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Investigation on the effect of UV-treatment on biofilm and microbial composition in drinking water distribution system

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Investigation on the effect of UV-treatment on biofilm and microbial composition in drinking water distribution system

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Abstract

Drinking water is one of the most important resources in modern society. One recently discovered problem is the effect of biofilm in the drinking water distribution system and another recently discovered problem is that UV-treatment produces a selection pressure on the microbial community. The effect of UV-treatment on biofilm and microbial composition in Norrvatten's drinking water distribution system in northern Stockholm was investigated. Presence of microorganisms was investigated with cultivations in three different media, Casein-Yeast extract (C-Y), Nutrient broth with added salt (NB + NaCl) and Reasoner's 2 A (R2A), at different temperatures. The ability to form biofilm was conducted with one six week spanning incubation of plastic scraps in C-Y media with water samples, and a five week spanning incubation of plastic scraps in R2A media. Molecular experiments were also conducted targeting five different groups of microorganisms like, ammonia oxidizing bacteria, Actinobacteria, *Pseudomonas*, *Sphigomonas* and coliform bacteria. It was noted that NB + NaCl and R2A were better media for the water samples and that the number of colonies was higher after the UV-treatment in the pump station compared to before the UV-treatment. It was shown that UV-treatment lowers the ability to form biofilm. Actinobacteria was identified in all mixed cultures except, C-Y outlet water (PP208) Jan 25℃, and in most pure cultures except C-Y after UV-treatment in DWDS (PP693) room (colourless colony), before UV-treatment in DWDS (PP691) R2A room (colourless) and C-Y Pipe 25℃ (pink colony). It was shown that the genus *Bacillus* was present in samples from the pipe (that was dug up). *Janthinobacterium* from samples with pipe material incubated in the inlet, outlet after UV-treatment water samples. *Pseudomonas* from samples after UV-treatment, in both the treatment plant and in the pump station. The results indicate that more thorough investigation is required in order to confirm the effect of UV-treatment in the drinking water distribution system extending the construction of the UV-treatment process.

Sammanfattning

Dricksvatten är en de mest viktiga resurserna i det moderna samhället. Ett nyligen upptäckt problem är biofilmens effekt på dricksvattnet i ledningsnätet och ännu ett annat nyligen upptäckt problem är att UV-behandling gör ett selektionstryck på den existerande mikrobiella kompositionen. Effekten av UV-behandling på biofilm och den mikrobiella kompositionen i Norrvattens dricksvatten nätverk i norra Stockholm undersöktes. UVs effektivitet av att minska antalet odlingsbara kolonier och selektionstryck undersöktes också. Förekomst av mikroorganismer undersöktes genom odling med tre olika media, C-Y, NB + NaCl och R2A, och under två olika temperaturer, rumstemperatur (≈20℃) och 25℃. Kolonierna räknades och kolonier med olika färger och utseende undersöktes genom förstoring. Från odlingen visades det att NB + NaCl och R2A media var bättre media för odla vattenprov och att antalet kolonier var högre efter UV-behandlingen i pumpstationen än innan pumpstationen. Förmågan att skapa biofilm utfördes med en sex veckors lång inkubation av plastbitar i C-Y media med vattenprov, och en fem veckors lång inkubation av plastbitar i R2A media. Det visade sig att UV-behandling minskar förmågan att skapa biofilm. Molekylära experiment genomfördes, där förekomsten av fem olika grupper undersöktes; ammonium oxiderande bakterier, Aktinobakterier, *Pseudomonas*, *Sphingomonas* och koliforma bakterier.

Aktinobakterier detekterades i alla blandade prov förutom C-Y utgående vatten (PP208) Jan, och i de flesta oblandade prov förutom C-Y UV-renat vatten (PP693) (färglös), R2A inkommande vatten in UV-rening PP691 rumstemperatur (färglös) and C-Y rör 25℃ (rosa koloni). För att detektera andra grupper behövs mer optimering av protokollet. Slutligen så 16S rRNA amplifierades extraktioner från utvalda enartiga kolonier och skickades för sekvensering. Där visade det sig att gruppen *Bacillus* fanns i prov från röret som grävdes upp. *Janthinobacterium* från prov med rörmaterial inkuberade i vattenprov. *Pseudomonas* fanns i proven efter UV-behandling, både från reningsprocessen och från pumpstationen. Resultaten pekar på att en djupare undersökningar behöver göras, för att kunna konfirmera UV-reningens effekt på dricksvatten i ledningsnätet.

Keywords:
UV-treatment, monochloramine, drinking water, drinking water distribution system, biofilm, heterotrophic plate count

Table of content

Abbreviations

Casein-Yeast extract	C-Y
Nutrient broth	NB
Reasoner’s 2 A	R2A
Drinking water	DW
Drinking water distribution system	DWDS
Drinking water treatment plant	DWTP
Extracellular polymeric substances	EPS
Ultraviolet	UV
Assimilable organic carbon	AOC
Disinfection by-products	DPSs
Quorum sensing	QS
Quorum quenching	QQ
Room temperature	RT
Ammonia oxidizing bacteria	AOB

Introduction

Importance of drinking water treatment

Water is a fundamental substance that is necessary for all life. WHO (World Health Organisation) guideline values state that humans need a minimum water of 2 l per day. In rural parts in many countries, the drinking water is often retrieved from private or communal wells, however in populated areas the drinking water distribution system (DWDS) needs to be more organized. To urban areas, water from fresh water sources, such as lakes and rivers have to be treated and then distributed to the population.(1)

Since water is consumed daily and in large volumes, the purity of the drinking water plays an essential role in human health. Contaminated drinking water (DW) is a major concern and health risk which especially affects the already vulnerable population such as the elderly, infants and the sick.(2) There are different types of contaminated DW such as chemical or biological, but in this study the microbial contamination was looked into. The microbial contamination can either originate from the intake water to the drinking water treatment plant (DWTP) and subsequently survive in the treatment process. Another way for microbial contamination is that there exist bacterial communities in the pipes of the DWDS and that bacteria gets shredded off from the biofilm and ends up in the DW. Only between 2000 and 2007 there have been 354 outbreaks of diseases related to microbial contamination of DW in Europe.(3) In Sweden there have been an outbreak of *Cryptosporidium hominis* in Östersund in 2010, where 27.000 inhabitants got infected and all inhabitants needed to boil their DW for 3 months. And another outbreak in Skellefteå the year after, where 20.000 inhabitants got infected and the inhabitants needed to boil their DW for 6 months.(4) Therefore it is highly crucial to treat the water for human consumption.

The water from the fresh water source is treated in a number of steps to remove organic material and microorganisms. The DWTP is therefore the most important part of the DWDS. However, the water then passes through the pipe network before reaching the consumer. Since the pipe network is not a sterile environment, the microbial flora in the DWDS can affect the drinking water.(5) The longer time the water needs to be transported in the pipe the higher chance of regrowth of microorganisms. Therefore, additional treatment steps in the outer parts of the DWDS can be implemented to reduce the number of microorganisms in the DW for human consumption.

Drinking water treatment plant (DWTP)

It is important that the DWTP is working to ensure that pathogens do not thrive in the treated drinking water. The process can be varied depending on the composition of raw water used for the drinking water treatment. The treatment process of Norrvatten’s treatment plant is depicted in figure 1.

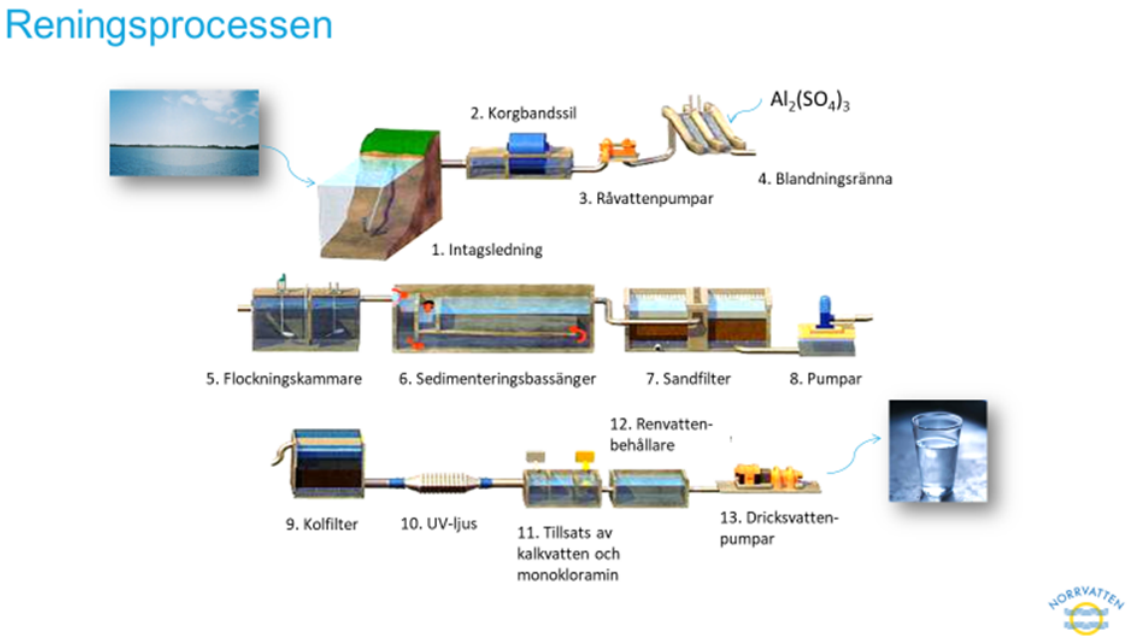


Figure 1. Shows the treatment process of Norrvatten's drinking water treatment plant: 1. intake line 2. wicker strainer 3. raw water pump 4. mixing groove 5. flocculation chamber 6. sedimentation pools 7. sand filter 8. pumps 9. carbon filter 10. UV treatment 11. addition of lime water and monochloramine 12. pure water container 13. drinking water pumps

In this study water samples from Norrvatten's DWTP and DWDS were examined. Therefore their treatment process will be explained. The incoming water to the DWTP is from lake Mälaren and the freshwater microbiota that survives the treatment can therefore become the foundation of biofilms in the DWDS. The intake water to the drinking water DWTP is either taken from a depth of 4 meters or 22 meters below the surface level depending on the season and present water quality. The intake water is only taken from 4 meters depth when there is ice cover on lake Mälaren. The first treatment step is that the incoming water gets filtered through a microfilter to ensure that large particles like fishes and algae are filtered off. Then the water is taken to a mixing groove where aluminiumsulfat is added, the mixture is moved into a flocculation or flotation chamber where particles such as organic particles and microorganisms gets flocculated with aluminiumsulfat. At the same time sodium metasilicate is added in order to make the flocculates bigger, which enhances the sedimentation. The next part is the sedimentation pools where the flocculates sink to the bottom, sediments and gets removed and if flotation is used, flocculates are removed by a surface scraper. Later the water is led to a 1.5 m thick sand filter where the flocculates which were not sedimented are removed. Then the water is led through a 2.5 m thick bed of granulated active carbon. The last treatment step is that the water is exposed to UV at ∼400 J/m^2. After that lime is added to adjust the pH to around 7.5. At the same time monochloramine is added to inhibit microbial growth in the DWDS. Then the water is stored in a storage tank and pumped into the DWDS.(6)

Disinfectants

Most DWTPs use flocculation, sand- and carbon-filters to purify the DW. However, disinfectants can also be used to purify the DW. They can especially help to maintain good water quality throughout the DWDS. Other disinfectants/treatment methods than the ones used in Norrvatten are using ozone or chlorine. Chlorine is very similar to monochloramine, except it has a different structure and is more reactive, which is further explained below. Ozone is a strong oxidant and will react with material in the water that is being processed, such as organic and inorganic material. This therefore removes microorganisms and odor/taste substances in the drinking water.(7)

One disinfection method that is used in Norrvatten's DWTP is to use UV-treatment. By exposing the DW to ultraviolet light the nucleotides inside the microorganisms can absorb the energy, which causes damage to the DNA, in the form of DNA lesions. The nucleotides absorb light at wavelengths between 200-300 nm, with an optimum around 260-265 nm. The nucleotide then enters an excited state where the formation of DNA lesions are possible. One such DNA lesion that could form is a pyrimidine dimer. Due to the structure of pyrimidines (Cytosine and Thymine) they are more sensitive to UV than purines (Adenine and Guanine).(8) The microorganisms have mechanisms to repair the damaged DNA, but if these repair systems fail, the microorganism may die.(9)

Another disinfection method that is also conducted at Norrvatten's DWTP is the addition of monochloramine. As with chlorine, monochloramines is a reactive molecule that will react with the microorganism to make it nonfunctional.(1) Monochloramine is a bit less reactive than chlorine, which makes it less efficient in certain ways. However, it also makes it last longer in the DWDS and forms less disinfection by products (DBPs). It is the DBPSs and the chlorine/monochloramine that give the distinct smell and taste of certain DW.(9)

Advantages and disadvantages of disinfectants

Since the different disinfectants used in DWTP work in different ways, they also have different advantages and disadvantages. All disinfectants have a downside, which is the selection of the microorganisms which are most resistant to the disinfectant being used. By combining different disinfectants the problem of selection will be minimized since there are fewer organisms that are resistant to both UV and monochloramine. The advantages of UV-treatment are that it is an easy and efficient method. Compared to the addition of monochloramine which requires high quantities of hazardous material, UV-treatment only requires UV-lamps and energy to irradiate the DW. Also the formation of disinfection by-products (DBPs) is less than when chlorine or monochloramine are used.(10)

It has been shown that UV-treatment can induce a viable but nonculturable state in the microorganisms.(11) Another disadvantage with UV-treatment is that it only controls the growth of microorganisms in one place. UV-treatment vastly reduces the amount of microorganisms in a certain place, however some microorganisms may become resistant and some spores can escape the radiation. Compared to chlorine and monochloramine which is added to the DWDS and follows with the water and continuously prevents microorganism regrowth. One advantage with monochloramine is that since it is less reactive

it can more easily penetrate the biofilm in the pipes of the DWDS. However, a disadvantage is that they contain amine which can act as a nutrient for ammonia oxidizing bacteria in the DWDS, since monochloramine breaks down to ammonium.(9)

Distribution network

Figure 2. Shows the municipalities where Norrvatten distributes drinking water to. The red circle is the DWTP at Görväln island and the blue circle is the approximate position of the pumping station with UV-treatment located on the DWDS. Exact locations of pipes and pumping stations are not shown due to security reasons.

As mentioned before, monochloramine is added to stop the growth of microorganisms in the DWDS. However to better understand the situation, Norrvatten's DWDS needs to be further explained. As mentioned, Norrvatten has their DWTP on Görväln island in Järfälla municipality. The drinking water is then distributed to municipalities in the northern Stockholm area. The municipality furthest away from the DWTP is Norrtälje (fig 2), since it is in the furthest ends in the DWDS there have been recurring problems of 3 and 7 days microbial cultivation levels higher than the standard limit, but still being acceptable values. Due to the distance and subsequent time the water has to be transported to Norrtälje, which can be as high as 7 days. Combined with the loss of monochloramine effect due to the breakdown of monochloramine in the DWDS, is the major reason why the cultivation results are higher in Norrtälje than at the DWTP. Therefore an UV-treatment has been installed in Norrtälje near the end of the drinking water network. The pipe material used in the DWDS also plays an important part. Three commonly used pipe materials are iron, copper and plastic. Both iron and plastic pipes can provide nutrients to microorganisms in the DWDS.

While copper pipes have a slight antimicrobial effect.(9) In Norrvatten's DWDS mostly iron, steel and plastic are used.

