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Biofilm formation in a large sized experimental set up of a drinking water distribution system consisting on various pipe materials

Abstract

In water distribution systems newly exposed areas, such as newly laid pipes are subjugated to biofilm growth. This growth have been associated with public health problems and has lead to the water treatment facilities seen today. In an attempt to better understand what mechanisms are important when it comes to drinking water, research involving simulated distribution systems is being used. These systems imitate real systems but are small and cannot fully recreate all the changing parameters normally seen. In this study, tubes out of the materials Polyethene (Pe), Polyvinyl chloride (PVC) and treated Pe (tPe) lined with Prebona protect was constructed into a large sized drinking water distribution system, to answer which material promoted the highest amount of biofilm. The developmental curves of the biofilm on different materials were compared in this large sized experiment with smaller sized versions done in other studies. Significance and differences were seen in a heterotrophic plate count (HPC) between three and seven day count in tPe tubes which indicated biofilm formation. When comparing materials with each other there were no differences seen regarding bacterial growth. In comparison to other studies (Manuel, Nunes, and Melo 2007, Tsetanova, 2006), which used pilot rig system, showed similar growth as in the present paper with a large sized system. The developmental curves appeared indistinguishable in this experiment, but to clarify this more rigs should be used and studied in an earlier stage. Also, characterization of biofilm by DNA sequencing would further highlight the differences between materials used. However, the comparison between large sized tubes in the distribution system and a pilot rig used by others suggests that the use of pilot rig systems are adequate.

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Introduction

It has long been an interest for ecologists to study how communities of plants and animals develop on newly exposed areas. Descriptions have been formed of how these organisms sequentially colonize and occupy a site to promote changes in biomass, productivity and diversity to finish in a steady state-community. The colonization leads to self induced gradual changes in a population which induces changes of the local environment. This does, in turn create new surroundings suitable for colonization whilst also disfavoring the previous inhabitants (Martiny et al. 2003).

It has been evident since 1930 that biofilms exist in potable water because of reported problems with regrowth of *Bacterium coli*. Years later, the presence of biofilm was associated with public health problems and even today they are subject to research due to the fact that biofilms can provide growths areas for pathogens, viruses and protozoa. The potable water research of biofilm development is of interest to drinking water companies. The main reason for this is because of the health effects associated with biofilms but also for aesthetic reasons such as taste and odor problems (Percival, Walker, and Hunter 2000:85-86).

The amount of bacteria increases in the distribution system of drinking water during transport between treatment plant and the consumer. This is the result of many parameters, such as disinfection decay, residence time of water, substrate uptake and corrosion deposits within the system. The overall biomass in the system is mainly attached to pipe walls. It is thought to contain 95% of the biomass while 5% is estimated to be in the water phase. This bacterial release into drinking water makes biofilm an important factor when it comes to water quality (Manuel, Nunes, and Melo 2007).

The process of which a biofilm is developed can be seen as a successional one, that starts out as a random attachment of organisms from the water body, readily creating a monolayer on the surface. Due to the low selection pressure initially for attachment because of spatial separation it is not farfetched to imagine a high level of diversity in this stage. The next attachment of cells will, by surviving on remnants of inhabitants or just by thriving in the protective environment created by the biofilm be able to colonize this area. In this extension of complexity and diversity the less competitive species may get out competed by the new colonizers. Continuing this trend, when the biofilm keeps developing, a reduction in diversity may happen when just a few competitors starts to dominate. However, during maturation, gradients form and internal recycling of resources leads to the creation of potential niches for survival. This leads to an increase of diversity which reflects in a complex spatial structure that holds many functional groups (Martiny et al. 2003).

It has been found that adhesion of bacteria associated with drinking water contributes in protecting microorganisms from chlorine disinfection. This would mean that mechanical cleaning of a system may lead to the release of problematic microbes. Even if this release is possible there is no routine examination in assessing biofilms present in pipe walls transporting drinking water. This might be because of the problems associated with distribution lines and the difficulty to sample these inaccessible places. The research in this area has generally led to documentation of biofilms cultivated in a laboratory environment with simulated distribution systems as pilot rig systems. This can only imitate a real distribution system to a certain degree due to all changing parameters in these kinds of systems (Percival, Walker, and Hunter 2000:86).

There are a lot of microorganisms in potable water and many of these are dormant or injured which could be the result of disinfection procedures such as chlorination. The degrees of which these organisms represent are unknown but there is a certainty that these organisms are metabolically active but unable to grow on culture media. The use of agar media for estimating the amount of bacteria in water is widely acknowledged to result in underestimation of the amount of bacteria. Quantification methods used can be counting in chambers, epifluorescence microscope or by HPC. Normally the dominating bacterium is gram-negative in potable water, such as *Pseudomonas* or yellow/brown pigmented *Flavobacterium*-like species. These types exists and normal harmless flora together with potentially harmful pathogenic organisms, such as for example *Legionella*. (Percival, Walker, and Hunter 2000:87)

The results of biofilms research in pipes are plentiful. They can give positive results in coliform tests, give increased demand of chlorine, give rise to opportunistic pathogen, change odor and taste, corrode pipes or discolor the water. Due to all of these possible effects, controlling, managing and researching the biofilm is an important task for companies working with distribution of drinking water. In order to get good potable water and a longer lag phase of regrowth a combination of measures should be implemented, such as mechanical cleaning and chlorination (Percival, Walker, and Hunter 2000:87-88) (Martiny et al. 2003).

