines can also be seen in the 1-week old biofilms, while in the older biofilms they are less detectable. This could be an indication that the older biofilm is the thicker. The filler particles can also be detected in the PVC within the biofilm. These seem to be encapsulated in the biofilm, which is shown in Figure 11.

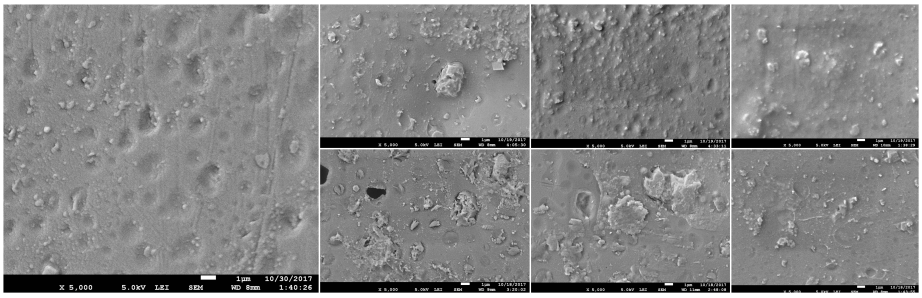


Figure 10: The large picture is the clean PVC. The upper row is the 1-week old biofilm from ATER, REF and AHE respectively. The lower row comprises 32-weeks old biofilms from ATER, REF and AHE respectively. The picture is taken in SEM at 5000x magnification. The bar represents 1 μm.

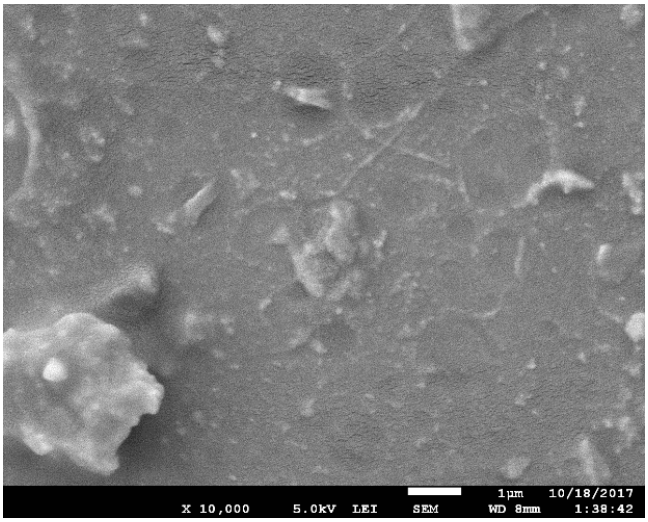


Figure 11: AHE 32-week-old biofilm, taken with 10000x magnification, showing encapsulation of particles.The bar represents 1 μm.

3.3 EDS

Next to the SEM imaging EDS was next used to find the composition of the biofilm and the PVC.

3.3.1 The composition of PVC

From within the PVC carbon, oxygen, chlorine, and calcium were detected by the EDS when the gold from the layering was filtered out. It also shows that the particles already detected with the SEM can be identified as an calcium oxide, as seen in Figure 12. On the surface of the PVC traces of aluminium, sodium and