Biofilm formation and growth in distribution network

Biofilm can be formed in the pipes of DWDS, as well as on most other surfaces. Biofilms are formed in a cycle of five stages. In the first stage microorganisms bind to the surface reversibly with the microbes flagella, pili, fimbriae, curli fibers and membrane proteins. Then the microbes start secreting extracellular polymeric substances (EPS) so they become irreversibly bound to the surface. The microbes become absorbed on either the surface or on each other. Later the microbes become embedded in the hydrogel of EPS, this provides a layer of protection which leads to the microbes able to resist disinfectants. The biofilm is further evolved by quorum sensing (QS) where cells release molecules that when received by other cells result in a change in cellular function. The biofilm grows bigger and the EPS gives the integrity of the biofilm. However, shear forces from the water can make parts of the biofilm to detach. The detached biofilm can then start a new biofilm further downstream.(12) These detached biofilm have been selected to a sessile lifestyle inside the biofilm and can therefore have genes coding for resistance for different disinfectants. However, they may lack genes necessary for a planktonic lifestyle, the detached biofilm therefore needs to find another surface or change to a planktonic lifestyle inorder to survive. If the sessile cells can change to planktonic lifestyle while at the same time continue to have resistance to disinfectants they can prove to be a big problem.(13)

Biofilm structure and microbial composition

One of the major problems with biofilms is that they are very persistent and hard to remove from the DWDS, which leads to that once a biofilm is formed in the DWDS it will most likely stay there. The biofilms can also secrete compounds that are associated with bad taste and odor or parts of the biofilm can shear off, which can lead to a microbial contamination. Moreover the biofilm can influence the fluid dynamics of the DWDS and acids can be secreted which can correlate the pipes.(14)

One major factor for biofilm formation is temperature. Generally a higher temperature give rise to a higher microbial growth. The composition of the biofilm also changes, due to the higher temperature selecting the microbes that are best suited for growth at that temperature. In a DWDS with a lower temperature (3-18℃) *Alphaproteobacteria* and *Actinobacteria* were the two dominant phyla. In iron pipes *Pseudomonas, Lysinibacillus* and *Flavobacterium* were abundant while in polyethylene pipes *Spirochaeta, Methylobacterium* and *Clostridium* were abundant.(15)

Biofilm microbial community

The actinobacterial cluster ACK-M1 and the closely related Sta2-30 actinobacterial cluster, are clusters of species that are a part of the Actinobacteria phylum. Both are a part of the microbial community of many freshwater lakes in northern Europe and in other parts of the world.(16,17) The phyla usually found in DWDS are alpha, beta-, gammaproteobacteria, Actinobacteria, Firmicutes and Bacteroidetes.(5) Other sources found that Proteobacteria,

Bacilli and Actinobacteria were the most common phyla in DWDS.(15) While the genera usually found are *Sphingomonas, Pseudomonas, Acidovorax, Brevundimonas, Acinetobacter, Methylobacterium, Microbacterium* and *Bacillus*. The biofilm is made up of a number of species that interact with each other in a complex way.(5) As found by many studies the *Proteobacteria* phylum is the most common phylum found in DWDS.(15,18,19) Certain species associated with the genus *Acinetobacter*, *Pseudomonas*, and *Sphingomonas* are chlorine resistant, pathogenic bacteria. They therefore pose a problem for DWTP and it is therefore important that the UV-treatment step can remove these bacteria.(18) As mentioned above the interaction between different species in a biofilm is complex. In the referred article it was shown that *Acinetobacter*, *A. deluvii* and *B. cereus* weakly promoted the formation of a multi-species biofilm. The same study showed that *M.laevaniformans* can inhibit the formation of *B.cereus, A. calcoaceticus* and *Salmonella typhi* by producing antimicrobial secondary metabolites and quorum quenching (QQ). While *Sphingomonas* have the ability to promote multi-species biofilm growth probably comes from that it produces important molecules for quorum sensing (QS) to increase biofilm growth. *B.cereus* and *M.laevaniformans* promoted the resistance against chlorine in the biofilm, regardless of species present.(5) Another study showed that *Acinetobacter, Burkholderia, Methylobacterium, Mycobacterium, Pseudomonas, Sphigomonas* and *Staphylococcus* are important in promoting biofilms.(15) Another study also state that in addition to *Pseudomonas*, the two genuses *Zooglea* and *Janthinobacterium* can express EPS at an higher level and therefore contribute to the biofilm formation.(20)

Kocuria rhizophila is a specie from the family *Micrococcaceae* found in environmental samples as well as on skin and have the ability to survive harsh environments and tolerate up 10 % NaCl.(21) *Micrococcus* is very closely related to *Kocuria*, and like species from that group have a high ability to survive harsh environments due to the ability to enter a dormant state.(22) The genus *Brevundimonas* is a newly created genus made up of mostly previous designated *Pseudomonas*. Found in most environments, such as soil and aquatic habitats, and have a high ability to survive, in different sources.(23)

Microbial community and UV-treatment

In the referred study it was shown that *Nitrosomonas* increased after UV-treatment and *Flavobacterium* decreased after UV-treatment. Both of these species were found in the biofilm and since *Nitrosomonas* is an important nitrifying bacteria it can affect the chemical composition of the drinking water. *Chitinophagaceae* is one family that decreases after UV-radiation however members of this family can break down the odorant 2-methylisoborneol (2-MIB) which is produced by *ACK-M1* of the order *Actinomycetales*.(8) Previous studies showed that *Nitrosomonas* which is an ammonia oxidizing bacteria could be present. Also that *Pseudomonas* and *Sphingomonas* could be present and that they contribute to the formation of biofilm. Lastly, many previous studies stated that many clusters of Actinobacteria exist in freshwater. The primer used in this study in the molecular part was one, to detect ammonia oxidizing bacteria, targets the 16S rRNA of ammonia oxidizers belonging to the betaproteobacteria class.(24) Two, to detect Actinobacteria and the primer used targets the V3 to V5 region of the 16S rRNA gene.(25) Three, to detect *Pseudomonas* and the primer targets the rpoD gene in *Pseudomonas* of the class of gammaproteobacteria.(26) Four, to detect *Sphingomonas* and the primer targets a highly

conservative part of the 16S rRNA sequence specific to *Sphingomonas*.(27) Five, to detect coliform bacteria with a primer that targets the LacZ gene.(28)

Methods

Sampling

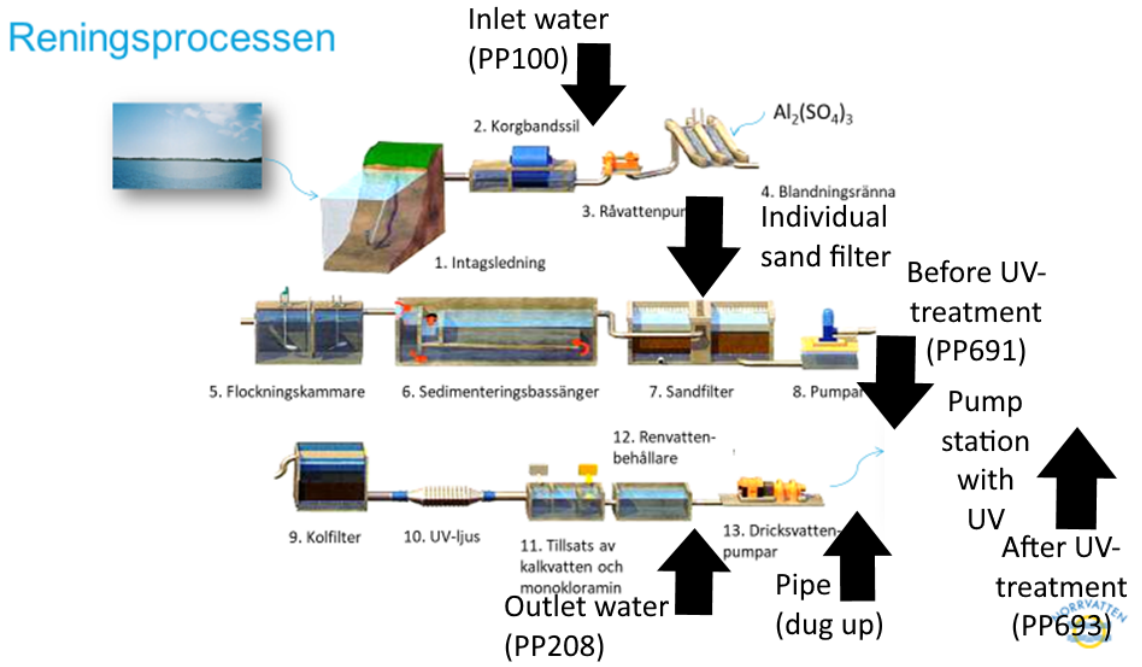


Figure 3. Describes the sampling points in this study. PP100 refers to samples taken just after the wicker strainer, SF4 refers to samples taken from a pipe leading out from sand filter 4, PP208 refers to samples taken from water exiting the pure water container, pipe refers to samples taken from the pipe that was dug up from the Görväln island, PP691 refers to samples taken from water entering the pumping station with UV-treatment in the DWDS and PP693 refers to samples taken from water exiting the pumping station with UV-treatment in the DWDS.

Water samples were collected on six points in the DW treatment process, at three different time points, Nov 2020, Jan 2021 and Feb 2021, can be seen in table 1 and fig 3. The water samples PP100 - PP693 were collected by Norrvatten, for the taps where it was not possible to have the water continuously running, the taps were burned to minimize contamination. Otherwise the water was sampled when the temperature was stabilized which took approximately 10 min of tapping. The water was then collected in sterile 0.5 L bottles which contained 20 mg/ml sodiumthiosulfate to bind the remaining monochloramine. The water samples were then stored in the dark 4℃ until used. The pipe sample was dug up from the ground on the 12/2-21, then cut into a handleable size and stored in the dark at 4℃ (fig 4.A). The inside of the pipe was scrapped or swapped when samples were needed. SF4 was scrapped in Norrvatten's DWTP on 23/2-21, then stored at 4℃. Pieces of SF4 samples were taken for extraction when needed. m100-m693 samples were taken on 25/2-21, when a piece of plastic pipe material which had been cut into smaller pieces and then treated with hypochlorite was added to each bag (fig 4.B). An explanation of the sampling sites in correlation to the DWDS can be seen in figure 3.

Table 1. Describes the time of sampling and the sample name corresponds to the treatment and distribution network.

Sample name	Date of sampling	Sampling place
PP100 Nov	5/11-20	Raw water in treatment plant
PP100 Jan	27/1-21	Raw water in treatment plant
PP100 Feb	22/2-21	Raw water in treatment plant
PP208 Nov	5/11-20	Outgoing water from treatment plant
PP208 Jan	27/1-21	Outgoing water from treatment plant
PP208 Feb	22/2-21	Outgoing water from treatment plant
PP691 Jan	27/1-21	Before pump station with UV-treatment
PP693 Jan	27/1-21	After pump station with UV-treatment
Pipe	12/2-21	Pipe from the Görväln island
SF4	23/2-21	From pipe leading out from sand filter 4, in treatment plant
m100	25/2-21	Pipe material incubated in raw water in treatment plant
m208	25/2-21	Pipe material incubated in outgoing water from treatment plant
m691	25/2-21	Pipe material incubated in ingoing water to pump station with UV-treatment
m693	25/2-21	Pipe material incubated in outgoing water from pump station with UV-treatment

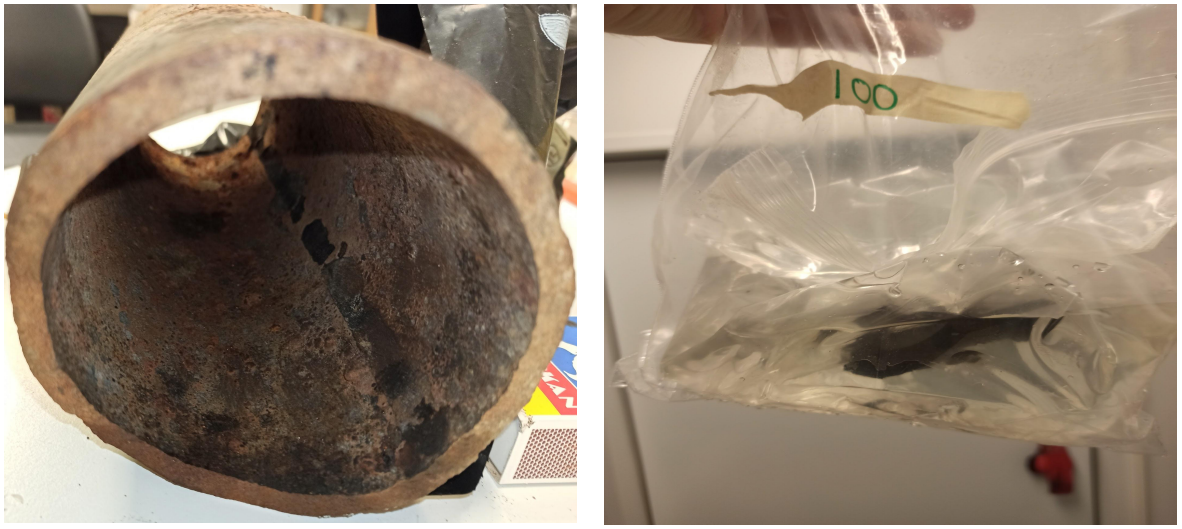


Figure 4. A Pipe from Görväln island, which was dug up on 12/2-21. B Pipe of plastic material incubated with an inlet (PP100) water sample.

Media and cultivation of microorganisms

A casein-yeast agar media (casein-peptone 6 g/L, yeast extract 3 g/L and agar 15 g/L) was mixed, autoclaved and poured into plates at the same time a liquid media without agar was prepared. Later a nutrient broth with 5 g/L NaCl media (nutrient broth 8 g/L, NaCl 5 g/L and agar 15 g/L) and a modified R2A media (yeast extract 0.5 g/L, peptone 1 g/L, glucose 0.5 g/L, K_2HPO_4 0.3 g/L, $MgSO_4 \cdot 7H_2O$ 0.05 g/L, sodium pyruvate 0.3 g/L and 15 g/L agar) was made, autoclaved and poured into plates. At the same time a liquid media without agar was also made.

The cultivation method resembles the method outlined in SS-EN ISO 6222-1.(29) However some differences between the cultivations in this project and those done in SS-EN ISO 6222-1 are that in the SS-EN ISO 6222-1 the cultivation is done at 22℃, while the cultivations in the project was carried out at either RT(20℃), 25℃, 30℃ or 37℃. In SS-EN ISO 6222-1 (which Norrvatten follows) the water sample is mixed with the agar solution, while in this project the samples were applied by spreading them on a solidified agar plate. The incubation time is also slightly different, in SS-EN ISO 6222-1 the incubation time is 68 h, but in the project it was 72 h.

Plating of water samples (see table 1) was done by adding 100 µl of water sample to a solidified agar plate, and then spread out by a raklar. For the pipe sample a cotton swab was soaked in a saline solution (9 g NaCl/L), then swabbed on the inside of the pipe and then spread across the solidified agar plate. The used cotton swab was then put into a 50 ml falcon tube with approximately 10 ml of liquid media. Plating and analysing microbial growth was done in the same way as with all three media. The plates were incubated at room temperature (RT) and at 25℃.The liquid media samples were incubated at RT (shaking at 70 rpm), 25℃ (shaking at 70 rpm) and 30℃ (shaking at 100 rpm). After 3 days a photo was taken of the plates that had colonies and they were subsequently counted, doing a heterotrophic plate count (HPC) After 7 days photos were taken on the plates that did not have any growth after 3 days but had growth after 7 days. The new colonies were counted,

and the plates stored in a fridge. For the plates with pipe samples only a photo was taken. For the plastic pipe material samples that was incubated in water sample, the pipe was swabbed by a cotton swab and then spread across a solidified agar plate. The plates incubated in either RT or 25℃.

Colonies that were different in morphology and color were investigated with a phase contrast microscope (Olympus CX31, Japan).

Biofilm formation

Five ml of each water sample (table 1) and 10 ml of C-Y medium in two experiments and R2A medium in two experiments were added to 50 ml Falcon tubes or a 12 well microtiter plate. To grow the biofilm on a plastic material (pieces of falcon tubes, fig 5), 6 pieces were placed into each tube. The samples were then incubated at 25℃ (70 rpm). After each week one plastic piece from each tube was collected and rinsed with MQ water. They were then placed into a mini petri dish separately, an unused plastic scrap was put into a mini petri dish as a control. This method was modeled after the method developed by Djordjevic *et al*(30). Two ml of 0.1 % crystal violet solution were added to each plastic scrap, the mini petri dishes were then tilted so most of the crystal violet was in contact with the plastic scrap. The plastic scraps were incubated for 15 mins, and then the plastic scraps were rinsed with MQ so that all excess crystal violet was washed away. Two ml 70 % ethanol were added to each sample and carefully mixed so the ethanol could come into contact with all the plastic. Followed by taking one ml of each sample, placed it into a cuvette and measured the optical density at OD 550 nm in a spectrophotometer (Biochrom WPA Biowave, USA), and an one ml 70 % ethanol was used as a blank. By subtracting the absorbance of the control the formation of biofilm could be estimated.

A similar experiment was conducted with a 12 well to grow biofilm. The same procedure was followed with the following exceptions: 20 µl sample and 1.88 ml liquid media was added into each well. The rest of the method was as outlined above.

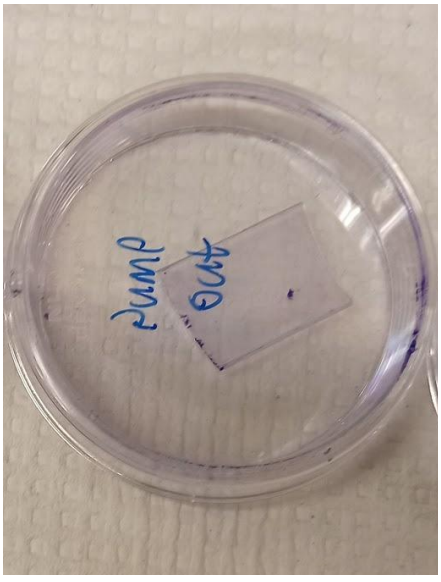


Figure 5. Showing a piece of plastic scrap, which was used to cultivate biofilm on.

DNA extraction

All samples except SF4 (sand filter material) were extracted by the following method while the pipe that was dug up was extracted by both methods. The extract from the mixed cultures came from the cultures that was used to form biofilm:
DNA was extracted from bacterial isolates randomly selected from an agar plate and mixed liquid media (to enhance the bacterial growth) using illustra genomic DNA extraction kit (GE healthcare) and the protocol was followed as described by the manufactures for gram-positive bacteria.

SF4 soil and pipe soil samples were extracted by the following method:
DNA was extracted from samples with Qiagen DNeasy PowerSoil kit (Qiagen) and the protocol was followed as described by the manufacturers.

The concentration of DNA was measured with a nanodrop to ensure that the extraction worked.

Polymerase chain reaction (PCR)

All PCR reactions were conducted with a MJ Research PTC-200 Thermal Cycler (MJ Research) and all gels except one gel for isothermal were run on a 1 % agarose gel, with 1 drop serva per 100 ml agarose mixture. For 30 min with a power pc 300 (BioRad), where 5 µl of the PCR product was loaded. The gels were then analyzed with a Gel Doc EZ imager (BioRad). The samples were diluted or concentrated to have a starting concentration of a DNA template of about 40-60 ng/µl.

The primers bAMOf (TGGGGRATAACGCAYCGAAAG) and bAMOr (AGACTCCGATCCGGACTACG) which targets the 16S rRNA of ammonia oxidizers belonging to the class betaproteobacteria.(24) were used in the PCR with the following settings and the expected size of the amplified product was 1.1 kb. The PCR mix consisted of 4.0 µl 5X reaction buffer, 1.0 µl forward primer (bAMOf 10 pmol/µl), 1.0 µl reverse primer (bAMOr 10 pmol/µl), 0.4 µl dNTP mix, 0.2 µl Phusion polymerase, 12.4 µl sterile water and 1.0 µl template DNA, with a total volume of 20 µl. The program was as follows: 98℃ 1 min 35 cycles of 98℃ 30s, 58℃ 30s, 72℃ 1 min and final extension with 72℃ for 10 min.