Recently materials like PVC and Pe has been preferred in drinking water distribution systems contrary to iron or cement based networks. These new pipes are easy to work with but there have been and still are questions to be answered regarding the effects surface material have on biofilm development. (Manuel, Nunes, and Melo 2007). Lower biofilm growth has been seen on Pe, PVC compared to various iron based material such as cast iron, cemented steel, asbestos-cement and cemented cast iron by some researches (Kerr et al. 1999, Niquette, Servais, and Savoir 2000). The reason for this was discussed to be due to the increase iron corrosion products which help in biofilm protection of disinfectants and flow rate effects. Wingender and Flemming (2004) however, found that there were no differences in colonization between the different materials (stainless steel, Pe and PVC).

Water has been transported in many different ways during the years of water distribution networks. New materials has been tried and also used in an effort of lengthening the lifetime and lower the workload of laying pipes. All this has been done to get as cost effective as possible and still delivering clean and safe water. In an era where recycling becomes all the more important the question arise. How about reusing the pipes already in the ground or pre treating new ones? This question requires a lot of research of course and in this experiment performed here one such type of material was used.

In this paper, real sized pipes were used in a continuous drinking water flow system. Three goals aimed to be answered by using this set up was: 1) What type of material promotes the highest development of biofilm. 2) How does the developmental curve of biofilms look like for different materials. 3) How does a large sized continuous flow reactor compare to the laborative smaller sized version.

Materials and methods

In order to study a system equal or similar to what can be found in distribution systems all over the world, it was decided to build up a large sized set up of pipes consisting of various materials. The pipe materials used were restricted to what suppliers had available and what seemed appropriate based on what pipes are used in distribution systems today. In this case Poleethene (Pe) and Polyvinyl chloride (PVC) pipes were selected for the experiment. For convenience, the pipes were pre cut and put together by various union connections depending on the pipe material. These connective parts had minimal surface area which would come in contact with the drinking water and thus would only add a minor area.

Unfortunately, undesirable results were obtained with a saw while preparing pipes which lead to a tubing cutting tool being used for a more accurate cuts in order to maintain clean and reproducible result. Each pipe was cut to 50 cm in length, Pe pipes had a diameter of 3.26 cm resulting in an inner surface area of 512 cm^2 while PVC pipes had a diameter of 3.1 cm and inner surface area of 486 cm^2 by the following formula:

$$Surface\ area = 2\pi rh$$

The volume of one 50 cm segment was calculated by:

$$Volume = \pi r^2 h$$

By adding the supportive turns into the calculations, total volume of the system was obtained and residence time could be calculated. The turns were 200 cm (Pe) and 50 cm (PVC) in length.

The water comes from the tap in V-huset LTH, Lund municipality. The water distribution is handled by Ringsjöverket which collects their water from Bolmen in Småland and in some cases Ringsjön, Skåne. In the water treatment plant the water undergoes a purification process in different steps. For example, the addition of chemicals induces flocculation in order to easily separate suspended solids. Rapid and slow sand filters are also used. When the water is clean and ready to be consumed it enters the distribution network where it resides until it reaches a water tap.

Two normal hoses were connected to the water tap in order to facilitate inlet and outlet functions. In the inlet a manometer were connected in order to control and maintain the same pressure in the system throughout the experiment. To get a high pressure, a valve was placed just before the outlet hose. The valve was adjusted to get a water residence time of 90 minutes.

This was calculated by formula:

$$Residence\ time = \frac{The\ capacity\ of\ a\ system\ to\ hold\ a\ substance\ (ml)}{The\ rate\ of\ flow\ of\ the\ substance\ through\ a\ system\ (\frac{ml}{s})}$$

Collecting outlet water and measuring the amount per minute ensured a residence time relevant to this kind of experiment.

By being able to have fixed pressure and residence time in the system it was ensured that preferences for growth was maintained. The temperature were not possible to regulate, instead it followed the temperature in the room which were around 19 degrees. The outlet hose were positioned at a high position compared to the distribution system to make the system eradicate water bubbles when active.

Two different kinds of pipe material were used: Pe and PVC. Six different series with each seven pipes were set up. There were four series of the Pe and two series of PVC. Two of the Pe pipe series were treated with a nano silver paint from a company named Prebona and thus treated as a separate pipe material.

Prebona is a relatively newly formed company that has specialized in the creation and sales of a product which they promise will give a protection against dirt and other pollutants on different surfaces. Without going into any technical details, the paint branded as Prebona Protect forms, when the water part is evaporated, a three dimensional structure which then covers the entire applied surface. A small amount of silver ions has been added in order to strengthen the three dimensional structure and these ions become strongly integrated into the structure. For more information regarding the company and the details regarding their product please visit their website listed in references (Hedlund 2012)

This set of pipes was individually prepared by pouring 25 ml of the silver solution into each pipe and by plugging the ends. Then the pipe was tilted in all directions and the solution was poured back carefully. The solution was weighed both before application and after application to be able to appreciate the weight of solution left in the tube. By getting the amount solution remaining in the pipe it was possible to estimate the sum of silver ions present per cm^2 in the tubes. The application of silver on the pipes was performed twice with a day in between allowing the pipes to dry in between treatment and the silver ions to receive the intended shape. The silver solution has a silver concentration of 1000 ppm or 0.1% which leads to equation below.

$$m_{Ag} = C_{Ag} * m_{Dispersion}$$

From this equation the amount of silver left after the water phase has evaporated was calculated. Silver concentration per cm^2 was obtained by dividing with the surface area. The resulting silver concentrations can be seen in table 1.

In preparing the tubes with Prebona Protect it was important to be certain that the resulting amount of silver were relevant to the investigation. Prebona reported that in a previous investigation (however pertaining to roofing tiles) dosages corresponding to 5 µg/cm^2 were used. This was seen to give effect regarding biological growth. However, since this investigation a higher dosage in the range of 10-15 µg/cm^2 of silver is recommended.