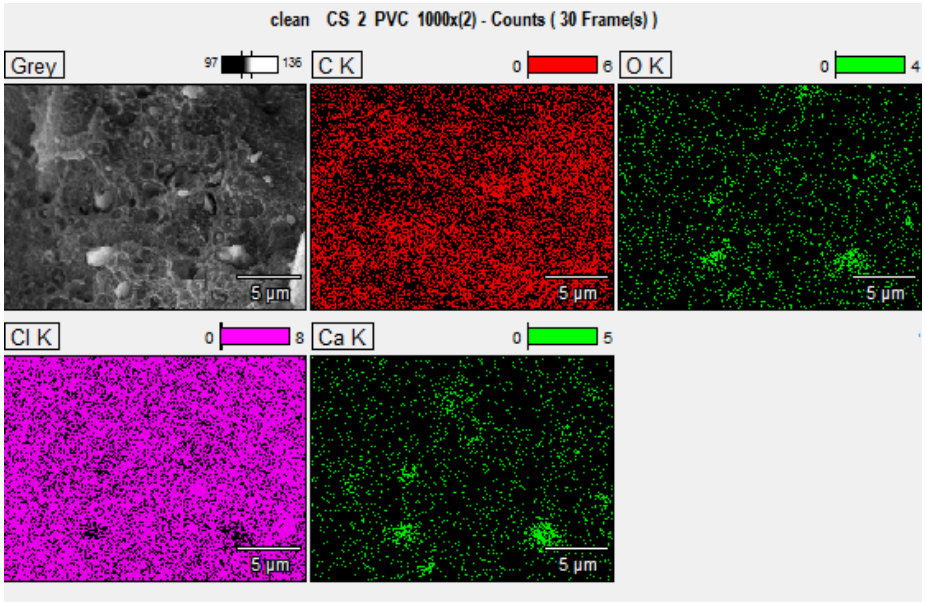


Figure 12: EDS image of the inside of the PVC taken with 1000x magnification.Showing a graphical representation of the different elements found in the PVC

silicon was also detected, this was not found in the PVC and is therefore most likely from an outside source. The results of the different EDS measurements were combined and averaged, as seen in Table 1, in order to approximate the composition of the PVS. Due to averaging and rounding the result does not amount to 100%.

Table 1: Atom percentage of the different elements found in the PVC pipe

Element	**Average atom percentage**	**Standard deviation of the average atom percentage**
Carbon	91.12	0.48
Oxygen	6.25	0.60
Calcium	0.23	0.04
Chloride	2.22	0.90

3.3.2 Composition of Biofilm

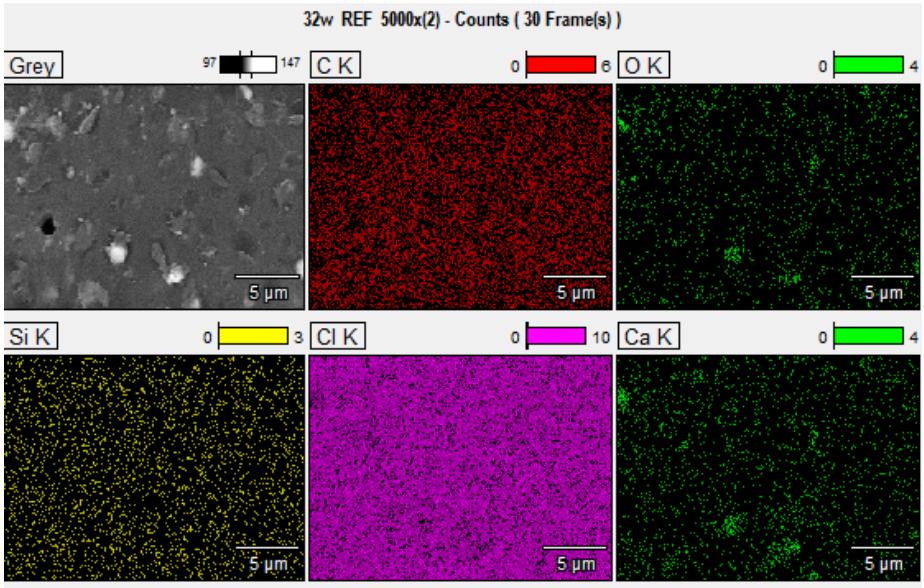


Figure 13: EDS of 32-week-old REF biofilm with 5000x magnification.

From the biofilm sample the EDS imaging detected mainly carbon, oxygen, chlorine and calcium, but also traces of aluminium, silicon, magnesium, sodium, barium, titanium, iron and copper. This does not mean that all those atoms are necessarily present; the readings of the machine could be in error, especially if only small amounts were detected. It might also be concluded that the particles found in the biofilm consist of calcium oxides, such as in the clean PVC samples. This can be seen in Figure 13. The results of week 32 on the biofilm are shown in Table 2 for the different setups.

Table 2: Averages of Carbon, Calcium, and Chloride week 32

Element	Atom percentage ATER	Atom percentage REF	Atom percentage AHE
Carbon	85.68	88.02	85.59
Chloride	7.50	7.78	9.13
Calcium	0.49	0.49	0.30

3.3.3 Correlation with age

Different variables were investigated in order to discover how they affect the composition found, and whether it is possible to detect the biofilm, or if it is only the PVC being detected by the EDS. This is performed by examining

the atom percentage of calcium and the ratio between the atom percentage of carbon and chlorine.

