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Exploration of how UV disinfection affects the microbial community in drinking water

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Popular science summary

Safe drinking water is essential for human survival, society and countless industries. The water that comes from the taps in Sweden is treated in large scale drinking water treatment plants to ensure this. The treatment plants use a multiple barrier approach to take care of different aspects, one of the more important being treatment of microbiological properties. However, these barriers are in continuous need of improvement due to increasing population and changes in climate. In 2011 there was a *Cryptosporidium* outbreak in Östersund with estimated costs for the society up to 220mSEK. Parasites such as *Cryptosporidium* and *Giardia* are resistant to chlorine, a method commonly used for drinking water disinfection.

The parasites *Cryptosporidium* and *Giardia* and other pathogens can however effectively be killed of with UV irradiation. This has led to an increase of the usage of UV in drinking water treatment plants over the world. Ringsjöverket, a drinking water treatment plant that provide water for parts of northwestern Skåne in Sweden, installed its very own UV aggregate as a part of the treatment process in 2016. UV has proven to be effective as a disinfection method and has the positive effect of not using chemicals. However, research has mainly been directed at investigating how specific pathogens behave after UV irradiation. Often the research has been conducted in labscale environments.

The aim of this thesis was to explore how the whole bacterial community is affected by UV irradiation. Water from Ringsjöverket, that had either been irradiated with Ringsjöverket UV aggregate or a labscale aggregate as well as untreated reference water was taken in early autumn 2017 and then stored at either 7 °C or 22 °C. The water was analyzed with three methods to see what happens up to ten days after UV irradiation. The first method, Heterotrophic plate count (HPC), is a method that counts the number of culturable bacteria in a water sample. It is a standard method for analyzing drinking water, but this has been debated recently since neither the total bacteria count nor the existence of harmful microorganisms can always be related to the results of HPC.

The second method that was used, flow cytometry (FCM), is FCM can accurately detect the number of bacteria in a sample through staining the DNA of the cells which then will emit a detectable light signal when shot with a laser. This method also allows detection of changes in the community since the relative amount of DNA for each cell also can be detected. FCM is increasing in popularity when it comes to analyzing drinking water due to its easiness to use and robust results. The last method, qPCR, analyzes the amount of DNA. This method is of great interest in detecting UV-related damage since UV irradiation damages the DNA and in theory makes it less detectable in qPCR.

Interestingly the HPC showed that the UV irradiated samples had more regrowth than the untreated reference. These results contradicted results from both FCM and qPCR that yielded higher amounts of intact cells and DNA respectively in the untreated reference after a couple of days. Elevated storage temperature of the samples had the effect of both increasing growth of the untreated reference but also contributing to a decline of bacteria in the UV irradiated samples. The instant effect of UV irradiation could only be detected with qPCR. The labscale aggregate proved to have a slightly larger effect compared to the full scale aggregate at Ringsjöverket. With increasing use of UV in water treatment plants further optimization of these methods is a good idea in order to ensure that the effectiveness of UV irradiation is sufficient.

Abstract

UV irradiation is getting increasingly popular as a disinfection step in drinking water production. Ringsjöverket, a drinking water treatment plant in Sweden installed a full-scale UV aggregate in December 2016. However, the effect of UV irradiation on drinking water has mainly been tested on specific pathogens in labscale environment. Heterotrophic plate counts are often used when analyzing drinking water despite the fact that the results may not always relate to the total bacteria population or the existence of harmful pathogens. This study investigated the effect of UV on the whole bacterial community with heterotrophic plate counts (HPCs), flow cytometry (FCM) and qPCR.

Untreated water directly before Ringsjöverket UV aggregate, water treated with Ringsjöverket´s UV aggregate and water treated with a labscale UV aggregate was analyzed. Water was stored in light-impermeable 20 l water containers at 7 °C and 22 °C. The water was then analyzed as a function of days after UV irradiation up to 10 days.

HPC resulted in that the UV treated samples showed less diversity in terms of morphology of CFUs, but also had more CFUs. This could imply that UV has a selective effect. FCM analysis of intact cells pointed to that the UV treated samples did not grow back, but also did not die off completely. This can be compared with an untreated reference where bacteria count increased from initial levels. The results from the intact cell analysis implies that UV inhibits the growth of bacteria but does not necessarily have a direct killing effect. Flow cytometric analysis pointed towards a decrease in the fraction of cells with high nucleic acid content compared to untreated reference. At higher temperature the decrease was followed by an increase a couple of days later. This implies that UV initially changes the bacteria community composition but that the effect is not permanent due to repair mechanisms or that some bacteria does not get affected by UV and then start growing. The instant effects of UV were not detected by HPC or FCM but seen with qPCR targeting the 16S rRNA gene. This method showed that untreated samples had more DNA that could be amplified, and that Ringsjöverket UV seemed to result in less DNA damage than the labscale aggregate.