The positioning of pipes can be seen in figure 1 and 2. As seen, the tubes are coupled together in series where two Pe pipes series come first and then two PVC series and lastly, tPe positioned in the end of the system. The reason to position these pipes in the end of the system was to ensure that no silver solution would come in contact with the Pe and PVC pipes. In order to get the tubes suitable for sampling, turns were added so that the tubes interesting for biofilm development could remain straight. The PVC type tubes were also of rigid structure and unable to bend which led to the use of 90° turns. In figure 3 it is possible to see the system after eight weeks in to the experiment, connected with inlet and outlet hoses.

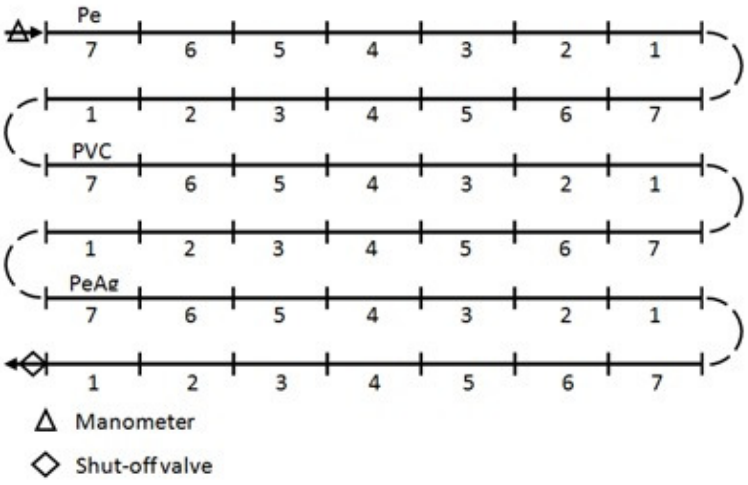

Figure 1: Schematic representation of the experimental set up.

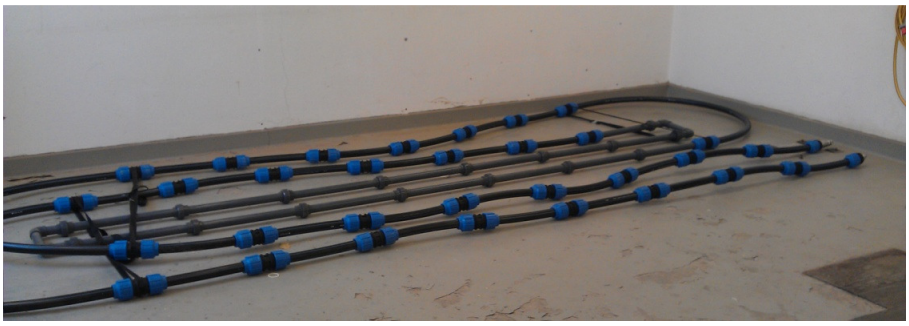

Figure 2: Photograph of the experimental set up prior to start.

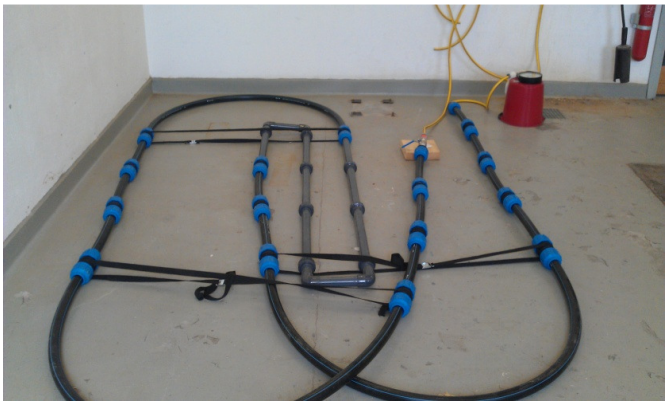

Figure 3: Photograph of the experimental set up after eight weeks in operation.

Before the system was activated it was treated with a 10 liter 0.1% sodium hypochlorite solution and flushed with 30 liters of water as common practice when disinfecting a distribution system. The pressure of the system was decided to be between 3-3.5 bar with a hydraulic residence time (HRT) of 90 minutes.

Two controls for each series were prefabricated and not added to the distribution system. However, they were treated with sodium hypochlorite solution and flushed with tap water prior to laborative treatments.

Once the system had been constructed and tested for leaks, water was free to flow through the system and the system was left running for roughly four weeks. Then it was time to gather the first pipe samples and in order to do this, the inlet water were closed and separation of the pipes begun after the pressure had gone down to 0 bar. To ensure that the Prebona solution did not get in contact with the other material types, these pipe samples were taken out of the system first. The pipe endings were sealed with caps and parafilm cuts which had been treated with alcohol prior to sealing.

The pipe samples, together with two of the controls were transported to the laboratory where treatments to gather bacteria samples were performed. Here, the same way of extracting the biofilm was used as Wingender and Flemming (2004) did for PVC pipes. However, it was decided to use 50 gram glass beads and 50 ml of water together with 2 minutes of shaking in order to get a large amount of the biofilm from the tubes into a glass container. This solution was later diluted using a dilution factor of 10^{-3} to 10^{-6}.

These dilutions were then plated in accordance to the spread plate method, for HPC on R2A agar. The colonies on the plates were counted after 3 days of growth in room temperature. After seven days they were also counted to appreciate the slow growing heterotrophic activity. By following the same schedule every week it was possible to ensure comparable HPC results of early biofilm development. Samples of all pipes and sampling weeks were saved in the freezer for future study.