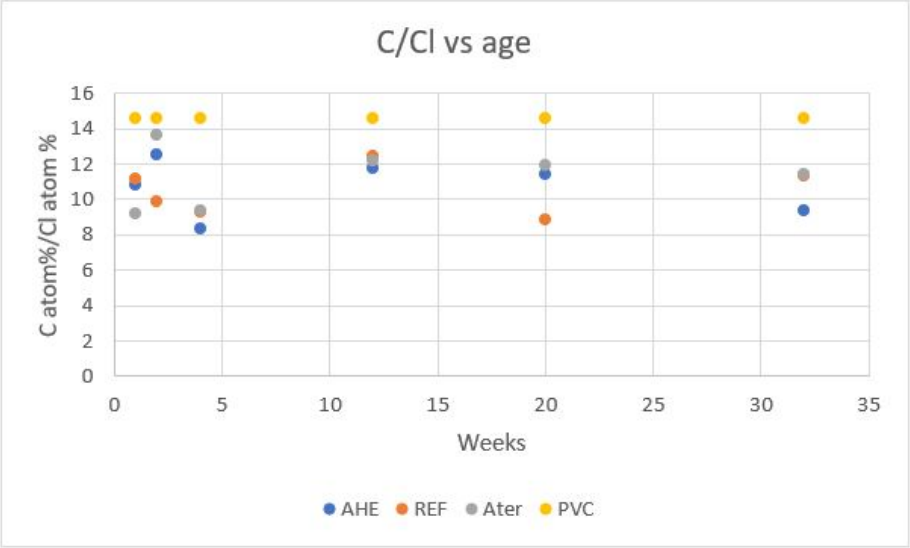


Figure 14: The ratio between the carbon and chlorine atom percentage for the different biofilm ages for the different system are showed. Next to the ratio of the PVC.

In Figure 14 the different ratios between the atom percentage of carbon and chlorine are showed for the different biofilm ages. In the graph in the first eye, no direct correlation is seen. To be sure this is the case the correlation factors of the different system is calculated. The correlation factor where -0.22, 0.13 and 0.21 for the AHE, ATER and REF system respectively. None of those values are high enough to say that any correlation between the ratio and age can be determined.

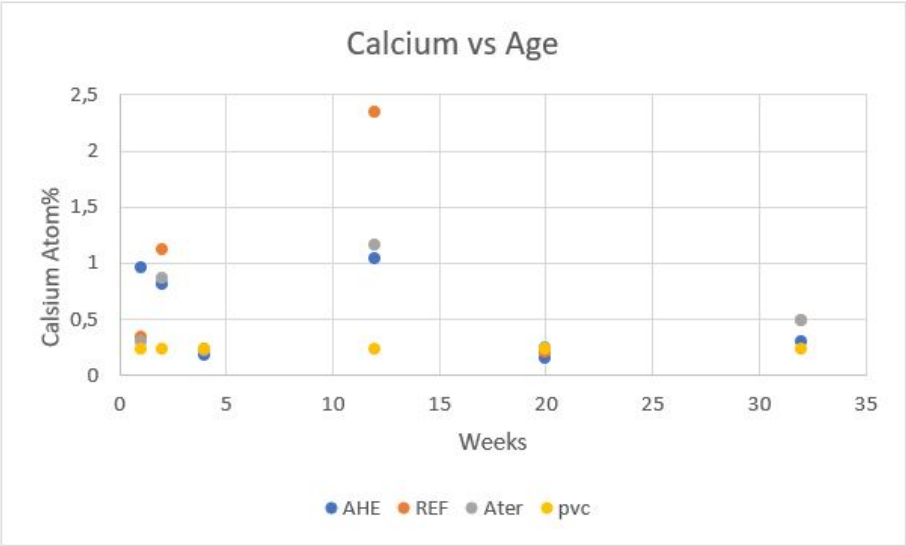


Figure 15: The Calcium atom percentage for the different ages of the biofilm ages for the different systems are shown next to the ratio of the PVC

In Figure 15 the different atom percentage of Calcium are showed for the different biofilm ages In the graph no clear correlation is seen. To ensure that this is the case the correlation factors of the different system is calculated. The correlation factor is -0.57, 0.006 and 0.01 for the AHE, ATER and REF system respectively. The values for ATER and Ref are almost zero. It can therefore be concluded that no correlation was found. Those values are sufficiently high enough to claim that any relationship between the ratio and age can be determined. The AHE seems to have a slightly negative correlation to both the age and amount of calcium; it is however still uncertain whether this trend is really present or whether it is a coincidence, especially as this is the only set up to show evidence of this trend.