The primers S-C-Act-235-a-S-20 (CGCGGCCTATCAGCTTGTTG) and S-C-Act-878-a-A-19 (CCGTACTCCCCAGGCGGGG) which targets the V3 to V5 region of the 16S rRNA gene specific to the Actinobacteria phylum (25), were used in the PCR with the following PCR settings and with an expected size of the amplified product around 640 bp. The PCR mix consisted of 4.0 µl 5X reaction buffer, 1.0 µl forward primer (ACT235f 10 pmol/µl), 1.0 µl reverse primer (ACT878r 10 pmol/µl), 0.4 µl dNTP mix, 0.2 µl Phusion polymerase, 12.4 µl sterile water and 1.0 µl template DNA, with a total volume of 20 µl.The program was as follows: 98℃ 2 min 35 cycles of 98°C 10 s, 70℃ 1 min and 72℃ 1 min. Lastly 72℃ for 10 min.

The primers PsEG30F (ATYGAAATCGCCAARCG) and PsEG790R (CGGTTGATKTCCTTGA), which targets the rpoD gene in *Pseudomonas* of the class of gammaproteobacteria (26), were used to amplify a gene fragment with size of 700 bp. The concentration was: 10 µl 5X reaction buffer, 2.5 µl forward primer (PsEG30F 10 pmol/µl), 2.5 µl reverse primer (PsEG790R 10 pmol/µl), 0.64 µl dNTP mix, 0.5 µl phusion polymerase, 37.86 µl sterile water and 1 µl template DNA, with a total volume of 50 µl. The program looked as follows: 98℃ 2 min, 30 cycles of 98℃ 10 s, 55℃ 1 min and 72℃ 90 s. Lastly 72℃ 10 min.

The primers SA429f (TAAAGCTCTTTTACCCG) and SA933r (TTTGGTGTACGAGGTGG), which targets a highly conservative part of the 16S rRNA sequence specific to *Sphingomonas* (27), were used to amplify a 500 bp long gene fragment. Concentrations 10 µl 5X reaction buffer, 1.0 µl forward primer (SA429f 10 pmol/µl), 1.0 µl reverse primer (SA933r 10 pmol/µl), 0.64 µl dNTP mix, 0.5 µl phusion polymerase, 41.86 µl sterile water and 1.5 µl template DNA, with a total volume of 50 µl. The program and concentration look as follows: 98℃ 2 min. 35 cycles of 98℃ 10 s, 50℃ 45 s and 72℃ 90 s, lastly 72℃ 8 min.

An isothermal PCR with the primers FIP (CAAGCCGTTGCTGATTCGCGTTTTTGACCTGACCATGCAGAGG), BIP (CCCTTCAGCAGCAGCAGACCTTTTACGCTGATTGAAGCAGAAGC), F3 (ACCATCGTCTGCTCATCCA) and B3 (TTAAACTGCACACCGCCG) which targets the LacZ gene (28), to get an expected amplified product of 100 bp. The formation of the product was also tested with fluorescence. 2.5 µl 10X reaction buffer, 0.888 µl FIP, 0.888 µl BIP, 0.111 µl F3 (taken from 1 pmol/µl), 0.111 µl B3 (taken from 1 pmol/µl), 3.5 µl dNTP mix, 1 µl bst polymerase, 2 µl $MgSO_4$ (both from new england biolabs), 10.5 µl sterile water and 1.5 µl template DNA, with a total volume of 20 µl. The gel was run for 45 min in a 2 % agarose gel. The following program and concentrations were used: 98℃ 2 min, 65℃ 60 min and 72℃ 10 min.

Sequencing

The 16S rRNA gene amplification was done for the selected bacterial colonies in order to find out the microorganism present in DWDS. The PCR mix was as follows: 4.0 µl 5x reaction buffer, 1.0 µl forward primer, 1.0 µl reverse primer, 0.4 µl dNTP mix, 0.2 µl polymerase enzyme, 12.4 µl sterile water and 1.0 µl extracted DNA. And the PCR program was as follows: 98℃ 30 s, (98℃ 10 s, 61℃ 30 s, 72℃ 90 s) for 30 cycles, then 72℃ 10 min and hold at 4℃. 5 µl of the product was run on a 1 % agarose gel for 30 min.

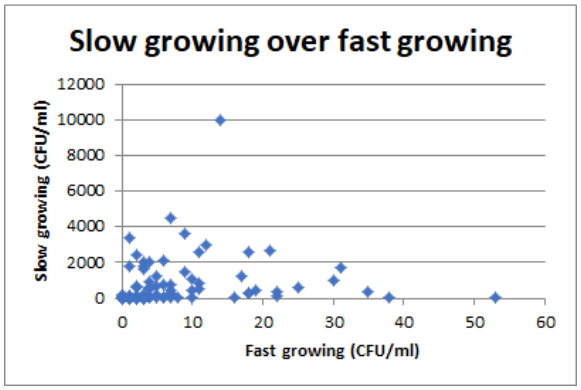
Data analysis from Norrvatten databank

Data regarding fast/slow growers, temperature, UV-treatment, conductivity and pH was compiled in an Excel sheet. After the data had been organized graphs were made.

Results

Data from Norrvatten

The data acquired from Norrvatten was dated between 2015 and 2017, with the samples being water samples collected before and after UV-treatment in DWDS, and when the UV-treatment was on or off in the DWDS. The data was processed and compiled in Excel, which can be seen in fig 6. No clear correlation can be seen between the colony forming units of slow growing (7 days) and of fast growing (3 days) microorganisms (fig 6.A). Figure 6.B shows a possible correlation between slow growers and temperature, the highest growth occurred at a temperature around 12℃. Figure 6.C shows slow growers over time with UV-treated samples marked for a possible correlation with UV, the correlation being that there is minimal growth with UV-treatment. Figure 6.D shows a possible correlation between slow growers and temperature with data from Synlab, where the data was collected from 2014 to 2020. There is a trend that the optimum temperature for growth seems to be around 10-15 ℃ as seen in figure 6.B and 6.D. In figure 6.B there is nearly no growth when the temperature is under 10℃. As can be noted is that the dataset from Norrvatten and Synlab varies.



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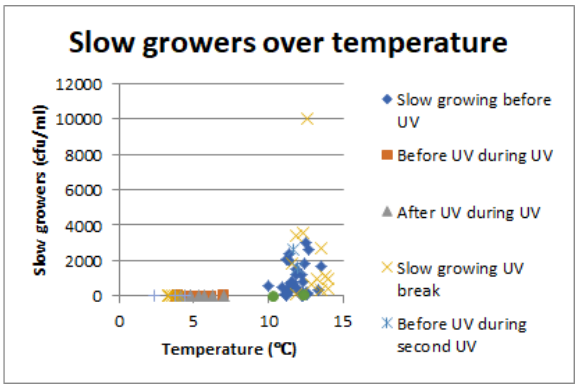

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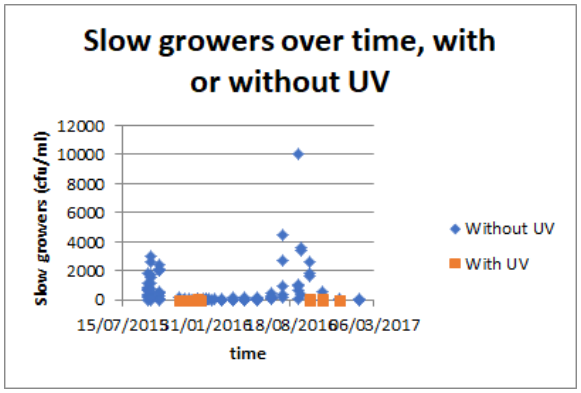

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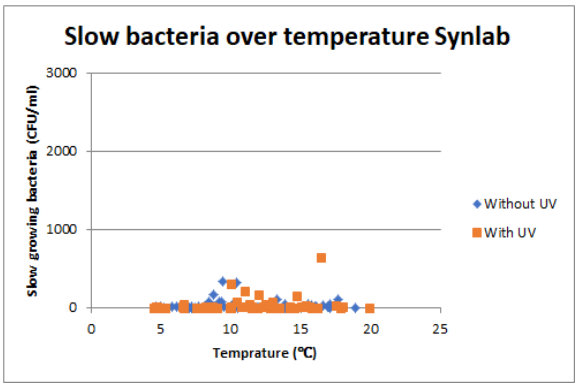


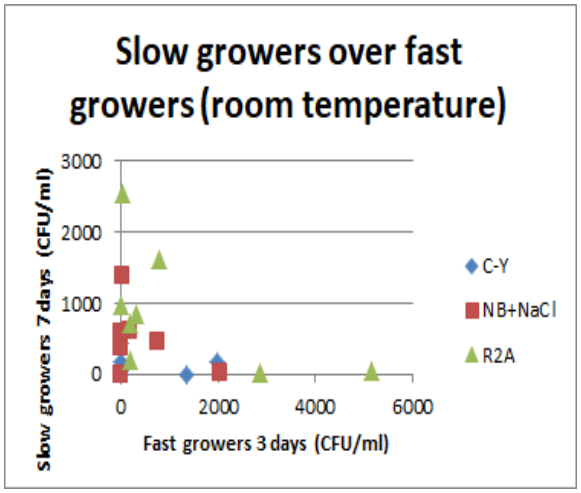
D

Figure 6. A, depicts slow growers (CFU/ml after 7 days) over fast growers (CFU/ml after 3 days), of water samples from Norrtälje cultivated on C-Y media over a period from 15/9-15 to 23/5-17 in Norrvatten. B, depicts slow growers over temperature, of water samples from Norrtälje, cultivated on C-Y media over a period from 15/9-15 to 23/5-17 in Norrvatten. C, shows slow growers over time, of water samples from Norrtälje cultivated on C-Y media over a period from 15/9-15 to 23/5-17 in Norrvatten. Where cultivation with and without when the UV-treatment is indicated.D, show slow

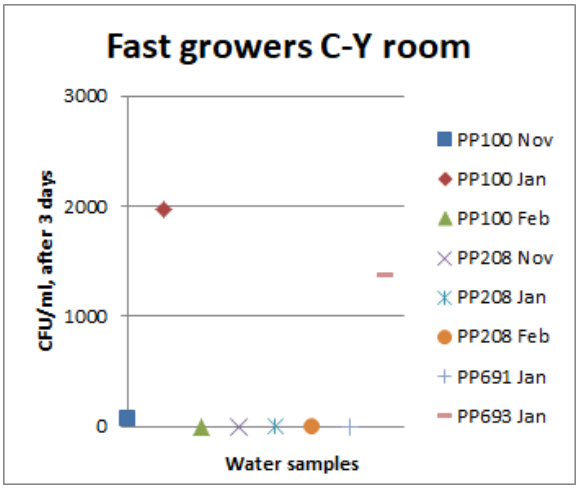
growers grown, of water samples from Norrtälje cultivated on C-Y media over a period from 29/12-14 to 2/12-20 in Synlab.

Cultivation

The cultivation microorganisms present in the water samples were analyzed (CFU/ml) by growing them in different media in the lab, which can be seen in fig 7-10. Fig 7.A describes slow growers over fast growers in the three different media. Among the different media, R2A showed a higher number of bacterial colonies followed by NB+NaCl and C-Y medium showing the lowest. Figures 7.B.C.D, are showing fast growers in C-Y medium at three different temperatures. As can be seen inlet water (PP100) Jan and after UV-treatment in DWDS (PP693) Jan have the highest numbers of colonies at RT and 30℃.



A



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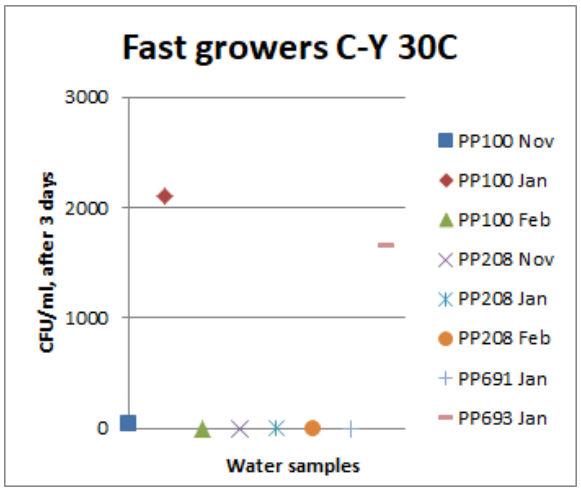

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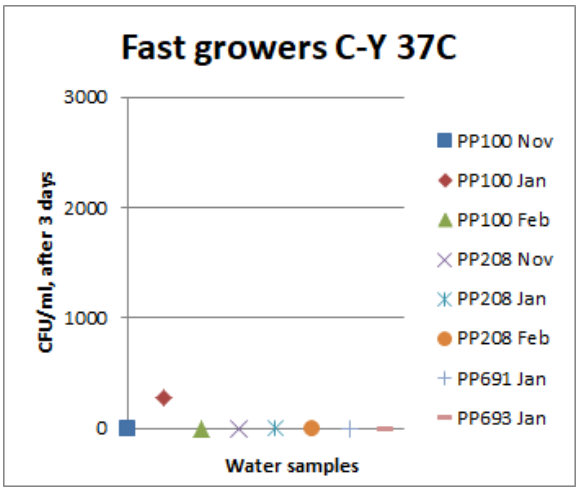
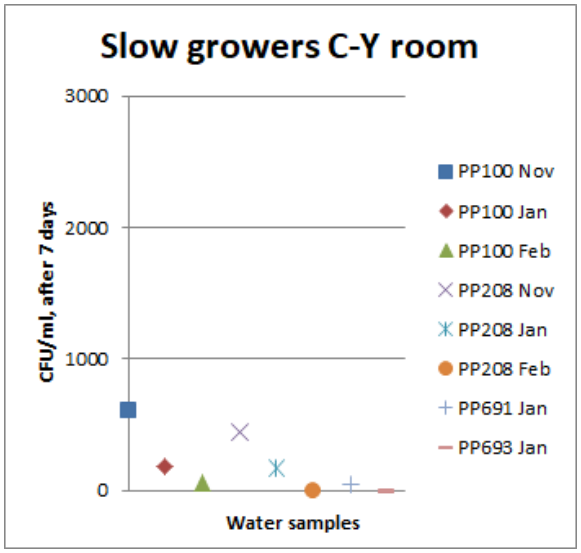


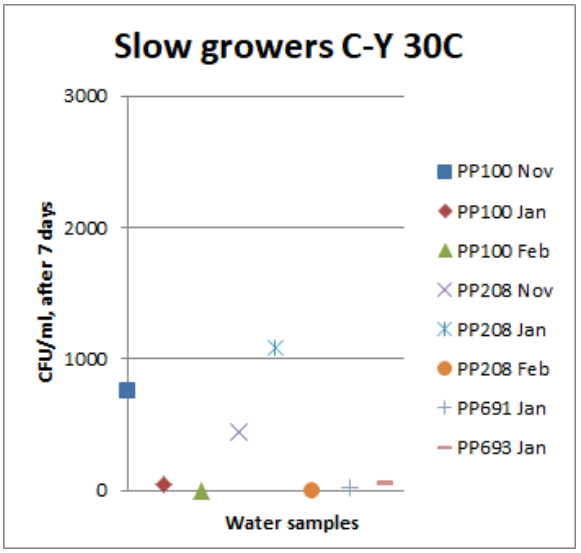
D

Figure 7. The number of microorganisms present in water samples collected by Norrvatten at Nov 2020, Jan 2021 and Feb 2021. A, depicts slow growers (CFU/ml new colonies after 7 days), cultivated on C-Y, NB-NaCl and R2A media, cultivated in KTH C-Y during 23/2-21 to 2/3-21 and NB + NaCl/R2A during 12/3-21 to 19/3-21, over fast growers (CFU/ml after 3 days), all at RT B, depicts fast growers from the different water samples grown in a C-Y media at RT C,depicts fast growers from the different water samples grown in a C-Y media at 30℃ and D depicts fast growers from the different water samples grown in a C-Y media at 37℃

Figure 8.A.B.C shows the slow growing bacterial colonies in C-Y medium observed 7 days after incubation. Higher number of colonies was observed in inlet water (PP100) sample Nov 2020 both at RT and at 30℃ followed by outlet water (PP208) Nov 2020 in RT but in PP208 Jan 30℃ had the highest number of colonies. In RT before UV-treatment in DWDS (PP691) had a higher number of colonies than after UV-treatment in DWDS (PP693), but the reverse was true in 30℃. The samples collected in Nov had the highest number of colonies followed by samples from Jan 2021, both inlet water (PP100) and after UV-treatment in DWDS (PP693) samples. Overall, there were very few colonies observed at 37℃ cultivations.