The total cell counts of the last three samples were also obtained by counting visible bacteria in a Bürker chamber. Because of the low amount of bacteria in each sample 40 chambers were counted for each sample.

Results

The treated set of pipes used in this investigation is summarized in table 1 and shows the amount of silver in both set and in each individual tube. The amount of silver varies between 3.85-5.74 µg/cm^2 over all the tubes and controls. The mean of silver in tubes was 4.95 µg/cm^2. This should, based on the experience of the Prebona Company be a good starting point and possibly be enough to see a difference in growth.

Table 1: Shows the amount of silver left in individual tubes per cm^2 after application and water evaporation.

tPe-tubes	m_{Ag} (µg/cm^2)	tPe-tubes	m_{Ag} (µg/cm^2)
Set 1		Set 2	
Control 1	4,66	Control 2	4,59
1	4,98	1	3,85
2	5,13	2	5,04
3	5,00	3	5,15
4	5,74	4	4,98
5	5,25	5	4,85
6	5,29	6	4,83
7	4,96	7	4,87

A representation of bacterial growth can be seen in figure 4 and HPC growth results of biofilm coming from different materials can be seen in figure 5, 6 and 7. Figure 5 shows cfu per cm^2 over time in Pe tubes with plates that had been incubated for three and seven days. In figure 6 same kind of data can be seen except this is PVC tubes and figure 7 tube material is of tPe tubes. In all materials something similar to a steady state around the magnitude of 10^6 cfu was seen and the seven day count was likely to be higher in comparison compared to the three day count.

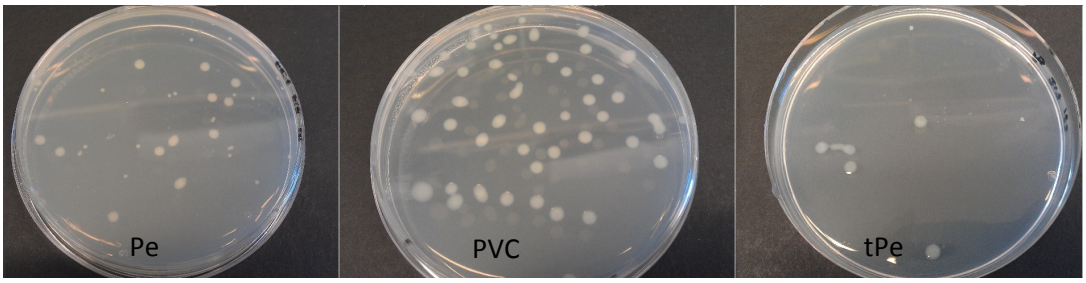

Figure 4: Shows a selection of the bacterial growth from HPC with a dilution factor of 10^{-3} that have been grown for 7 days.

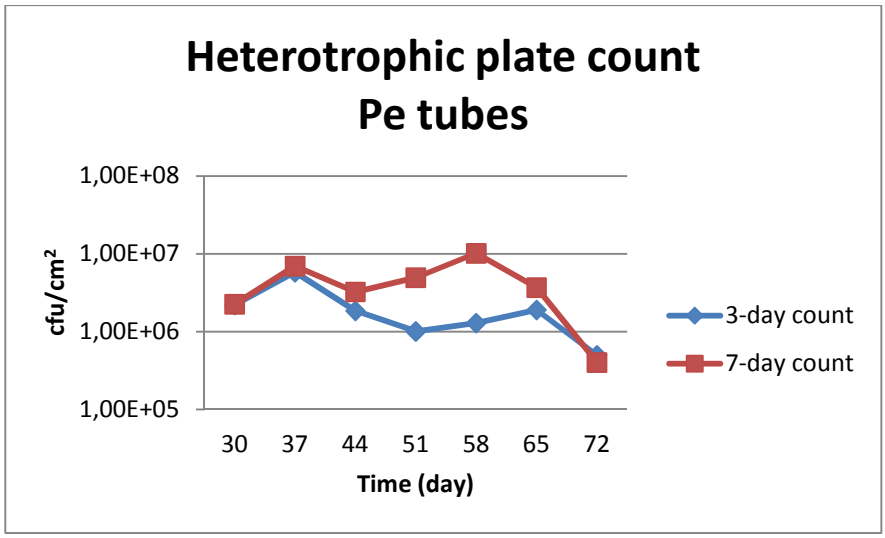

Figure 5: Quantification of cells attached to Pe tubes as biofilm per cm^2 by HPC.

As seen in figure 5 with Pe tubes, the seven day count appear to be almost as much as ten times the three day count at day 58. The brief cfu separation ends when the second to last sample was taken. The overall tendency in figure 5 is that both counts follow the same incline trend while seven day count has a higher bacterial count.

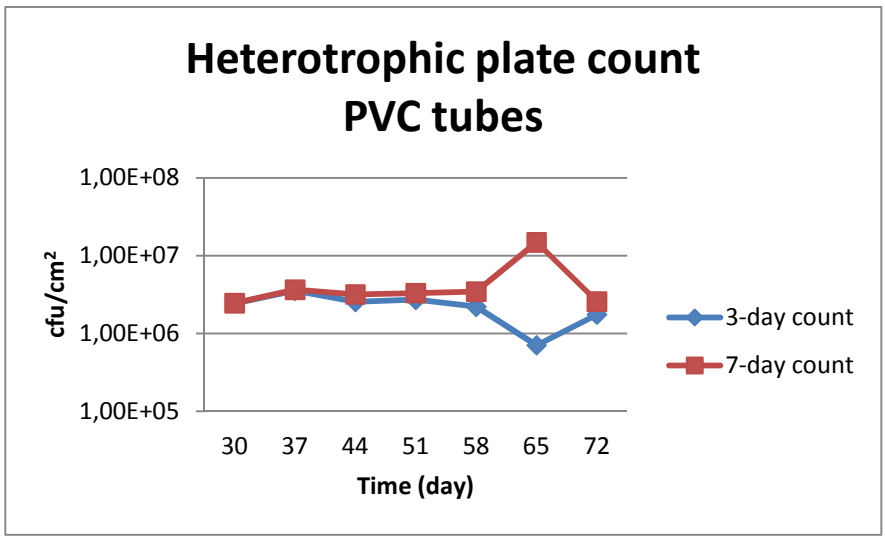

Figure 6: Quantification of cells attached to PVC tubes as biofilm per cm^2 by HPC.