Further studies could be directed at investigating different dyes for flow cytometric analysis, such as dyes that targets bacteria at different rates depending on metabolic activity. Also, detection of UV damage through qPCR could be improved through using longer amplicon length. To get even further insight in how the taxonomic composition of bacteria is changed by UV, the amplicons from qPCR can be sequenced.

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Abbreviations

DWDS	Drinking water distribution system
FCM	Flow cytometry
HPC	Heterotrophic plate count
qPCR	quantitative polymerase chain reaction
HNA	High nucleic acid
LNA	Low nucleic acid
MBA	Microbial barrier analysis
NGS	Next generation sequencing

1 Introduction

Drinking water with sufficiently high quality is important for sustaining humans. A multitude of microbial barriers such as chlorination, slow sand filters, etc are used in water treatment plants to ensure safe, clean and palatable drinking water (*Produktion av dricksvatten - Svenskt Vatten*, 2016). UV was reported to first be used as early as 1906 in Marseille (Svenskt Vatten, 2009). Lately UV as a disinfection process has been used more frequently and during 2016 Ringsjöverket installed its own full-scale UV-disinfection aggregate. One of the main reasons behind UV treatment increasing in popularity is that growth of the parasites *Cryptosporidium* and *Giardia* are effectively prevented with UV treatment while they are resistant to chlorine (Hijnen, Beerendonk and Medema, 2006). Also, with UV treatment potentially less chlorine is needed which gives the positive effect of distributing less chemicals in the environment. In Sweden UV-treatment received attention due to a *Cryptosporidium* outbreak in Östersund 2010 where 30 000 people got affected (Sydvatten, 2016).

Cryptosporidium is believed to be one of the disease bringing microorganisms that will become more common as a result of global warming (Folkhälsomyndigheten, 2010). This gives increased incitement to gain more knowledge of how effective parasite treatment, such as UV, works in regard to the whole drinking water distribution network. The increase of viruses and protozoa such as *Cryptosporidium* is to some extent happening already (Roffey *et al.*, 2014).

Ringsjöverket (Sydvatten AB) has numerous water treatment steps to ensure that the water is free of particles and doesn´t smell or look unpalatable but most importantly microbial safe. At the end of the water treatment there is a disinfection step which since 2016 consist of UV irradiation. After the UV there is a final disinfection through adding chlorine (*Ringsjöverket | Sydvatten*, Accessed 2018).

The cost of the *Cryptosporidium* outbreak was evaluated and estimated to 220 mSEK by the Swedish Defence Research Agency (Totalförsvarets Forksningsinstitut, FOI) (2011). *Cryptosporidium* outbreaks in Galway, Ireland, with a population of 90 000 cost 38-47 mSEK and the *Giardia* outbreak in Bergen, Norway, with a population of 260 000 (of which 60 000 received water from the treatment plant with contamination) cost a 47 mSEK. The outbreak in Milwaukee, USA, with a population of 1,6 milllion (of which 880 000 received supply from contaminated treatment plant) cost 96,2 mUSD$ at 1993 years value. The Milwaukee incident also caused 63 deaths (Lindberg, Lusua and Nevhage, 2011).

It is estimated that a pathogen outbreak in the drinking water distribution system (DWDS) for a community with 20 000 inhabitants has a total cost of 136 mSEK and a community with 60 000 inhabitants a cost of 415 mSEK. Note that cost estimates of the effect on various functions in society are taken from examples in Sweden (Törneke and Engman, 2009).

Heterotrophic plate counts still are one of the most common ways to analyze water, despite the fact that it is known that less than 1% of drinking water bacteria show up on these plates since only the culturable bacteria show up. Also, the method requires substantial time, both in labor and in waiting for results (Gatza, Hammes and Prest, 2013). Furthermore, it is also a poor indicator for specific harmful organisms and the result of growth from a sample population may differ depending on conditions and setup of the method (WHO, 2017). Updating the routine methods used for monitoring the safety of drinking water might be necessary to ensure validity of results.