A



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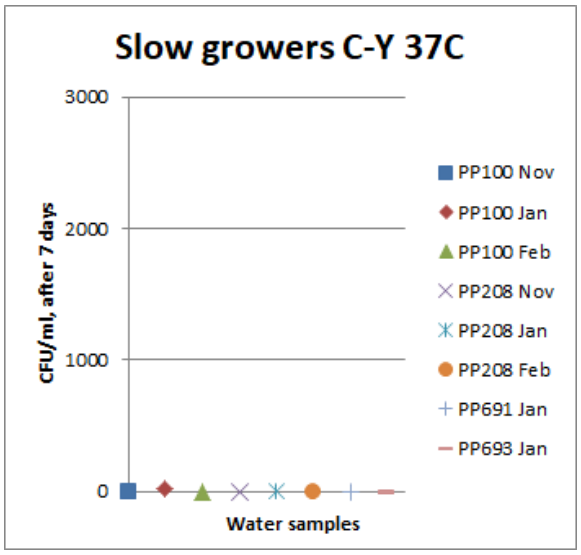
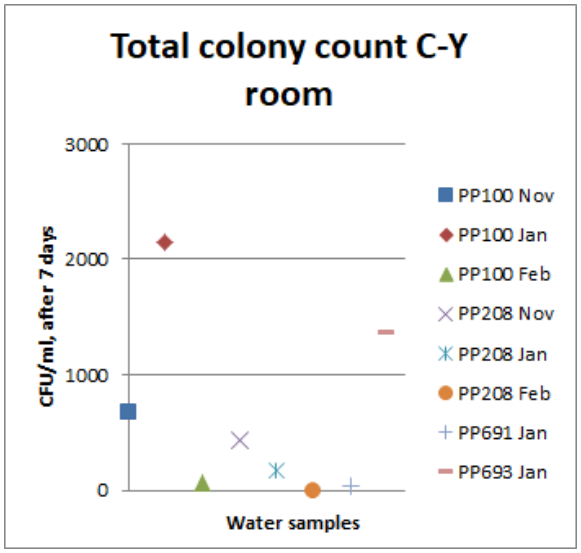


C

Figure 8. A, depicts slow growers from the different water samples grown in a C-Y media at room temperature B,depicts slow growers from the different water samples grown in a C-Y media at 30℃ and C depicts slow growers from the different water samples grown in a C-Y media at 37℃

Figure 9 shows the total number of colonies cultivated in each media, by adding the number of colonies formed after 3 days and the number of colonies formed after 7 days. By comparing fig 9.A.B.C it is shown that there is a higher number of colonies with the R2A

medium compared to the NB+NaCl and C-Y media. The highest count of a water sample in R2A medium is outlet water (PP208) Feb with a count just above 5000 CFU/ml, in the two other media the highest count is only slightly over 2000 CFU/ml. The number of colonies is not the only thing that is different between the three media. In the R2A medium there were only three water samples that did not get more than 1000 CFU/ml, for NB+NaCl media that number is five and for C-Y six. By comparing fig 9.B.C.D.E it is shown that a higher temperature gives a higher number of colonies. For NB+NaCl the same trend could be seen, were at 25℃ the highest number of colonies with sample after UV-treatment in DWDS (PP693) was slightly below 3000 CFU/ml while at RT is was slightly above 2000 CFU/ml. In R2A 25℃ four samples had less than 1000 CFU/ml and in NB+NaCl 25℃ six samples had less than 1000 CFU/ml. As can be noted when comparing fig 9.B and D, is that in NB+NaCl media at RT outlet water (PP208) Feb is the sample with the second highest number of colonies, while at 25℃ it is the sample with the second lowest number of colonies.



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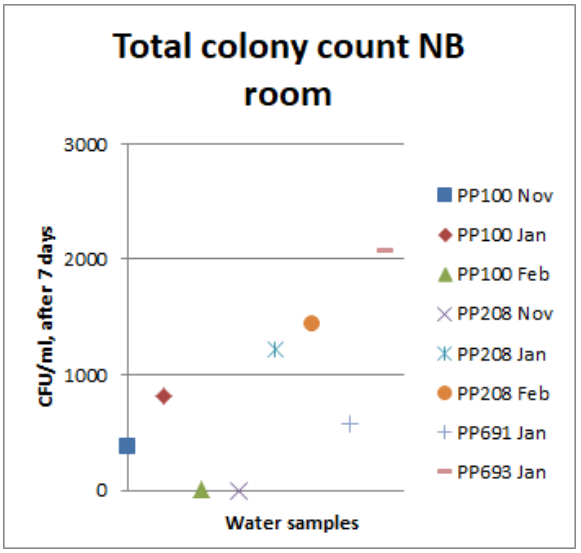

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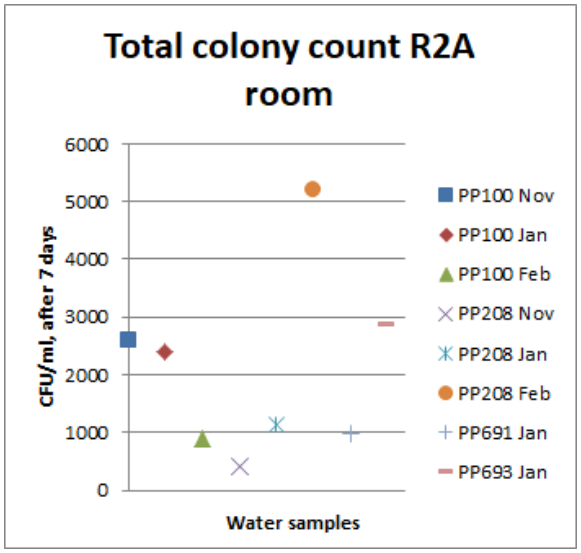

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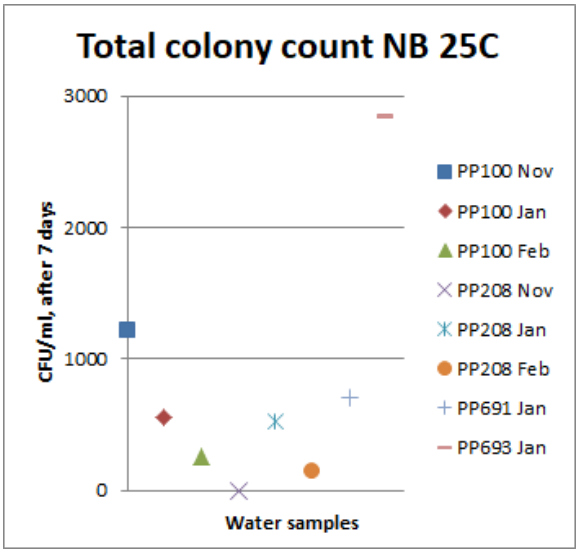

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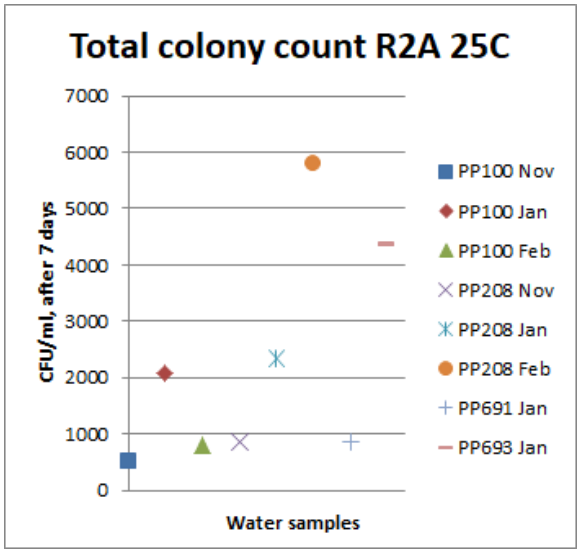

E

Figure 9. A, depicts the total CFU/ml after 7 days from the different water samples grown in a C-Y media at RT B, depicts the total CFU/ml after 7 days from the different water samples grown in a NB + NaCl media at RT C,depicts the total CFU/ml after 7 days from the different water samples grown in a R2A media at RT and D depicts the total CFU/ml after 7 days from the different water samples grown in a NB + NaCl media at 25℃ and E depicts the total CFU/ml after 7 days from the different water samples grown in a R2A media at 25℃

Figure 10, shows photos taken of cultivated bacterial isolates. Figure 10.A shows a plate of R2A medium with a sample from the pipe, swabbed and incubated for 3 days at RT. As can be seen it forms a film of mycel-looking structure. It was identified as *Bacillus mycoides* based on 16S rRNA gene sequencing (table 2). Figure 10.B shows a plate of C-Y media with a four time re-plated sample from m100 (pipe material in ingoing water sample) incubated for 7 days at RT. As can be seen it forms a violet pigment that in high enough concentrations turn more black like. It was identified as *Janthinobacterium lividum*, based on 16S rRNA sequencing (table 2). Figure 10.C shows a re-plate of sample C-Y PP208 (out from treatment plant) Feb incubated at RT, which was identified as *Pseudomonas fluorescens* based on 16S rRNA sequencing (table 2). Lastly, figure 10.D shows a plate with R2A with sample after UV-treatment in DWDS (PP693) Jan, incubated at 25℃. As can be seen it forms a white-yellow film, It was identified as *Brevundimonas mediterranea*, based on 16S rRNA sequencing (table 2).

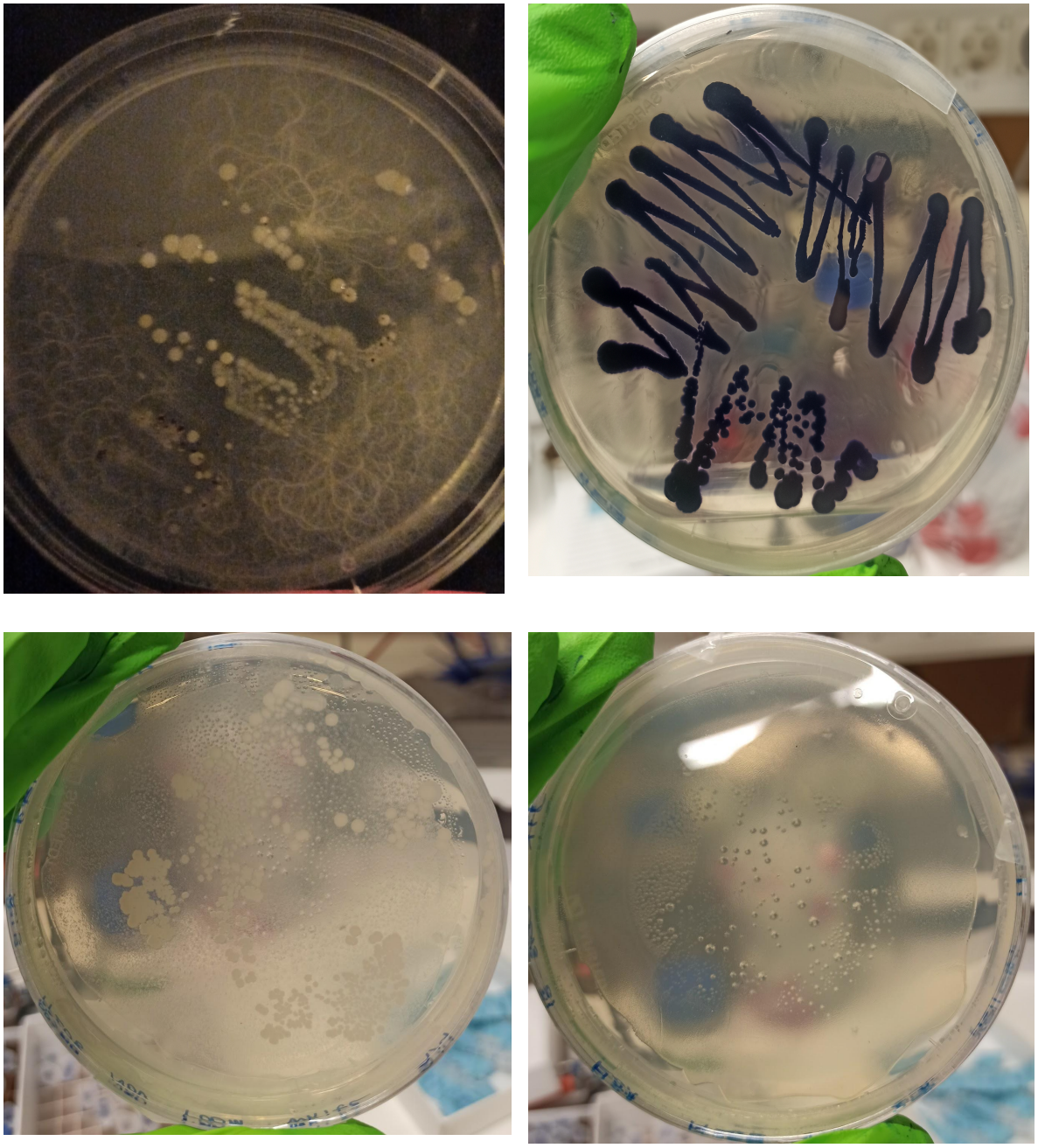

Figure 10. A shows plate with bacterial colonies isolated by swabbing the pipe that was dug up, then incubated in R2A medium at RT after 3 days, B shows four time re-plate with m100 C-Y, the original was from swabbing the pipe material incubated in PP100 sample. Then it have been incubated in C-Y medium at RT. C shows a re-plated plate with PP208 Feb sample incubated in C-Y at RT, D shows a re-plated plate with PP208 Jan sample incubated in R2A at 25℃.

Biofilm formation

The biofilm formation by microbial consortia in the water samples were analyzed by crystal violet staining method (fig 11). Figure 12.A is a compilation of results from 6 weeks of incubation of plastic scraps in C-Y media, with lines between the points for ease of seeing the trend, showing a slight wave shape. Most of the samples followed a wave shape. Different samples followed different curves, before UV-treatment in DWDS (PP691) Jan,

outlet water (PP208) Jan and PP208 Feb (all incubated in C-Y media) increased their biofilm during the first and second week to later decrease (fig 13.A). Outlet water (PP208) Nov, inlet water (PP100) Jan and after UV-treatment in DWDS (PP693) Jan (all incubated in C-Y media) had a quite developed biofilm after one week of incubation, which decreased after three weeks of incubation and then started to increase again during week four-five depending sample, to then decrease again. On the other hand, there was a change in the samples collected in Jan 2021 and Feb 2021, where the inlet water samples (PP100) showed less biofilm formation, whereas the outlet water (PP208) showed comparatively more biofilm growth.

Figure 12.B is the results from incubation in a 12 well plate instead of plastic scraps after 1 week in C-Y media. It is interesting to note that the higher biofilm formation was found in the outlet water samples (PP208) collected in Nov 2020in one week. There is a difference in biofilm formation between plastic scraps and a microtiter plate.

Figure 12.C is a compilation of results from 5 weeks of incubation of plastic scraps in R2A media, with lines between the points for ease of seeing the trend showing a slight sinus curve shape. For the samples incubated in R2A media most samples except before UV-treatment in DWDS (PP691) Jan, outlet water (PP208) Nov and PP208 Jan had a quite developed biofilm after one week of incubation and then decreased. While the aforementioned samples in R2A media showed the opposite behaviour by starting with a not so developed biofilm and then developing it during two-three weeks of incubation and then decreasing (see figure 13.C). This shows a trend in biofilm growth on the plastic scrap surface by attachment and detachment process.

Figure 12.D is the results from incubation in a 12 well plate instead of plastic scraps after 1 week in R2A medium. When comparing before UV-treatment in DWDS (PP691) and after UV-treatment in DWDS(PP693), the PP693 sample formed less biofilm in the C-Y medium compared to the R2A medium. Overall, R2A medium showed lower biofilm formation compared to C-Y medium.

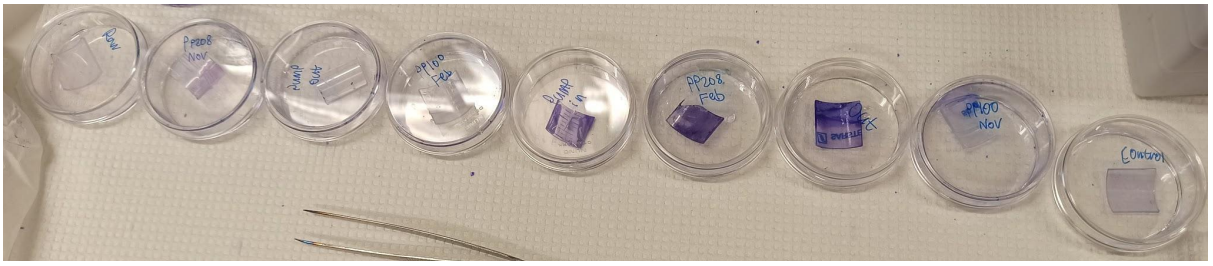

Figure 11. Shows stained plastic scraps, before addition of 70 % ethanol.