The amount of cfu per cm^2 seen in PCV tubes (figure 6) did not differ as much as with Pe tubes except with a larger then 10-fold difference in the day 65 sampling. This sample showed a large increase for the seven day count and lowered three day activity which was the opposite of that seen in figure 5 at the same day.

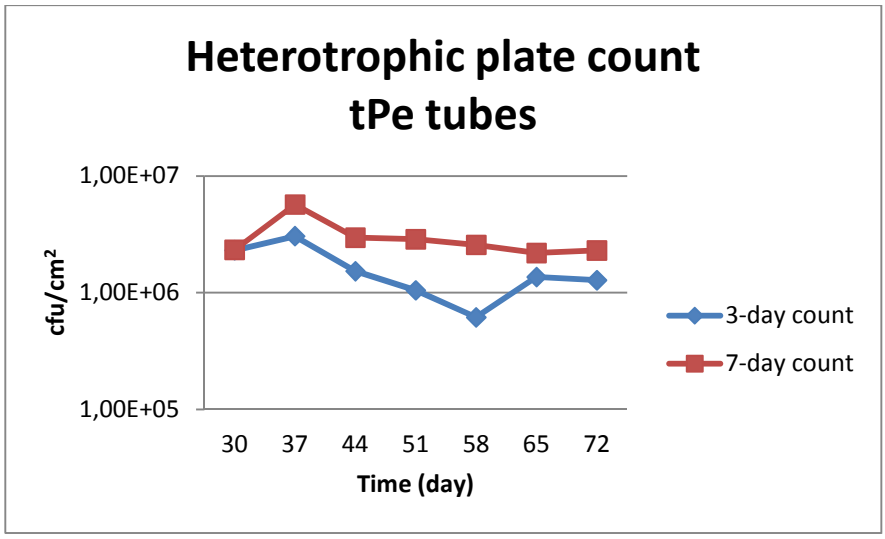

Figure 7: Quantification of cells attached to tPe tubes as biofilm per cm^2 by HPC.

In the tPe tubes (figure 7) a steady amount of cfu per cm^2 can be seen with the seven day count being higher compared to the three day count. These were no large (in comparison to figure 5 and 6) changes in the bacterial regrowth. There was a slight overall decrease in growth seen in this figure.

Table 2: Paired student t-test comparing three day counts with seven day counts

	P=
Pe	0.11
PVC	0.24
tPe	0.005

As seen in table 2, student t-tests regarding three day and seven day growth can be seen. There were a tendancy (p=0.11) of difference in growth on Pe tubes. PVC biofilm growth showed a higher p-value of 0.24. The tPe tubes however, showed a significant statistical difference of p=0.005 of growth between the two growth option.

To be able to compare the different tube materials the data have also been added to figure 8 and 9. All the three day HPC (figure 8) shows an increase in growth during day 37. Then a trend where all materials show an overall decrase in growth is seen. The rest of the samples did not reach up to the growth seen during day 37 again for all the repective tube materials. PVC biofilm seem to follow an overall decreasing trend together with tPe.