3.3.4 The correlation between the different elements

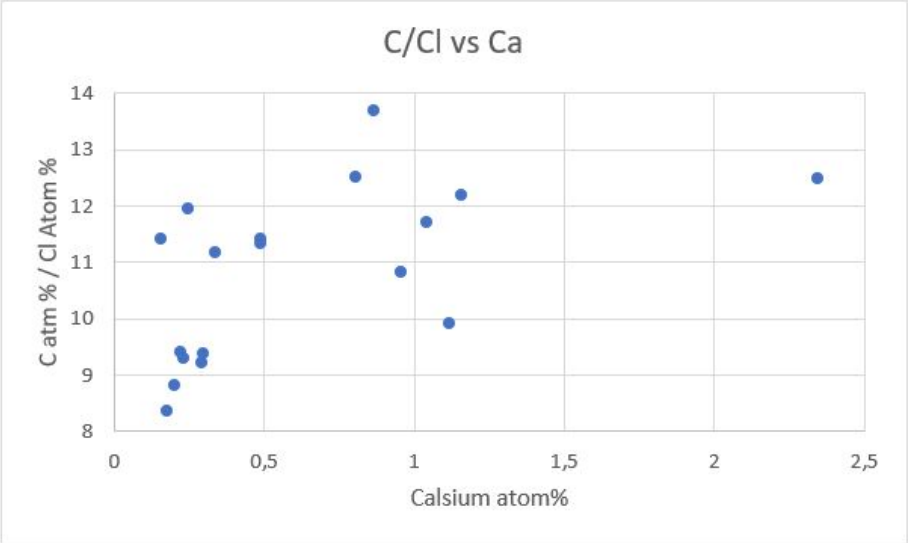


Figure 16: Ratio of carbon and chlorine atom percentages plotted against the values for calcium atom percentage

The correlation of the ratio between the carbon and the chlorine atom percentage to the amount of calcium was examined, as shown in Figure 14. With a correlation factor of 0.54, a slightly positive relation was found, indicating that the more calcium, the more carbon compared to chlorine can be detected. Whether this was due to the calcium changes the measurement, or due to coincidence, or due to the biofilm growing faster with calcium, is difficult to say. If we examine the division element, as shown in Figure 16, we can see how the calcium covers up the chloride signal from the PVC, although the same trend is not observed for carbon. This could be an explanation of this apparent trend.

3.3.5 The difference between the different setups

To discover whether there is any significant difference between the systems a students T-test was done concerning the averages ratios and the calcium atom percentages, it has been assumed that age had no significant influence on any of the values and all values of all ages have been averaged. This is because no correlation had been found in this research. All student mean differences are higher than the the chosen critical P value, and can be found in Table 3, and therefore no significant differences between the averages for the different carbon to chlorine ratios or the calcium atom percentages between the systems.

Table 3: P values for the different comparisons with the students T-test

Comparison	P value Ca/Cl	P values Calcium
AHE vs REF	0.85	0.36
ATER vs AHE	0.42	0.85
ATER vs REF	0.34	0.31
ATER vs PVC 1	0.03	0.02
Ref vs PVC 1	0.02	0.04
AHE vs PVC 1	0.01	0.01
ATER vs PVC 2	0.21	0.01
REF vs PVC 2	0.23	0.03
AHE vs PVC 2	0.13	0.01

3.3.6 Difference between the Biofilm and PVC

To compare the clean PVC samples with those of the biofilm, a students T-test was also used. The averages previously used for the different setups were again used and compared to the PVC averages.

The T-tests showed that the biofilm samples had a significantly lower carbon to chlorine ratio than the clean PVC, as well as a higher calcium concentration. This can be seen in Table 3 where the P-values were lower than the critical P-value for the first comparison of biofilm with the PVC. This is the reverse of what would be expected; one would expect carbon in the biofilm but no chloride, which would show a higher carbon to chlorine ratio in the biofilm. In addition, a high carbon to chlorine ratio compared to high calcium is also the reverse of what was found in the biofilm. One possible explanation of this is that the rougher surface of the broken PVC makes it difficult to obtain accurate measurements of the carbon due to shadowing. An indication of this can be seen in Figure 16. Therefore, the same T-test was performed without the data from the cross-section. With this new T-test no significant difference could be found between the C/Cl ratios for either the PVC or the biofilm, as seen in the second comparison in Table 3.