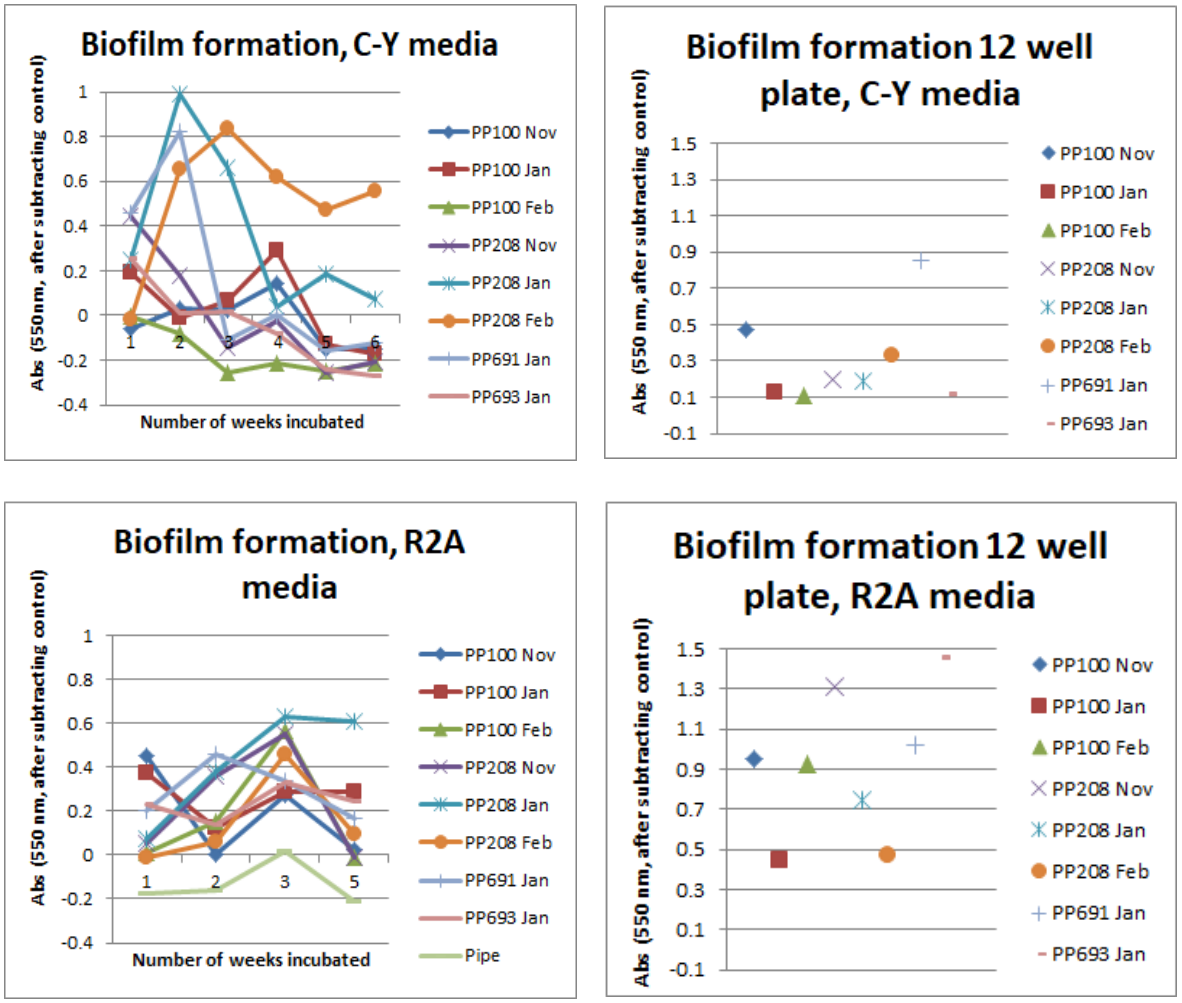

Figure 12. Analysis of biofilm formation on plastic scraps and microtiter plate for all eight water samples in C-Y and R2A media. A and B depict absorbance at 550 nm over a period of six weeks of biofilm formation with eight different water samples grown in plastic scraps and one week on microtiter plates in C-Y medium. C and D depicts absorbance at 550 nm over a period of five weeks with nine different water samples in plastic scraps and one week on microtiter plates in R2A medium. In this experiment no measurement was done during week 4, however a duplicate was done on week 5 and the average is shown in the graph.

Molecular methods

Five different PCR:s were conducted, with five different primer sets as described in the method section. The presence of these bacteria that the primers target was investigated during the literature study. The bacteria being targeted were all found in DWDS or in freshwater environments and therefore believed to exist in the water samples. Also they either have important properties like, formation of biofilm, survival of UV-treatment or metabolic properties such as ammonia oxidizing. In the PCR reaction targeting ammonia oxidizing bacteria, the 16S rRNA of ammonia oxidizers belonging to class betaproteobacteria is targeted.(24) For the PCR targeting Actinobacteria the V3 to V5 region of the 16S rRNA gene is targeted.(25) In the PCR targeting *Pseudomonas* the rpoD gene in *Pseudomonas* of the class of gammaproteobacteria is targeted.(26) In the PCR targeting *Sphigomonoas,* a highly conservative part of the 16S rRNA sequence specific to

Sphigomonoas is targeted.(27) Lastly in the isothermal PCR the LacZ gene of coliforms is targeted.(28) The DNA was extracted from randomly isolated colonies from different water samples and mixed cultures grown by incubating water samples with different media.

To easier interpret the pictures, the sample names were added in the picture and the DNA ladder was included for size comparison. Figure 13 is the result of the amplification of pure culture for producing amplicons for ammonia oxidizing bacteria. Isolates from after UV-treatment in DWDS (PP693) Jan 30℃ showing possible bands at the appropriate size of 1.1 kb.

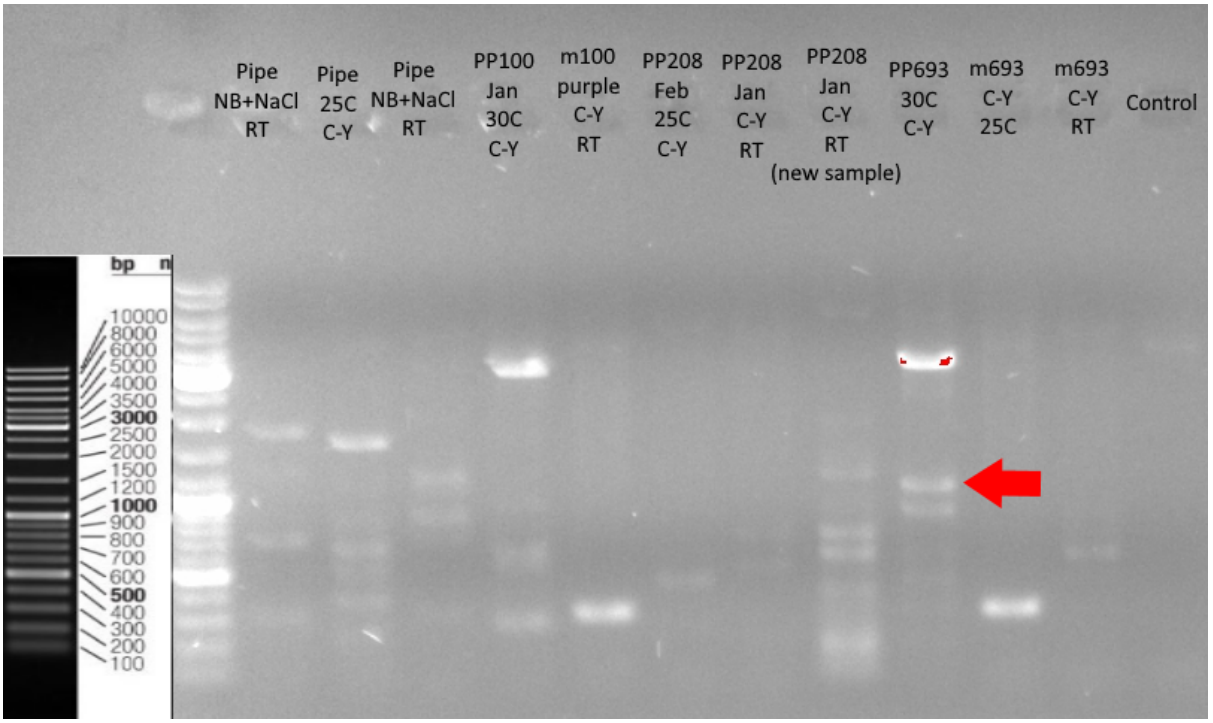

Figure 13. Depicts the picture of 1 % agarose gel after electrophoresis for 30 min of PCR detecting ammonia oxidizing bacteria, with an expected size 1.1kb.

Figure 14, is a PCR on mixed cultures for amplifying the produced amplicons for Actinobacteria with primers S-C-Act-235-a-S-20 and S-C-Act-878-a-A-19, with all samples and the negative control (no template DNA added in the PCR reaction) showing a band at the appropriate size of 640 bp. It is interesting to note that microorganisms belonging to the phylum Actinobacteria could be detected in all the samples from inlet water to after the DWTP till the DWDS after UV-treatment, also see fig A.9 and A.10.

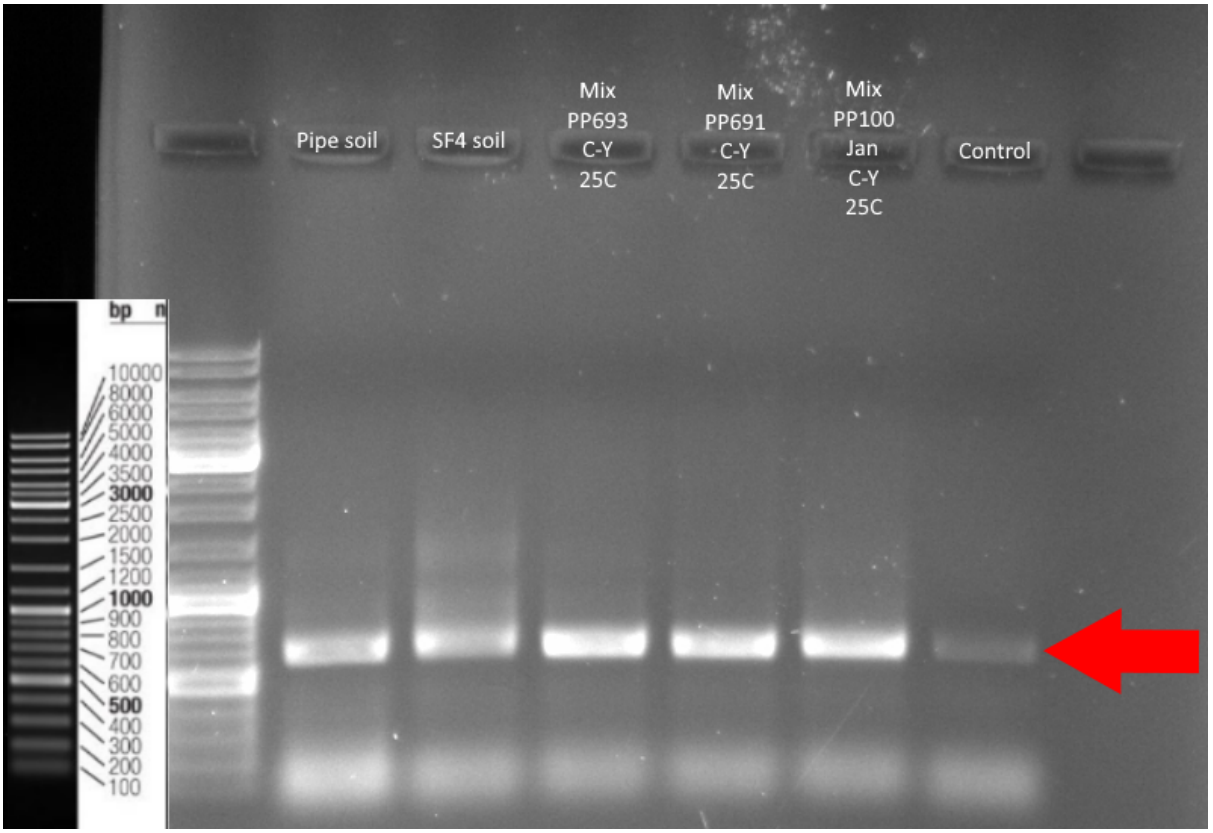

Figure 14. Depicts the picture of 1 % agarose gel after electrophoresis for 30 min of PCR detecting Actinobacteria, with an expected size 640 bp.

Figure 15 is a PCR on pure cultures for producing amplicons when Actinobacteria is present. The samples C-Y m208 (pipe material incubated in outgoing water) in RT and 25℃ formed intense bands with the size of 640 bp. The rest of the tested extracts of pure cultures except C-Y after UV-treatment in DWDS (PP693 RT colourless, R2A PP693 RT colourless and C-Y pipe 25℃ pink had an amplified band with the size of 640 bp with varying intensity, however less intense as the aforementioned two samples. In this gel and to a lesser extent in other gels, the gel is slightly bent. More gels are found in the appendix.

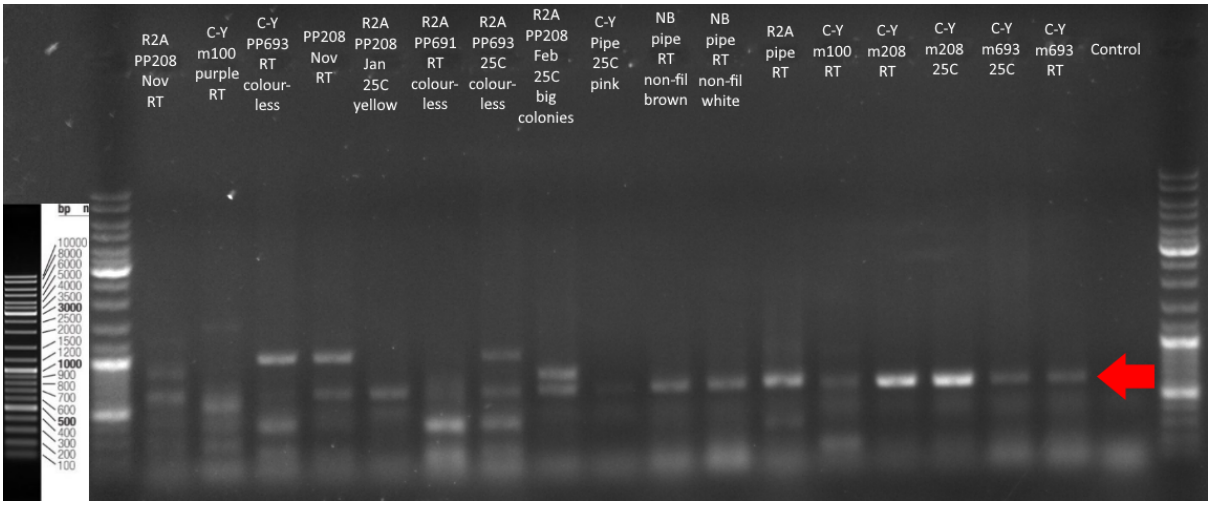

Figure 15. Depicts the picture of 1 % agarose gel after electrophoresis for 30 min of PCR detecting Actinobacteria, with an expected size 640 bp.

The gene amplifications for *Pseudomonas* and *Sphingomonas* genus and the isothermal PCR for detecting coliform bacteria did not show any reliable amplifications and are therefore not presented here (see appendix).

Sequencing

Table 2 shows the sequencing results from the pure culture samples that were isolated randomly and amplified 16S rRNA amplified and sent to Eurofins for sanger sequencing. The samples outlet water (PP208) Jan room C-Y, after UV-treatment in DWDS (PP693) Jan 30℃ C-Y, inlet water (PP100) Jan 30℃ C-Y, outlet water (PP208) Nov room C-Y and R2A PP693 25℃ colourless did either get an unidentified bacteria or the received sequence were too short to get a corresponding sequence with high confidence. In some of the sequences different species had the same percentage of identification, they are therefore shown with a slash (/).

From the sequencing (table 2) three major genus could be found, these being *Bacillus sp*, *Janthinobacterium sp* and *PSeudomonas sp* all of them having a percentage of identity over 98%. In all the sequenced pipe samples and R2A PP2208 (outgoing water) Nov RT pink, *Bacillus sp* were found to exist, with *Bacillus mycoides* being the most common in that the genus. The other genus is *Janthinobacterium sp*. As can be seen in table 2, only sequencings of pure culture samples from where the pipe material was incubated in different water samples were identified as that genus. *Janthinobacterium sp* was found in pipe material incubated in inlet water (PP100) and outlet water (PP208) and after UV-treatment in DWDS (PP693).

The last major genus is the *Pseudomonas sp*. Which was found on plates from water that have been treated with UV, namely outlet water (PP208) and after UV-treatment in DWDS (PP693) table 2. Pseudomonas *sp* do not have the ability to form endospores, however they seem to be able to survive the UV-treatment. Other than microorganisms from one of the three genus mentioned above, three other genus were also identified, see table 2. These were *Micrococcus sp* which belong to the Actinobacteria phylum, was found from an isolate from C-Y inlet water (PP100) Jan 37℃, *Kocuria rhizophila* which also belongs to the Actinobacteria phylum, was identified in C-Y m208 (pipe material incubated in outlet water) 25℃. Lastly, Brevundimonas *sp* from R2A outlet water (PP208) Jan 25℃ from a yellow colony on the plate.

Table 2. Shows the results from sanger sequencing of 16S RNA samples received from Eurofins.

Sample name	Comments, from looking at replated plate	Identification of microorganisms	Per Ident (%)	PCR result of Actinobacteria + positive - negative (+) inconclusive
C-Y m100 (pipe material incubated in	Impure colonies with purple	*Janthinobacterium lividum* / *Janthinobacteri*	99.58	(+)

inlet water) room		*um tructae /Janthinobacterium svalbardensis*		
C-Y m100 (pipe material incubated in inlet water) room purple	Pure with purple colonies	*Janthinobacterium lividum / Janthinobacterium tructae*	99.75	+
C-Y PP100 Jan 37℃		*Micrococcus aloeverae*	99.22	+
R2A PP208 Nov room red	Mixed culture, three different, ones is *Bacillus mycoides*	*Bacillus mycoides / Bacillus cereus*	99.3	+
R2A PP208 Jan 25℃ yellow	FIlm, not so yellow	*Brevundimonas mediterranea*	99.06	+
R2A PP208 Feb 25℃ big colonies	Film	*Pseudomonas fluorescens*	99.9	+
C-Y m208 (pipe material incubated in outlet water) 25℃		*Kocuria rhizophila*	99.58	+
C-Y m208 (pipe material incubated in outlet water) room		*Janthinobacterium lividum / Janthinobacterium tructae /Janthinobacterium svalbardensis*	99.58	+
C-Y Pipe room		*Bacillus mycoides*	99.51	+
C-Y Pipe 25℃		*Bacillus mycoides*	98.83	+
C-Y Pipe 25℃ pink	Many small yellow colonies	*Bacillus sp*	99.41	-
NB NaCl Pipe room non-filamentous	Film, seems to be some *Bacillus*	*Bacillus sp*	99.43	+

brown	*mycoides*			
NB NaCl Pipe room non-filamentous white	Film, seems to be some *Bacillus mycoides*	*Bacillus mycoides* / *Bacillus thuringiensis*	99.75	+
C-Y m693 (pipe material incubated in UV treated water) 25℃		*Janthionobacterium agaricidammosum*	99.41	+
C-Y m693 (pipe material incubated in UV treated water) room		*Pseudomonas fluorescens* / *Pseudomonas proteolytica*	99.41	+
C-Y PP693 room colourless	Colourless film	*Pseudomonas gessardii* / *Pseudomonas fluorescens* / *Pseudomonas cedrina*	99.81	-

Discussion

The present study was conducted to evaluate the effect of UV treatment in the drinking water distribution system (DWDS). In this study biofilm formation and cultivation of microorganism in different media was conducted followed by molecular analysis of groups of organisms which are potential to be found in the DWDS and compared with data from Norrvatten. Data from Norrvatten showed that there is a trend that UV decreases the cultivable microorganisms in the water samples, fig 6.C. But when comparing the number of colonies with or without UV in fig 6.C and D, the results are contradicting, because in fig.D no correlation can be seen. By comparing fig 6.B and D the highest number of colonies exist in temperatures between 10-15℃. The seasonal changes with temperature therefore have a big effect on the number of colonies obtained from cultivating the water sample, since the water temperature in the pipes is fluctuating between 5 and 20℃ depending on the season (fig A.1). What can be noticed is that the water samples were analyzed by two laboratories, and it seems to get different results from similar water samples during the same time, with the difference being so high as about 9000 CFU/ml. Other trends that could be seen are that the number of slow growers grew best when the conductivity is between 37-38 ms/m (fig A.2). The number of cultivable microorganisms grew best when the pH was between 8 and 8.3 (fig A.3), however since the pH follows the logarithmic scale there is a large difference between the 8 and 8.3.