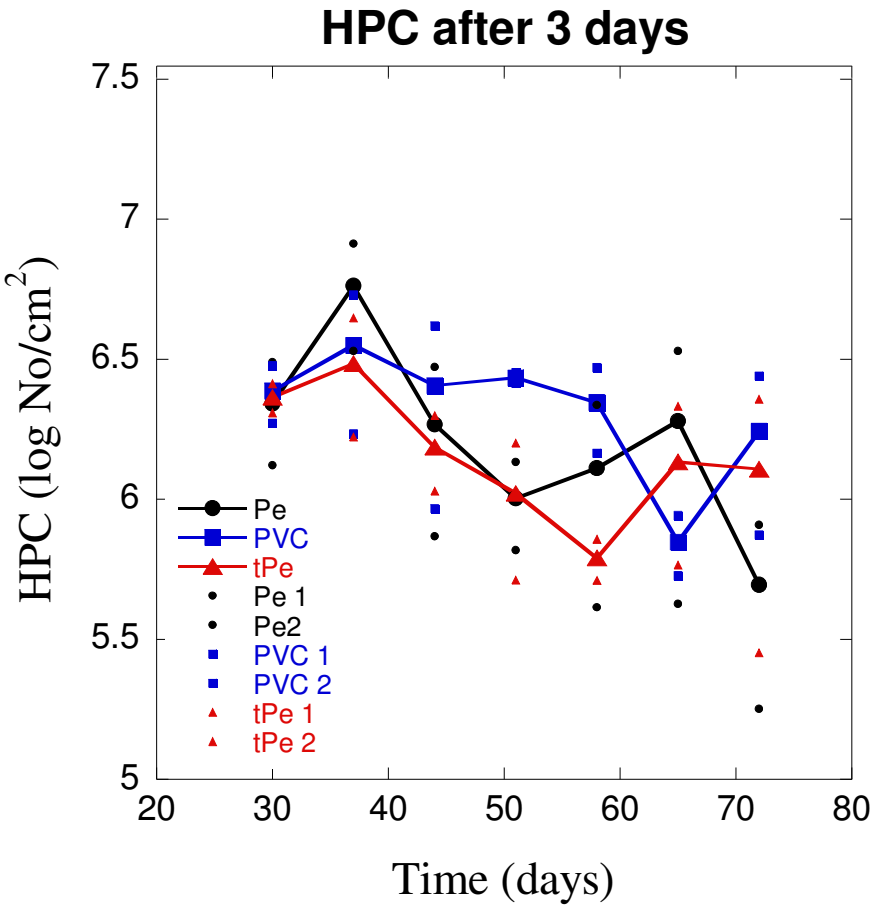

Figure 8: Biofilm quantification and comparison of tube materials after three days of growth on heterotroph plates. Smaller symbols and 1 and 2 denotes 2 replicates, with the large symbol connected by lines is the mean.

Similar to day 37 in figure 8, day 37 in figure 9 also show an increase in comparison to day 30. However there is a less evident trend after this. Pe pipe biofilm has a clear decrease during the last sample. The trend for both PVC and tPe is that of a steady state without much variation. There is one sample (day 65) however where PVC tube biofilm shows an uncharacteristic variation in the different replicates.

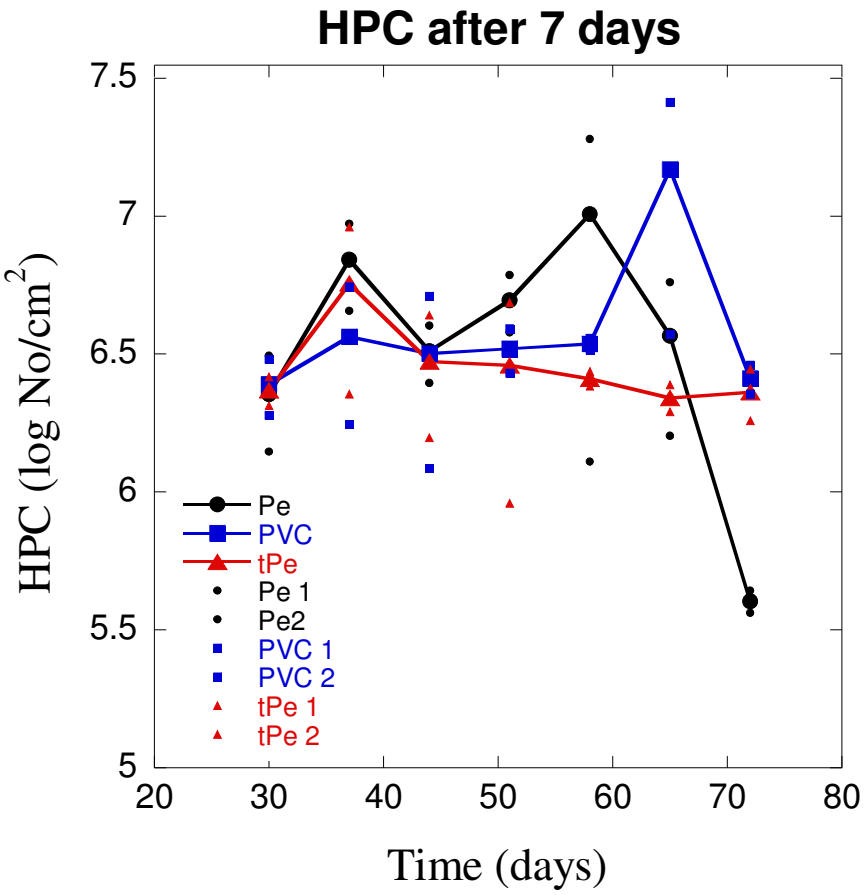

Figure 9: Biofilm quantification and comparison of tube materials after seven days of growth on heterotroph plates. Smaller symbols and 1 and 2 denotes 2 replicates, with the large symbol connected by lines is the mean.

Student-t-tests were made in order to test if there were and significant difference between the different tube materials. As seen in table 3, the p-values varied between 0.07-0.92 which is a huge span. It is safe to say that there was no significant difference between Pe and PVC tubes when it comes to HPC of their biofilm.

Table 3: Paired student t-test comparing cfu per cm^2 in different tube materials to each other.

	Three day count (p)	Seven day count (p)
Pe and PCV	0.58	0.92
PVC and tPe	0.07	0.38
Pe and tPe	0.20	0.30

As total cell counts also were performed for three of the samples taken, these are presented in figure 10. The total cell counts appear to have been around one magnitude lower than the HPC presented previously. As seen in the figure, Pe and tPe seems to follow the same cell count where day 65 shows an increase and day 58 and 72 is about the same. PVC biofilm, also in the same magnitude seem to be somewhat higher in count day 58 and 72 but lower day 65.