The calcium level was however still significantly lower in the PVC than in the biofilm. This could indicate that the water contributes additional calcium. This has previously been indicated for biofilm grown on TiO_2/Ti composite electrodes by research undertaken by Bennani et al [2]

4 Discussion

The results show that it is difficult to find any particular trends in the data. This of course can be for quite different reasons, and this, together with the valency of the data, will be discussed in the following section. Due to time limitations each sample only had limited repetition, making a significant variation in the results. It would therefore have no doubt proved beneficial if more time had been available to increase the number of EDS measurements for each sample.

For the temperatures the data for some periods is missing. This means that we are forced to make assumptions about the relevant prevailing conditions. This especially an issue for the younger biofilm that only grew during that period. For the older biofilm the data covers more of the growth time, although this period has less influence as it was at the beginning of the sampling.

A further point of attention is that the oldest sample investigated was only 32 weeks old. If this is compared to drinking water that can be up to 100 years old, then this is a relatively short time. It is therefore a difficult issue because the results of the biofilm could be quite different when it ages even further. A study was undertaken that showed that several years may be required before a steady state is achieved. This suggests that the results of in this study may not be applicable for older biofilm [15].

There was no apparent correlation between the variables found in the results. This may be due to no correlation being present, or to the method used for investigating the biofilm. The SEM and EDS also had some limitations.

The most significant limitation with the EDS is that it only examines elements that are present and does not distinguish specific molecules . This made it difficult to distinguish the biofilm from the PVC due to the same elements being present. An attempt was made to resolve this by examining the ratio between carbon and chlorine. This however did not work. One solution could perhaps be to grow biofilm on another material that chemically differs more from biofilm than the PVC. This may lead to a more accurate conclusion. However, it has been proven that biofilm on PVC is significantly different than on other materials [4, 14]. This suggests that other methods of analysing the structure of the biofilm might have been more efficient.

Another limitation was that carbon appeared to be sensitive to shadowing due to the roughness of the surface. This meant that the carbon-chlorine ratio differed greatly and could be one of the reasons for no trend being found between the ratios for the carbon and chlorine atom percentage, and also for the age and between the biofilm samples and the PVC.

In addition, when particles of calcium were present the EDS had difficulty seeing through it. This was especially a problem for the chlorine. This may be the reason for the correlation between the amount of calcium and the carbon-chlorine ratio.

Some traces of aluminium, natrium and silicon were found on the surface of the PVC. These traces were not seen on the inside of the PVC, suggesting that those elements result from outside contamination. This indicates that the samples are not sterile. This is of course logical from the PVC being a commercial

product. The contamination could have come either from the cutting process or from one the machines used to make the PVC.

On the biofilm aluminium, silicon, magnesium, natrium, barium, titanium, iron, and copper were found. This may have come from the water, but some contamination was already present on the PVC, possibly from the cutting process. A further significant fact is that some elements present in trace amounts can be misread by the EDS detector. However, due the elements not being found consistently, or in any significant quantity, it is difficult to say what is actually present in the biofilm and what arises from outside contamination concerning such small amounts. However, all of these materials have been found in previous EDS samplings performed in other biofilm research initiatives, such as that undertaken by Little et al., in which aluminium, silicon, magnesium and iron were found in biofilm grown on commercially pure copper surfaces after exposure to estuarine water for four months. Aluminium, silicon, magnesium, natrium, titanium, iron and copper were also found in biofilm grown on 304 stainless steel surfaces after exposure to estuarine water. This could indicate that some of the elements did indeed originate from the biofilm itself, initially coming from the water [11]. Additionally, research by Liu et al. has found metals on the surface of drinking water distribution[13].

5 Conclusion

The SEM imaging showed some evidence of ticker biofilm of a higher age and the difference between those samples with and those without biofilm was noticed.

This was not confirmed by the EDS, in which no trend was found between the different biofilm ages, the setups, or between the biofilm samples and the clean PVC and the indicators. However, there was a higher amount of calcium found in the Biofilm samples than in the PVC. It would be interesting to further examine why this should be the case.

It can be concluded that the SEM with EDS is not an efficient way to investigate the biofilm, and no definite conclusion was arrived at in this study. Some improvements could be made by using a different pipe material than PVC, as this would render the carbon from the biofilm more detectable. Another option would be to combine the SEM results with other ways of examining the biofilm structure, such as confocal fluorescence microscopy. Both of these possibilities do however have additional limitations.

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