The cultivation method of SS-EN ISO 6222-1 (29) was adopted to the lab conditions with minor changes. After analyzing figure A.1 and cultivation with C-Y, the temperatures 20 and 25℃ were used in the project for forthcoming cultivations. The growth of microorganisms on the NB + NaCl medium and the R2A medium was higher than when the C-Y medium was used, with R2A having the highest growth. The R2A media is a defined media that emulates the water environment. Because the medium is similar to the environment which the water samples come from there is no shift for the organism when they are incubated on R2A medium. It was unexpected that microbes from water samples grew better on R2A and NB + NaCl medium than on C-Y medium, since SS-EN ISO 6222-1 uses the C-Y medium. In the C-Y cultivations there was more growth in inlet water (PP100) samples than the outlet water (PP208) samples, indicating that the DWTP removes cultivable microorganisms in the process water. For the NB + NaCl and R2A cultivation the difference between inlet water (PP100) and outlet water (PP208) was not clear. Because, which sample produced the most colonies switched between cultivations in RT and 25℃. When comparing the 3 days C-Y cultivations from this project to the one done by Norrvatten (table A.1), Norrvatten got no growth for samples after the treatment, while in this project water sample from after UV-treatment in DWDS (PP693) had countable colonies. This difference could be due to different incubation temperatures, time or handling of the samples. The growth of microorganisms with samples after UV-treatment in DWDS (PP693) is higher than sample before UV-treatment in DWDS (PP691). This was true for all media and all temperatures. This leads to believing that the UV-treatment in the pumping station has the opposite effect and increases the amount of cultivable microorganisms. This seems unbelievable, other factors such as reservoirs or excess biofilm formation around the UV-lamps could also be an explanation to the higher number of cultivable microorganisms. The morphology of the colonies and microorganisms were also studied, but it was hard to see any significant difference, especially under the microscope. Another factor is the time since the samples

were taken to analyze. The microorganisms in water samples from November may have been reduced due to limited nutrients.

What is most noticeable in the biofilm formation is that very little biofilm was formed by the pipe sample. One explanation for this is that the microorganisms in the biofilm in the pipe are specialized to a sedentary lifestyle of being part of a biofilm. So when they are dislocated from the biofilm they may not be able to adapt and survive in a non-sedentary lifestyle and form a new biofilm. With the C-Y media the outlet water (PP208) samples except for PP208 Nov have a more developed biofilm than the samples with inlet water. When comparing before UV-treatment in DWDS (PP691) and after UV-treatment in DWDS (PP693), the PP693 sample formed less biofilm than the PP691 sample. With the R2A the PP208 samples have a more developed biofilm than the inlet water (PP100) samples except for PP100 nov which have a more developed biofilm. Compared to the samples in C-Y media PP693 have a more developed biofilm than before UV-treatment in DWDS (PP691). (fig 13.A) The contradicting results made it hard to draw a conclusion. However, it can be said by some certainty that the ability to form biofilms seems to increase after the treatment process and that the biofilm decreases after the UV-treatment. This may be due to that sheard of biofilms from the treatment plant can enter the drinking water which may have an enhanced ability to form biofilm. Another explanation could be that the environment of the drinking water is worse for growth than the raw water, which leads to an advantage to biofilm forming microorganisms. Only one measurement was made for each sample/week. A test to show the robustness of the method by doing a duplicate measurement with samples incubated for five weeks in R2A media gave standard deviation as high as 0.36 (table A.2).

Molecular analysis was conducted from the water samples mixed with different media and isolated pure cultures. This amplification of specific genes targeted five different groups of microorganisms found in DWDS or in freshwater environments and therefore believed to exist in these water samples. They either have important properties in the formation of biofilm, survival of UV-treatment or metabolic properties such as ammonia oxidizing bacteria (AOB). Only C-Y after UV-treatment in DWDS (PP693) Jan 30℃ showed amplification of AOB genes around 1.1 kb. But in further tests with the same samples no band with the size of 1.1 kb were found (fig A.8). One explanation to why there are no band indicating AOB is because the medias used (C-Y, NB+NaCl and R2A) did not promote the growth of AOB. Because the media used did not contain a high concentration of ammonia and that other microorganisms could maybe outcompete the AOB. However, in the water samples and in the DWDS there should be AOB, since the disinfectant monochloramine is added which breaks down to ammonia which the AOB uses as nutrient. The second PCR that was conducted was to amplify Actinobacteria to produce a 640 bp long product. A problem with the method was that the negative control (without added DNA template) also produced a band at 640 bp. Therefore it was hard to know if the produced band was from present Actinobacteria, contamination of one of the reagents or something else. In the case where the negative control gave a band, the intensity of the remaining bands were very high and there were no unspecific bands (fig 15). All mixed samples except the outlet water (PP208) Jan C-Y. (fig A.13) formed a band at 640 bp. Table 2 shows that the PCR to amplify Actinobacteria seems to be very broad and may amplify other phyla, since only two samples, C-Y inlet water (PP100) Jan 37℃ and C-Y pipe material incubated in outlet water (m208) 25℃ were samples that got identified as belonging to the Actinobacteria phylum by both specific PCR and by 16S rRNA sequencing. The other samples showed positive occurrence

of Actinobacteria in the PCR but not in the 16S rRNA sequencing. It could be that the primer pair was too unspecific or because of contamination or unpure isolates. The third PCR showed that no *Pseudomonas* was present (fig A.12). From previous results from Norrvatten, *Pseudomonas* should be present in the DWDS which was also shown by the 16S rRNA sequencing further below. Either the PCR setting didn't work or there were no *Pseudomonas* present in the samples that were tested. However, when mixed samples were tested by the PCR, a faint band around 700 bp from the well of C-Y mix PP693 Jan 25℃ can be seen (fig A.13). All the pure isolated samples identified as *Pseudomonas* with 16S rRNA sequencing were from the after UV-treatment (PP693) or outlet water (PP208), it is therefore plausible that *Pseudomonas* existed in C-Y mix PP693 Jan 25℃. More thorough testing and optimization needs to be done, before a conclusion can be made. The fourth PCR that was made to see if *Sphingomonas* was present. However as can be seen in figure A.11 no band was visible. Since *Sphingomonas* is a genus and not a phylum like Actinobacteria it was thought not worth investigating more. More optimizations of the PCR was needed. An attempt was made to test an isothermal PCR method which was an upcoming method for water sample analysis. An isothermal PCR would enable PCR reactions to be done without a thermocycler. However after two tests with two different PCR settings no product could be amplified. It was concluded that the method needed more optimization.

Not all different looking colonies from the plates were sent to sequencing, therefore the sequencing does not give a full picture of the cultivable microorganism. From the 16S rRNA sequencing (table 2), three major genus could be found, *Bacillus sp*, *Janthinobacterium sp*, and *Pseudomonas sp* all of them having percentage identity over 98 %. In all the sequenced pipe samples and R2A outlet water (PP208) Nov room pink, *Bacillus mycoides* were found to exist. *Bacillus mycoides* form characteristic colonies which can possibly enable biofilm growth due to its ability to form filamentous colonies. One specific property of *Bacillus sp* is that they form endospores. Since the endospores have a higher capacity to survive UV-treatment these species could exist in the DWDS and form biofilms.(31) The other genus is *Janthinobacterium sp* which is a soil bacteria that produces purple pigment (fig 10.B). As can be seen in table 2, *Janthinobacterium* was only found on pipe material that was incubated in water samples, there is a possibility that the pipe could have been contaminated. However, it was treated with hypochlorite to remove any contamination from skin contact. The other major genus found is *Pseudomonas sp*. The results are interesting since the samples with *Pseudomonas* have been treated with UV, namely the outlet water (PP208) and after UV-treatment in DWDS (PP693), see table 2. Due to the finding of *Pseudomonas* in these samples, it seems to be able to survive the UV-treatment. *Pseudomonas* is an important genus of microorganisms in the formation of biofilm and therefore is a very important genus of microorganisms to carefully analyze in the DWDS. Other than microorganisms from one of the three genus mentioned above. There were also three other genus found (see table 2). These were *Micrococcus sp* which was found from a isolate from C-Y inlet water (PP100) Jan 37℃, *Kocuria rhizophila* C-Y m208 (pipe material in outgoing water) 25°C and lastly *Brevundimonas sp* from R2A outlet water (PP208) Jan 25℃ from a colony that was yellow. Due to *Micrococcus sp* ability to survive harsh environments it may have the ability to survive the treatment process. However the genus was only found from an isolate from the inlet water (PP100). *Kocuria rhizophila* exists in the environment and can therefore have entered the water sample from lake Mälaren. It is also a microorganisms that can survive harsh environments, which can be one reason why it can survive the treatment process. Lastly there is *Brevundiomonas* which is a genus that is

closely related to *Pseudomonas*. Since *Pseudomonas* were present in the samples it is therefore not surprising that the closely related *Brevundiomonas* also is present.

Conclusion and further perspectives

Results obtained from this project showed that the UV-treatment in the DWDS does not have a direct indication to reduce the number of culturable bacteria. Since both the cultivations that were done during the project and the compiled data from cultivations done by Synlab showed that UV-treatment has no direct indication to reduce the number of culturable bacteria. The biofilm formation was lower in the UV treated samples compared to water coming into the UV-treatment. In some randomly selected isolates there was detection of the presence of Actinobacteria such as *Micrococcus sp* and *Kocuria sp*. However most of the species were from the *Bacillus sp*, *Pseudomonas sp* and *Janthinobacterium sp* genus based on 16S rRNA gene analysis. To be able to draw a definite conclusion about UV's role, more samples from before and after UV-treatment in the treatment process needs to be investigated. So that only UV's effect will be examined at not the rest of the steps of the treatment process. Also more samples from before and after the UV-treatment in the DWDS (PP691 and PP693) needs to be investigated, as well as duplicates or triplicates to get a better foundation to make a definite conclusion. In conclusion further research needs to be conducted in order to say if it is viable to expand the usage of UV-treatment along the DWDS. A further topic to build upon could be the interaction between pathogenic microorganisms and an existing biofilm in the DWDS.

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Figure 1, 2 and 3. Same source as 8 (Norrvatten, https://www.norrvatten.se/dricksvatten/dricksvattenproduktion/reningsprocessen/ (obtained on 12/2-21)

Appendix

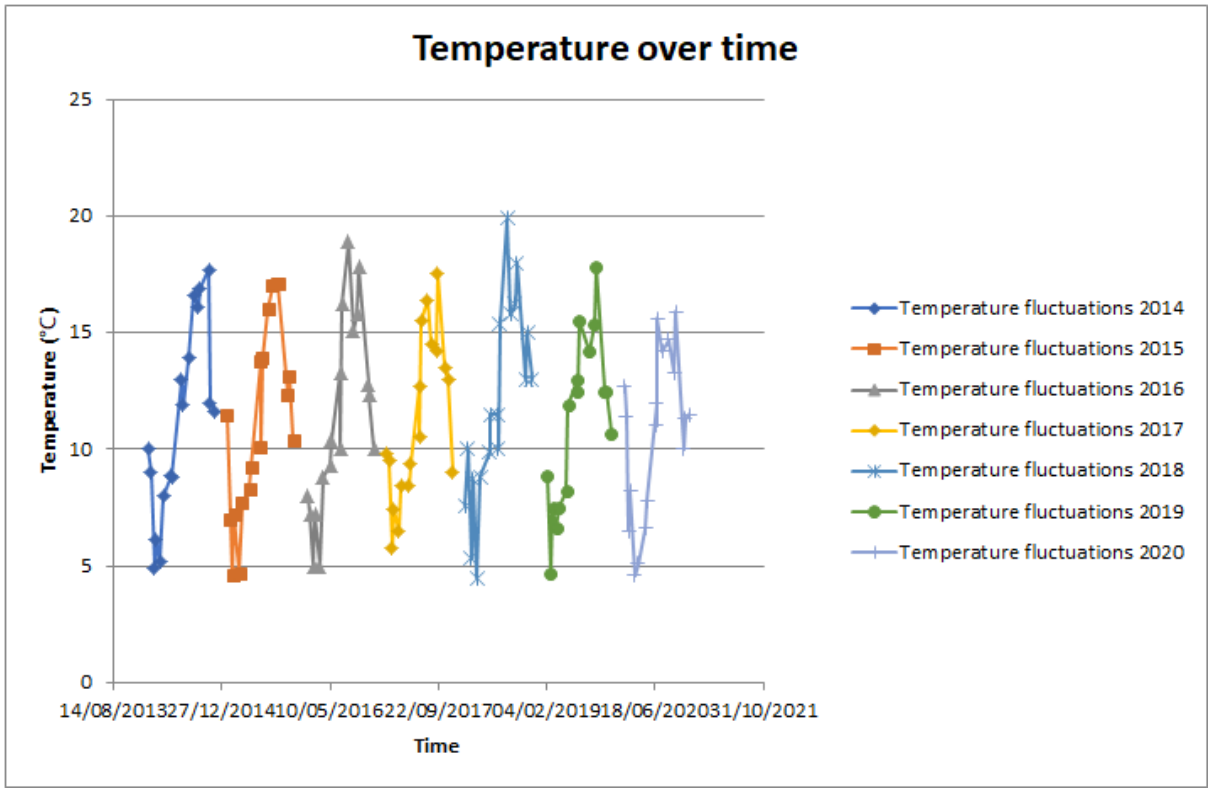


Figure A.1. Depicts the fluctuation of temperature in Norrvatten's DWDS.

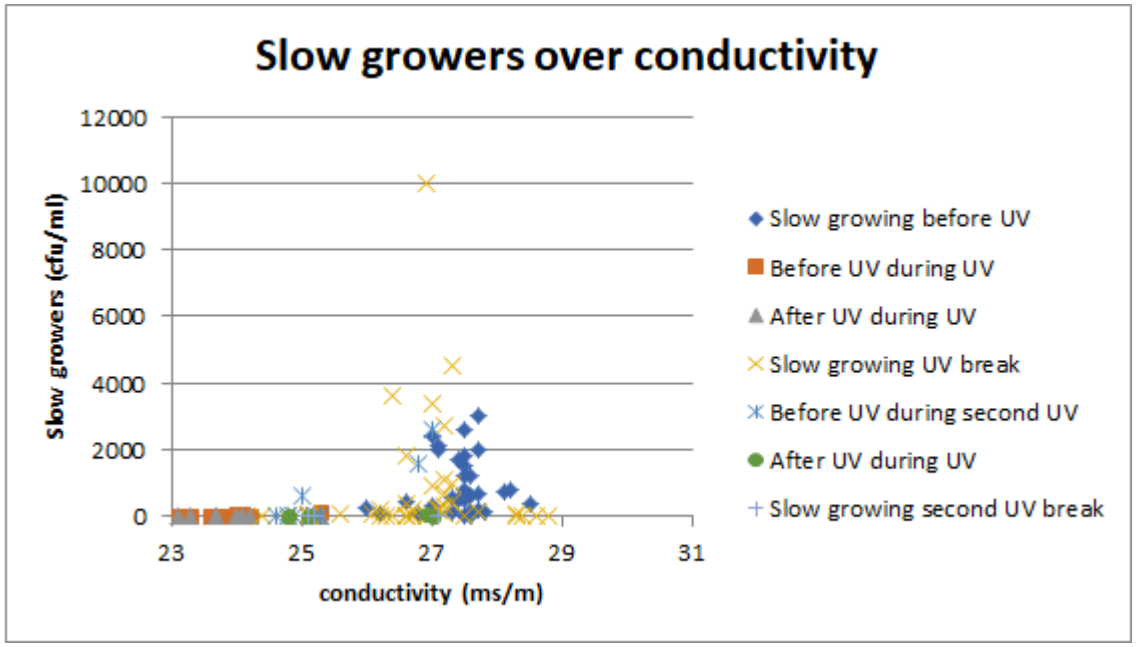


Figure A.2. Shows slow growers over conductivity (ms/m), with cultivations being done at Norrvatten.