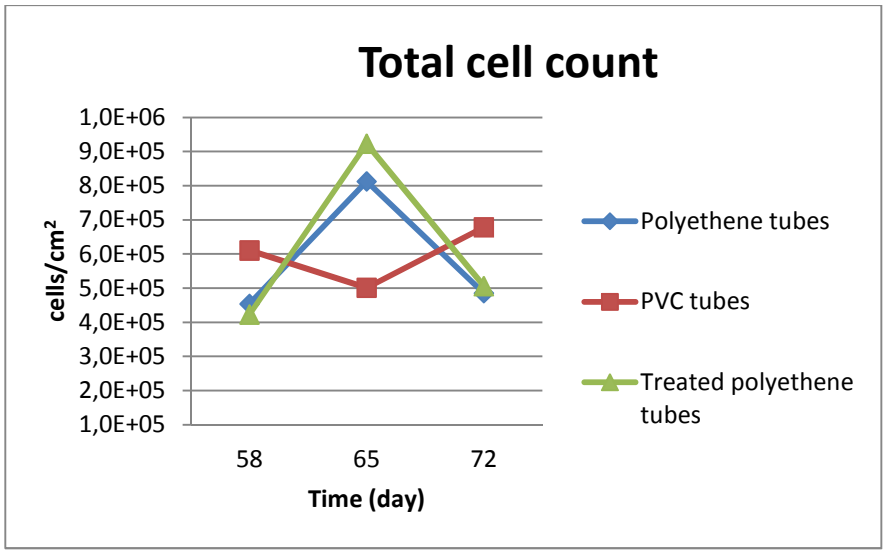

Figure 10: Total cell count quantification and comparison of different tube materials.

Discussion

Biofilms were grown in ordinary polymeric pipes (Pe, PVC and tPe) with drinking water flowing through and then total cell count and HPC data were obtained. The tube model simulating a drinking water distribution system used in this investigation ran continuously for 72 days with the exception of sampling days when tube sections were extracted. These extractions were timed to approximately one to one and a half hour in duration. During this time, the biofilm did not dry out but it is not impossible to imagine it having an effect on the results regarding biofilm buildup and subsequently the amount of growth seen. It is also noteworthy that the biofilm is best regarded as young biofilm since the maximum age is 72 days old and actual drinking water distribution systems and other investigations such as Martiny et al. (2003) and Wingender and Flemming (2004) were in use for years. This would mean that the investigation will mirror a biofilm in early developmental stages and prone to fluctuation in growth.

Although great care was given to ensure constant pressure in the distribution system by taking readings off the manometer, pressure did vary over time, especially hours after sampling. This could have been avoided using a more sensitive shut-off valve so that the pressure did not depend on how open the tap was. Also, considering that the residence time was 90 minutes and the water varied by room temperature and room temperature varied with outside spring temperature it is likely that biofilm growth is increased compared to an actual drinking water distribution network. In the first schematics of the system, the turns consisted of the samples. This was not possible due to the rigid structure of the tubes. In the case of Pe tubes, longer tube sections were added to turn the structure. The impact of these sections on growth is probably low. It is however possible that there is a higher growth rate downstream of the system because of how sampling was carried out but since parallel set ups would take too much space and water the serial system was decided upon.

The amount of silver ions in the tPe tubes as seen in table 1 corresponded well with a study that Prebona performed previously. This other study, although not based in water showed reduced growth with their recommended amount of silver ions (5 µg/cm^2). This recommended amount has later been increased, but it was decided to continue with the values seen in table 1. If experiments like these are done with Prebona protect in the future it is suggested to increase the amount of times the surfaces are painted in order to increase the silver ion content. Also important, is the drying process. In order to make an even layer of silver ions it is suggested to turn the tubes when drying and if possible to do this continuously. In this experiment, the tubes were turned only two times per paint session and this could have a negative impact on the effectiveness of the Prebona protect solution due to the fact of an uneven distribution.

The plates seen in figure 4 are a good representation of how most of the plates used looked like and also shows the colonies phenotype. Regardless of material type used to produce the biofilm the dominating colony

appeared milky in color. The different colony sizes also seen appeared in all type of biofilms. It is expected that the seven day count has a larger amount of cfus only because the slow growing bacteria manifests as colonies after a longer period of time. This corresponds well with the figures 5, 6 and 7 however in figure 6 no great separation can be seen. Since HPC is generally considered to end up in an estimation of cfu concentration below the actually concentration value it is possible to use seven day count to lower this error. The reason for variance in three and seven day count between figure 5, 6 and 7 may lay in the growth material its self or just random variation. In figure 5 which have an increase of slow growing bacteria (seven day count) may have a biofilm that favors these individuals during day 37-58 and a shift some day which shows up in sample 65.

In the case of figure 6 there is very little variation between three and seven day count. The decrease of three day count and large increase is interesting. This could have been some kind of change within the biofilm however it could also be some kind of error because during the last sample, the quantification level previously seen returned.

Figure 7 is the only set in the experiment that showed any significance as seen in table 2. In this case it appears that there was more growth during the seven day count or lowered growth during the period of the three day growth. There were however no large growth variation during the experiment. Considering that there were significance and no large variation, it is possible that the treatment of the tPe tubes did have some effect on the biofilm during the entire experiment favoring slow growing bacteria or disfavoring opportunistic ones.

In figure 8 tube material biofilm is plotted against each other after three days of growth. It can be seen that the materials seems rather equal except that the tPe overall appear low. The overall decrease seen in figure 8 could be because that the first sample was taken at day 30 and that there had been a period prior to this with a large increase in biofilm. However it is widely known that HPCs only allow a portion of a large pool of potential colony forming units to survive on agar and thus possible to concieve that the decrease in growth is because a shift in biofilm dwelling bacteria which are unable to grow on the type of agar used in this experiment.

There is an increase for all material biofilms growth between day 30 and 37. The reson for this could be because the first sample shows a too low growth. During the first samples the approximate dilution rate was unknown and resulted in less usable petridishes contrary (due to over growth) to all the other samples taken. There is also a visible biofilm increase period seen for all materials some time during second half of the experiment. These tests was performed during the spring with the start in march and ending in june. It is possible that temperature played a greater role later in the experiment leading to this increase in growth.