Research aimed at investigating the effectiveness of UV disinfection has mainly been directed at specific pathogens and often in labscale environment. Therefore there is a need to explore how the whole microbial community is affected by UV irradiation.

1.1 Hypothesis/aim of project

The aim of the project was to explore how UV irradiation affects the whole community of the most common microorganisms in drinking water. The most appropriate methods of analysis for monitoring a microbial community after UV treatment will also be identified and investigated. The methods heterotrophic plate count, flow cytometry and qPCR were used for the investigation.

This was done through conducting 3 data collections, one pre-experiment with data collection from tap water from Kemicentrum, Lund and two data collections from Ringsjöverket with different storage conditions. The sample collections were analyzed with heterotrophic plate count, flow cytometry and qPCR on daily intervals after UV irradiation up to 10 days.

1.2 Scope

The project was conducted in 20 weeks time. Due to time constraints of conducting each experiment it was deemed that 3 experiment runs could be done.

1.3 Disposition

The introduction part serves to give the reader an idea of drinking water production and the related future challenges. The background, material and methods and results will mainly be divided into the three different analyzing methods that has been used in this study, heterotrophic plate count, flow cytometry and qPCR. The discussion and conclusion part will sum up all of the above.

2 Background

2.1 Drinking water and UV

Water from taps that are meant to produce drinking water is classified as a foodstuff. Similar to other foodstuffs rules based on the principles of HACCP (Hazard Analysis ad Critical Control Point) are used to evaluate risks in drinking water (Svenskt Vatten, 2015). Microbial barriers are defined as a preparation step in a drinking water treatment plant that prevents the existence of pathogenic organisms such as bacteria, viruses and protozoa (Svenskt Vatten, 2015). The barriers work through either removal or inactivation of the organisms. Artificial infiltration of surface water, chemical flocculation followed by filtration, slow sand filter, ultrafiltration and disinfection by chlorine, ozone or UV are all examples of barriers. The demands on microbial disinfection barriers are examples of the HACCP-based routines. Svenskt Vatten has together with Norwegian water authorities, Norsk Vann, developed a tool called Microbial Barrier Analysis (MBA) as a part to verify and validate the HACCP-system. The MBA Sweden uses is a way of evaluating the raw water and determine how much treatment needs in order to get decent drinking water. Microbial Risk Analysis (MRA) is also used. MRA is a simulation tool for evaluating the microbial risks for drinking water (Abrahamsson, Ansker and Heinicke, 2009). World Health Organization follows similar guidelines (WHO, 2016).

Disinfection barriers needs to have high enough dose to be effective. The dose of UV disinfection is not as easily compared with doses of other chemical disinfection methods. Doses of chlorine and ozone for example are according to Livsmedelsverket (2014) calculated through multiplying C (concentration of chemical) with t (time of contact). These so called Ct values can most closely be related to an UV-dose given in J/m^2 (in Europe). According to German and Austrian standards, 400 J/m^2 gives a good barrier effect in most cases. The calculation of the doses is based the intensity of the UV-light times multiplied with the exposure time. Also, the transmittance of UV irradiation, how much UV-light that are able to pass through a certain amount of water, needs to be taken into account. In reality the exact values of UV-doses are difficult to obtain since the intensity of UV-light varies throughout the aggregate. Also, the flow of water, which determines exposure time, is not the same throughout the flow volume of the aggregate. (Livsmedelsverket, 2014). This way of calculation the UV-dose correlates with what was given from the manufacturer of the labscale UV-aggregate. They also point out that there is an ageing factor that needs to be included in the calculations as new UV-lamps are more effective than old. The calculations are included in appendices 1 and 2.

UV-light in disinfection mostly refers to UV-C light with a wavelength ranging from 150-280 nm. The light bulbs normally used are either low-pressure light bulbs which specifically emits radiation at 254 nm or medium pressure light bulbs that has higher intensity but a wider spectrum Svenskt Vatten (2009). Disinfection methods, such as chlorine and ozone, inactivates the bacteria through oxidation according to Hijnen et al (2006). UV, however, mainly disinfects through penetrating the cell and damaging the DNA and thus inhibiting replication of DNA. (Hijnen, Beerendonk and Medema, 2006). This different mechanism of disinfection can allow for inactivation of the parasites *Cryptosporidium* and *Giardia*, which are resistant to chlorine.

More specifically UV light causes lesions in the DNA through formation of pyrimidine dimers (Oguma *et al.*, 2001). See illustration of pyrimidine dimers in Figure 1.