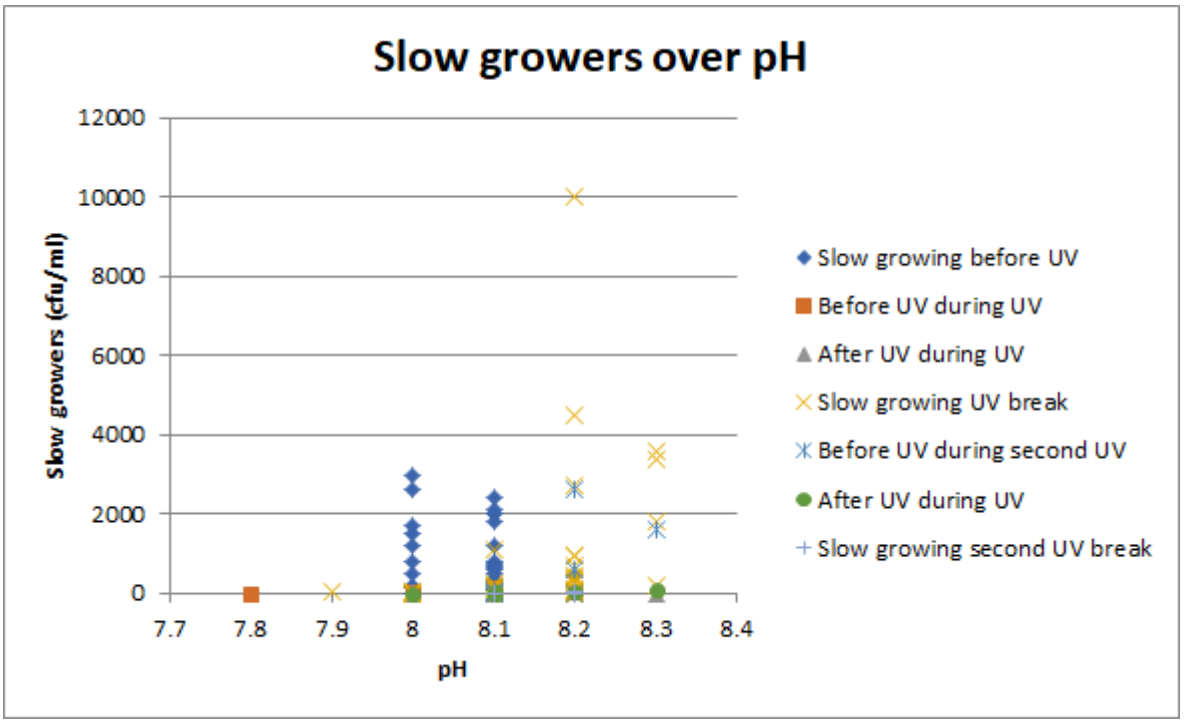
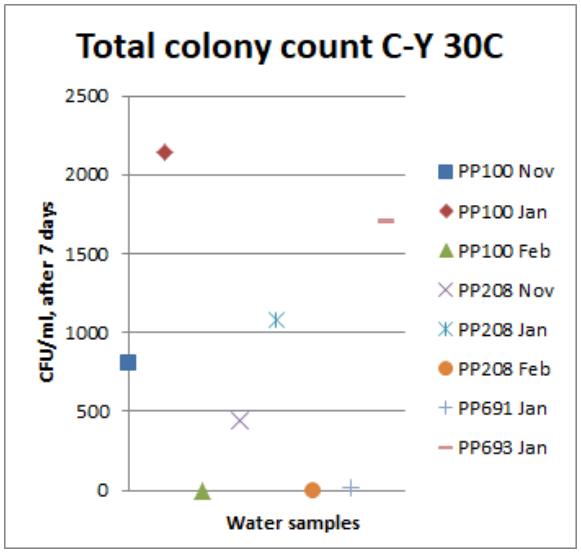


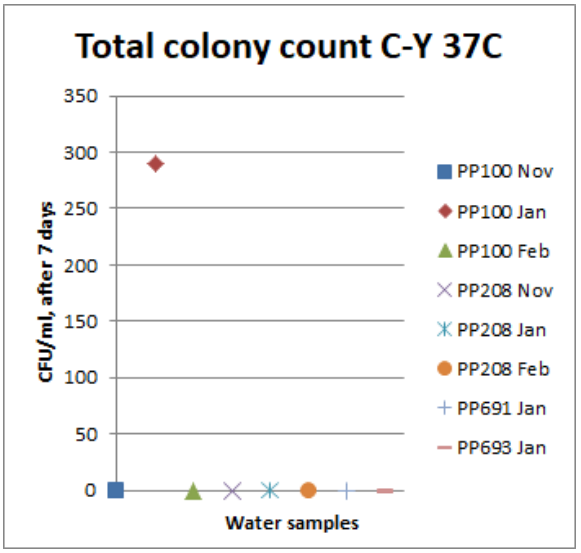
Figure A.3. Shows slow growers over pH, with cultivations being done at Norrvatten.

Table A.1 Showing CFU/ml of 3 day old cultivations on C-Y media done by Norrvatten with the same water samples as investigated in the project.

Sample	22°C
PP100 Nov	-
PP100 Jan	520
PP100 Feb	120
PP208 Nov	1
PP208 Jan	0
PP208 Feb	0
PP691 Jan	0
PP693 Jan	0

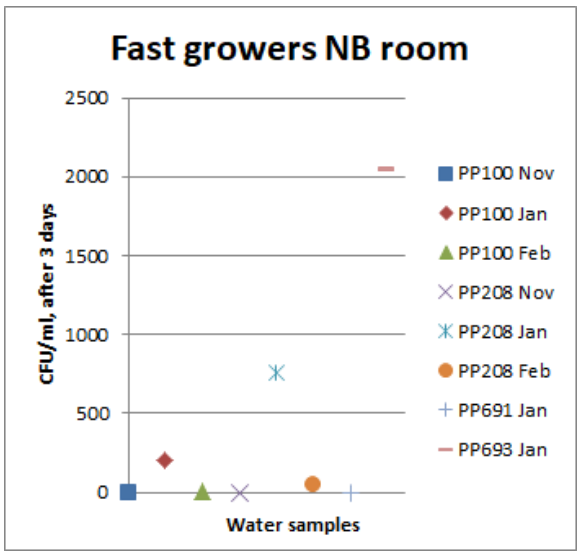


A



B

Figure A.4 A,depicts the total CFU/ml after 7 days from the different water samples grown in a C-Y medium at 30℃ and B depicts the total CFU/ml after 7 days from the different water samples grown in a C-Y medium at 37℃



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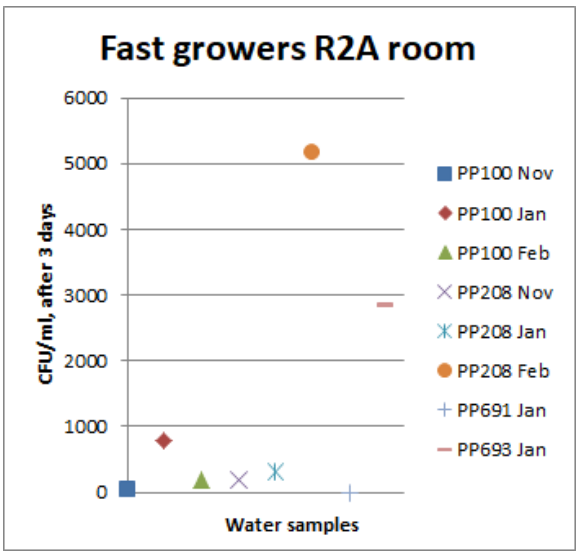

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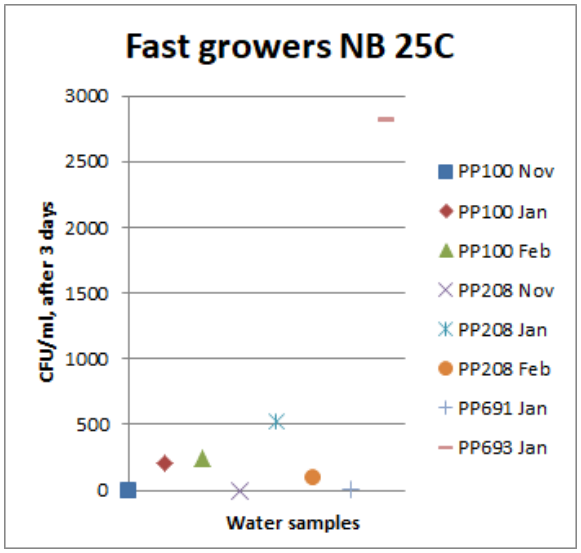
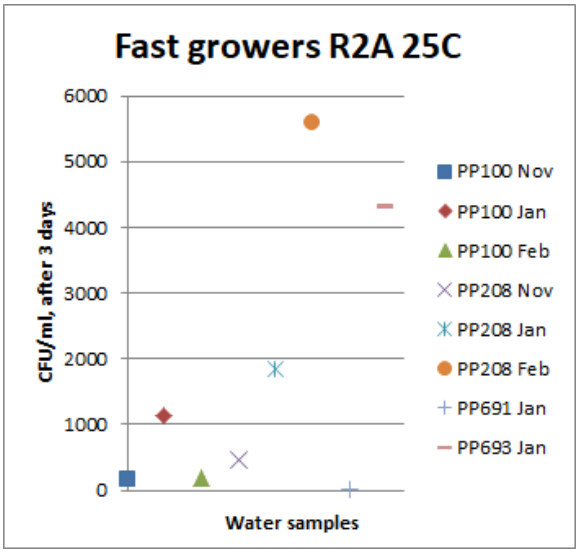
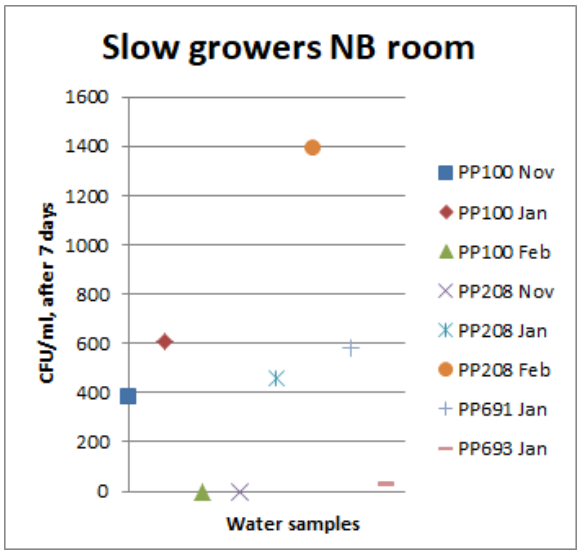


C



D

Figure A.5 A, depicts fast growers from the different water samples grown in a NB + NaCl media a at room temperature B,depicts fast growers from the different water samples grown in a R2A media at room temperature, C,depicts fast growers from the different water samples grown in a NB + NaCl media at 25℃ and D depicts fast growers from the different water samples grown in a R2A media at 25℃



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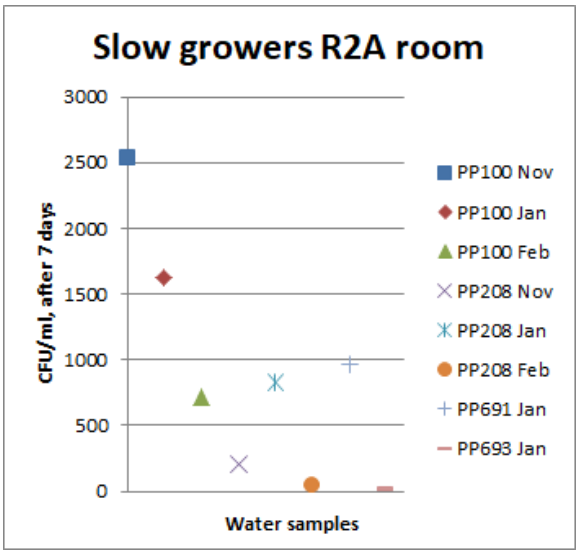
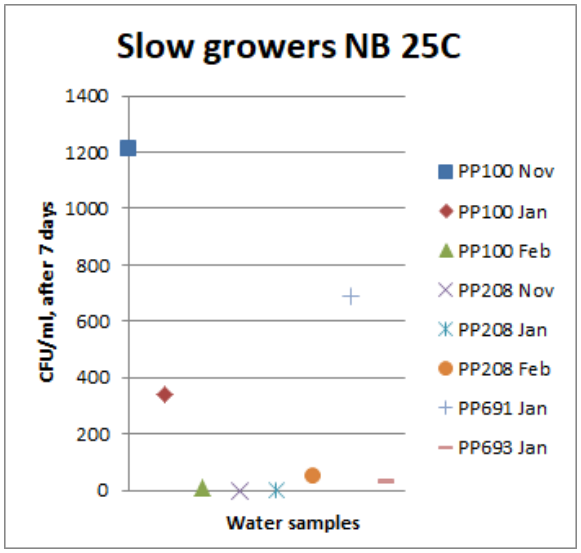


B



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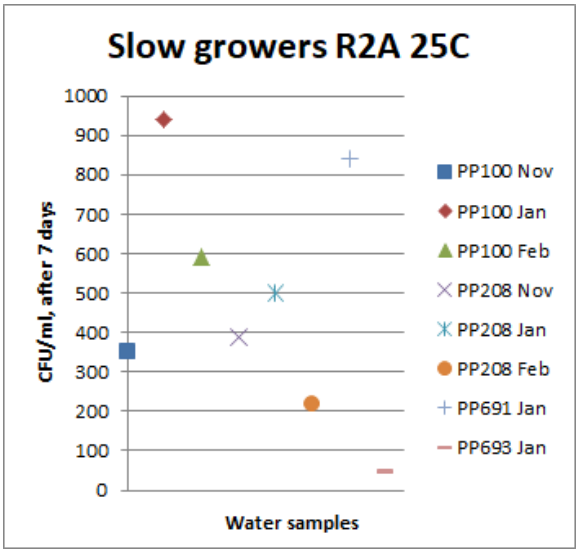
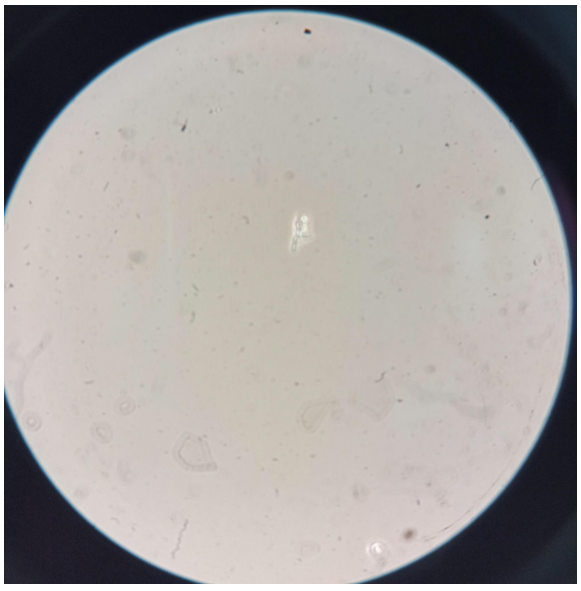
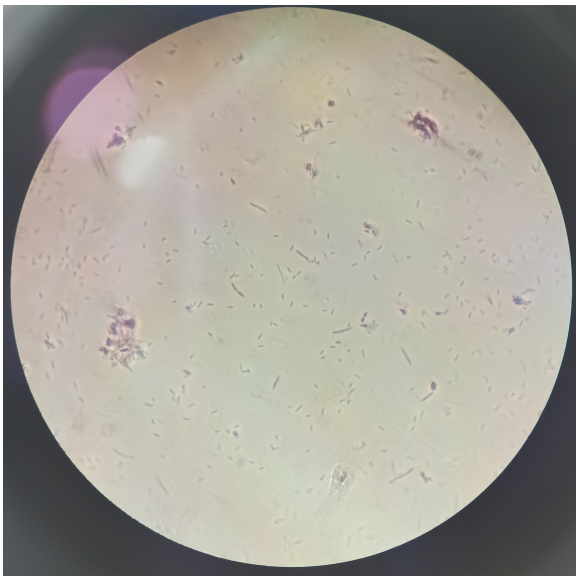


D

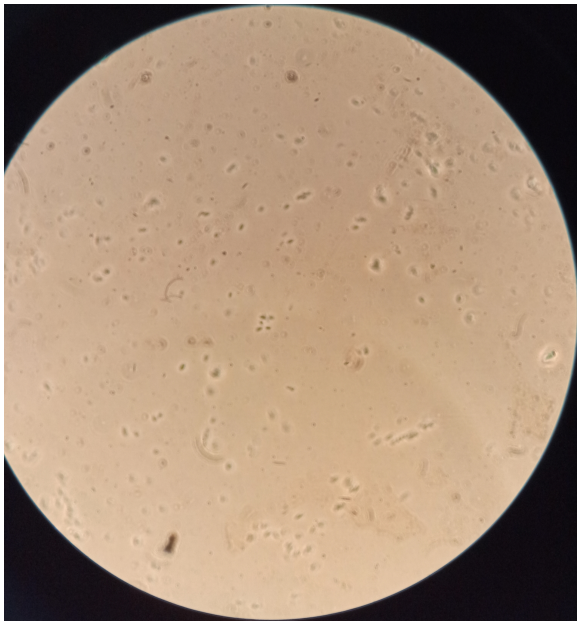
Figure A.6 A, depicts slow growers from the different water samples grown in a NB + NaCl media a at room temperature B,depicts slow growers from the different water samples grown in a R2A media at room temperature, C,depicts slow growers from the different water samples grown in a NB + NaCl media at 25℃ and D depicts slow growers from the different water samples grown in a R2A media at 25℃