In the seven days of growth picture (figure 9) both Pe and PVC appear to have a cfu increase towards the end of the experiment while tPe is a lot steadier. For all compared materials it can be said the quasi-steady state is not affected to a great extent by the pipe material which the biofilm is grown at. This idea is strenghened by table 3. No significance level could be seen in the three or seven day count except in one case, the difference between three day PVC and tPe which was $p=0.07$. A comparison between Pe and tPe also did not result in any significance, however with more samples and time it is not unlikely that this could become significant. The variance between Pe and PVC seems caotic. There seem to be no trend to be found which also was strengthened by a p-value of 0.58. All these findings are in accordance with Manuel, Nunes, and Melo 2007 and Kerr et al. 1999 who saw no significant difference in biofilm growth on different polymeric materials and Tsvetanova 2006 saw lower growth in polymeric pipes compared to stainless steel and carbon steel. No study referred to in this paper tested any nano or silver surface as material. This surface cover used here, tPe, which has never been tested could in some way be hindering adhesion or growth on the surface. However, considering that with nano structures there are many unknowns, investigations regarding health and safety issues should be a priority in all cases, whether it be drinking water or rain water.

Figure 9 which show the slow growing heterotrophs shows less variance compared to figure 8 which showed faster growing bacteria. As in figure 8 the tPe biofilm also stands out in figure 9. Similar as before the tPe biofilm is constantly in the lower region of cfu/cm^2 but in this figure it looks to have less variance compared to all the other materials. Perhaps the initial colonization of the tPe tube is different and also later leads to difference in the slow growing community which could lead to less fluctuation in growth. With this being said, there is however no significance between the materials. Pe and PVC biofilms exhibit the same patterns with increase of biofilm in the second half of the experiment just to end with a decrease. Why this can be seen is however unknown.

The total cell count showed a magnitude of cells approximately 10-fold lower than that of the HPC which is to be expected without any dye. By not using any kind of dye and only counting in bürker chamber mistakes has most likely been made continuously when counting. With only three samples counted it is difficult to extract relevant information and in figure 10 it appears that tPe tubes does not have lower cells/cm^2 then the other materials as with most HPC in this paper. While extracting biofilm it is possible that the tPe tubes also released some of the applied nano paint surface which could possibly have gathered grime and thus giving rise to a higher number counted in microscope due to the fact that no bacterial dye was used.

The type of system used in this investigation can be compared to continuous reactors used in other investigations, such as Manuel, Nunes, and Melo (2007) which used two types of continuous reactors. They reason that the cause for a higher values of for example R_2A cultivable cells in the continuous reactors compared to a batch reactor was due to a much higher dilution rate or retention time compared to microbial growth rate, which stimulates adhesion. Manuel, Nunes, and Melo (2007) had similar results as presented in this investigation although the cell numbers appeared to be slightly higher reaching up to the magnitude of 10^7 for one continuous flow reactor. This indicates that using a large sized network of pipes compared to a smaller equivalent is of little use and that continuous flow reactors are good indicators of the situation present in real distribution networks. With this in mind, a large sized batch reactor is likely to also yield lower cells per cm^2 and similar results as seen in Manuel, Nunes, and Melo (2007).

Similar to this study, Tsetanova (2006), also studied different pipe materials and examined the biofilm formation process in PVC, Pe, stainless steel and carbon steel pipes. The biofilm was then subject to different tests including the number of heterotrophic bacteria by R_2A growth. The biofilm was grown under a longer period (0-150 days) compared to this study and it was shown that the highest bacterial density was in carbon steel while PVC and Pe showed the lowest growth. It was discussed that the pipe material influenced the biofilm growth in a strong manner during the initial phases, however unclear where this initial phase ends it would appear that around day 75 the influence became negated (Tsvetanova 2006). This would mean that in the experiment in this paper that kind of initial effect would likely to be undistinguishable due to this experiment only had 72 days of growth. If samples had been taken after 75 days it is possible that the differences would become even less evident.

In Manuel, Nunes, and Melo (2007) various plastic materials such as PVC and high density Pe was tested in different potable water reactors. After only a week of growth, an early steady state was seen around 10^6-10^7 cfu/cm^2 which is the same quantity of cultivable cells seen in this paper. Manuel et al (2007). took their first sample already after only 10 days and saw the bacterial amount already around the magnitude stated contrary to the first sample taken in this paper, which was after 30 days. Depending on if you are interested in very young biofilm development or older you need to keep this in mind when sampling. These results do correspond well with each other but using a real sized distribution system, as used here, it would not be appropriate to take as many samples needed to investigate the earliest attachment of bacteria, due to the difficulties during sampling.

Furthermore, Manuel, Nunes, and Melo (2007) also say that there was no effect on biofilm growth potential based on growth material. This was also what was seen in this paper, between PVC and Pe with the reservation of treated Pe which gave an indication of lower growth. This indication could possibly become even more significant if Prebona protect surface content were increased. In order to understand the effect material has on biofilm growth many variables must be looked into and analyzed. These can be hydrodynamic conditions, nutrient release from tube materials or the biofilm composition that different materials give rise to amongst others.

In conclusion and in an attempt to answer the questions stated before the project started (seen in the introduction) it is difficult to decide which of these materials used here promoted the highest development of biofilm because they all promoted similar amounts. There was however some difference in growth in the tPe tubes. The appearance of a developmental curve was also a difficult question partly due to the amount of biofilm had already been established after 30 days of growth and in part because this experiment did not extend into characterizations of the different biofilms in question. In order to answer this question in more detail parallel rigs with multiple sets of the same material would be a good beginning in addition to characterization by DNA sequencing. In order to simplify this work, smaller systems are to prefer and according to data gathered from references to yield approximately the same results as a large piped system would.

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