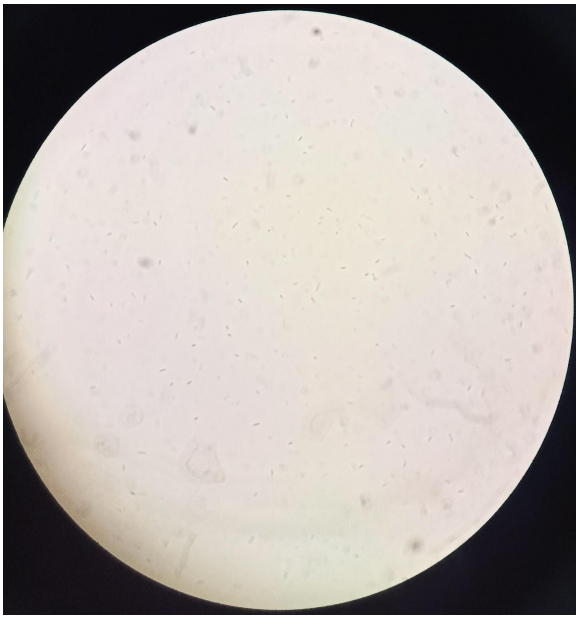
A



B



C



D

Figure A.7. A shows magnification of R2A pipe at room temperature after 3 days, B shows magnification of h four times replated m100 C-Y at room temperature after 7 days, C shows magnification of PP208 Feb R2A at room temperature after 3 days, D shows magnification with PP693 Jan C-Y at room temperature after 3 days

Table A.2 showing absorbance measurements (at 550 nm) on biofilm formation test in R2A media after 5 weeks of incubation

Sample	First measurement	Second measurement	Standard deviation
PP100 Nov	0.0864	-0.0316	0.0834
PP100 Jan	0.1871	0.3812	0.1372
PP100 Feb	0.0866	-0.1137	0.1416
PP208 Nov	0.1321	-0.1519	0.2008
PP208 Jan	0.6077	0.6034	0.0030
PP208 Feb	0.2402	-0.0473	0.2033
PP691 Jan	0.2853	0.0411	0.1727
PP693 Jan	0.2102	0.2722	0.0438
Pipe	0.0414	-0.4654	0.3584

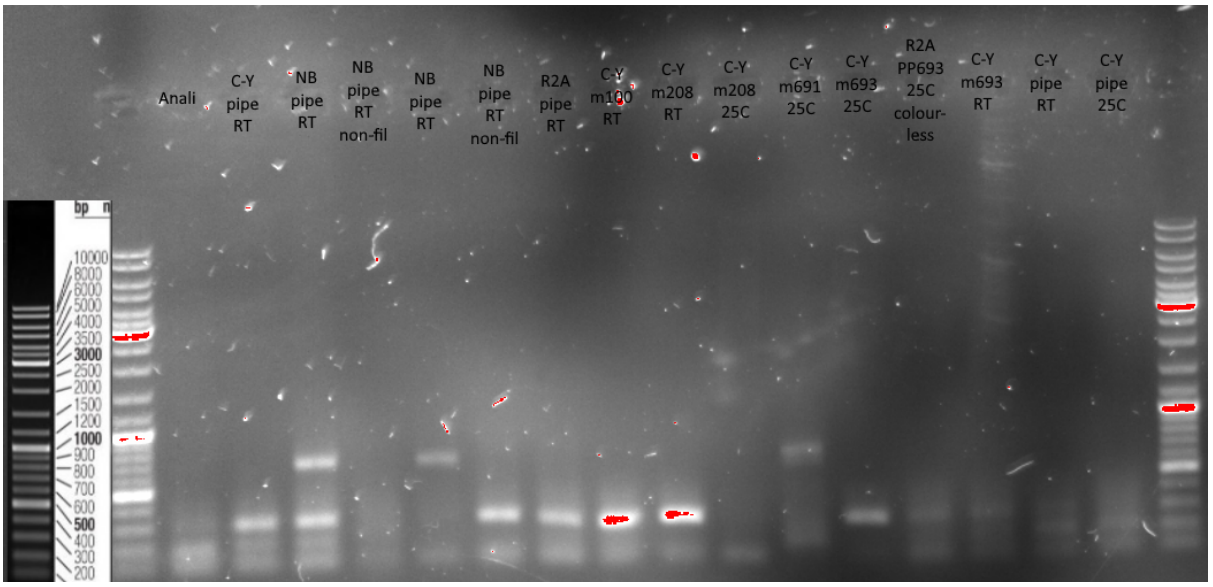


Figure A.8. Depicts the picture of 1 % agarose gel after electrophoresis for 30 min of PCR designed to detect ammonia oxidizing bacteria , with an expected size 1.1kb, carried out on 22/4-21.

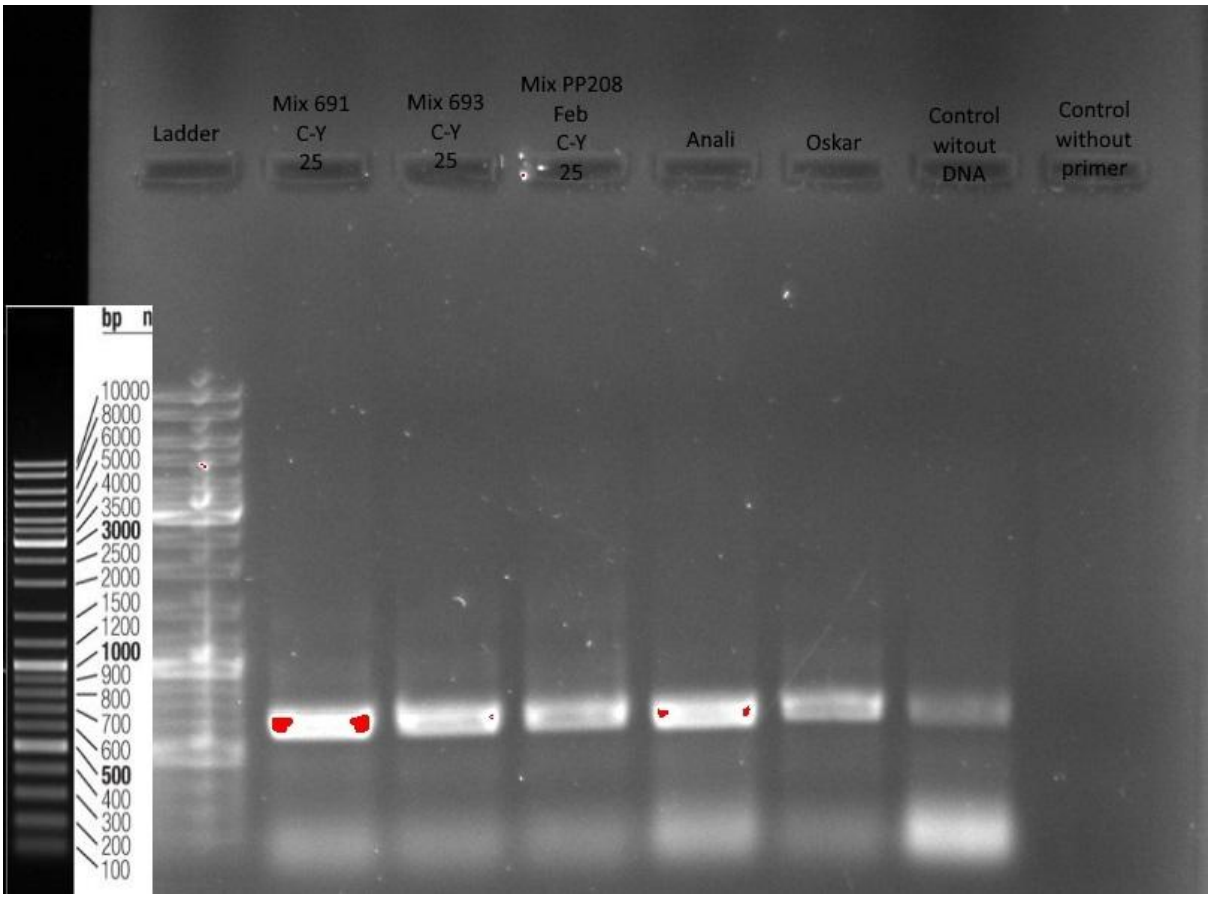


Figure A.9. Depicts the picture of 1 % agarose gel after electrophoresis for 30 min of PCR designed to detect Actinobacteria, with an expected size 640 bp

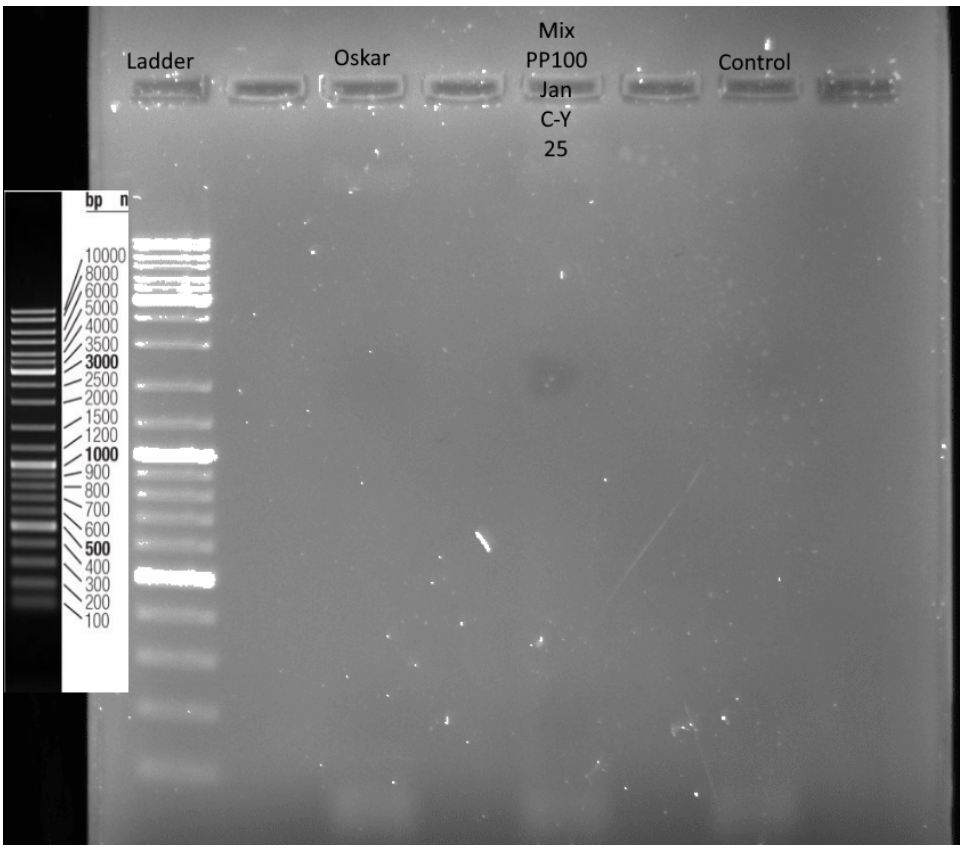


Figure A.10. Depicts the picture of 2 % agarose gel after electrophoresis for 45 min of Isothermal PCR designed to microorganisms with LacZ gene, expected size 100bp

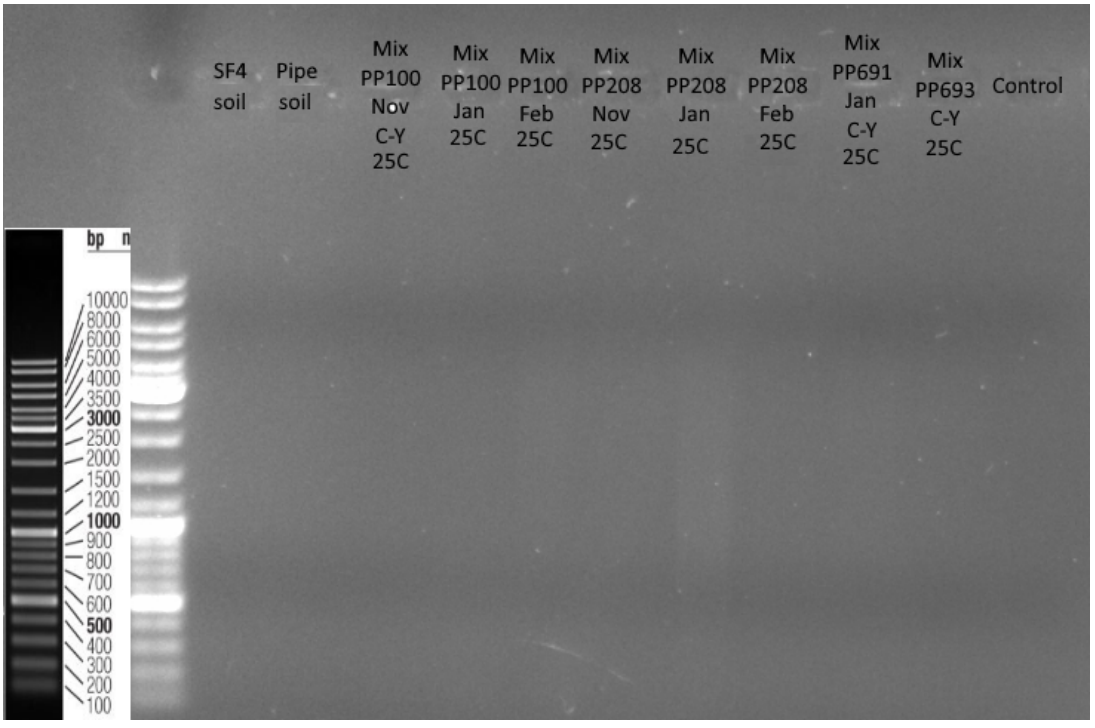


Figure A.11. Depicts the picture of 1 % agarose gel after electrophoresis for 30 min of PCR design to detect Sphingomonas, with an expected size 500 bp

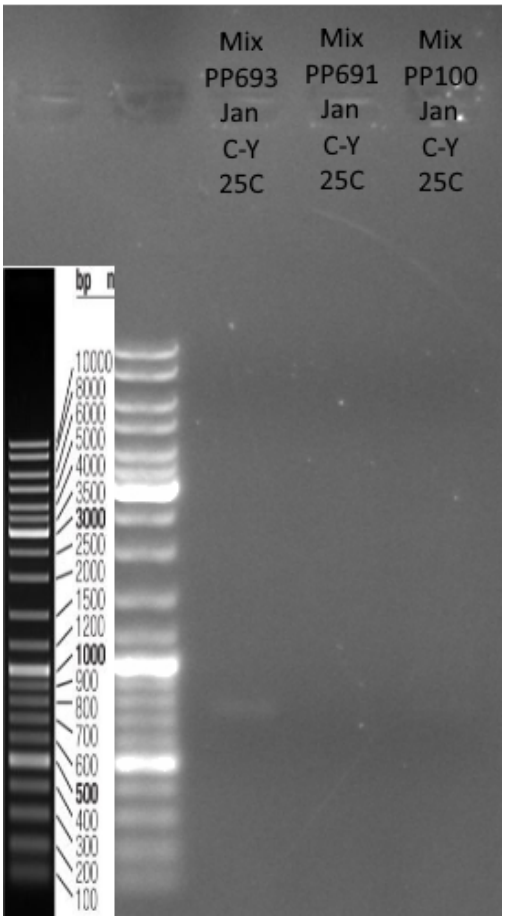


Figure A.12. Depicts the picture of 1 % agarose gel after electrophoresis for 30 min of a re-run of a PCR product which is supposed to be Pseudomonas where 10 µl product was added to the gel expected size 700 bp

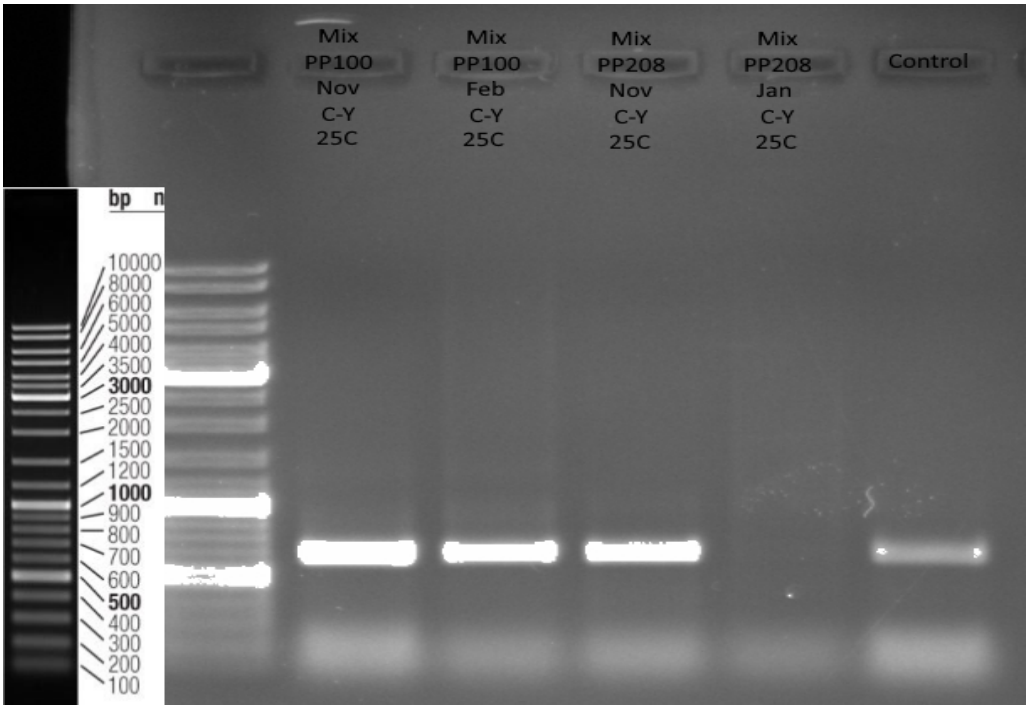


Figure A.13. Depicts the picture of 1 % agarose gel after electrophoresis for 30 min of PCR designed to detect Actinobacteria, with an expected size 640 bp