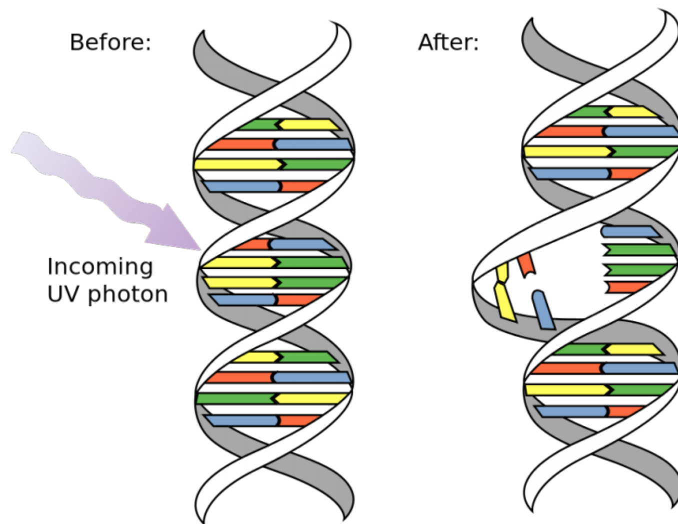

Figure 1. Picture illustrating pyrimidine dimer (from https://en.wikipedia.org/wiki/Pyrimidine_dimer).

2.2 Heterotrophic plate count

HPC has been used in the previous 100 years in the analysis of drinking water. All over the world this method is being applied to evaluate efficiency of treatment steps and monitoring the DWDS (Siebel *et al.*, 2008, p1). HPC evaluates the number of organisms that are able to grow on oligotrophic agar plates with without the presence of inhibitory agents or any other selective factor. Depending on the procedure setup, the plates are incubated up to 7 days, making the method somewhat time consuming. The method has a wide detection range of microorganisms, as opposed to methods that analyses specific pathogens. The use of HPC functions as an indicator in disinfection treatment steps which serve to keep number of microorganisms low. The actual value of colony forming units on the plates (CFUs)s are not as important as changes in number of CFU (WHO, 2017).

Even though the medium used in HPC are meant to be non-selective, there is always a selection due to the fact that the different bacteria of a sample will be able to grow at different rates depending on conditions such as incubation time and temperature. The type of medium can also be varied in terms of being high or low in nutrients. Allen et al (2004) writes that for water-bacteria a low nutrient medium is more favorable. R2a agar is mentioned as being the most common medium in heterotrophic analysis of drinking water. R2a was developed to have low-nutrient and low-ionic strength in order to favors organism with water based lifestyle (Allen, Edberg and Reasoner, 2004).

One of the major issues when it comes to evaluating the bacteria content in drinking water is the concept known as the plate count anomaly. This refers to that some bacteria can´t be cultured on agar plates but still are present and viable. It is estimated that only fractions as low as 1% of biospheres can be cultured (Epstein, 2013). This anomaly is also present in drinking water. The type of genera that can be enumerated with HPC depends on factors such as origin of water, season of the year, water treatment type and incubation temperature (Allen, Edberg and Reasoner, 2004).

2.3 Flow cytometry

Flow cytometry is mentioned as a fast and robust method for accurately detecting concentration of bacteria in water samples. This technique can analyze total bacteria cell count within the hour and has been increasingly used as a means of monitoring the microbial contents in drinking water (Gatza, Hammes and Prest, 2013). It analyzes through injecting sample suspensions into a stream of sheath solution through a narrow tube. Through this so called hydrodynamic focusing the particles and bacteria can pass one by one through a laser beam. The particles will then fluoresce at different wavelengths and intensities depending on what dyes and how much dye that have been pre-stained into them. Since bacteria have different properties than the debris particles one can get an accurate count of the total bacteria when using dyes that target specific characteristics of bacteria, such as DNA molecules.

One staining protocol that is described by Gatza et al (2013) and that has been used in this report is a staining protocol with SYBR® Green that stains into double stranded DNA. Propidium Iodide, is a dye that stains into the DNA and RNA of cells but unlike SYBR® Green can only enter cells with intact cell membrane integrity due to be a larger, charged molecule. Bacteria cells stained with SYBR® Green fluoresce and emits green light at λ_{max}=520 nm (FL1) while cells stained with Propidium Iodide fluoresce higher red light (FL3) Staining with both SYBR® Green and Propidium Iodide will then allow differentiation of intact and dead cells through using gating strategy. Figure 2 describes this phenomenon. (Gatza, Hammes and Prest, 2013).

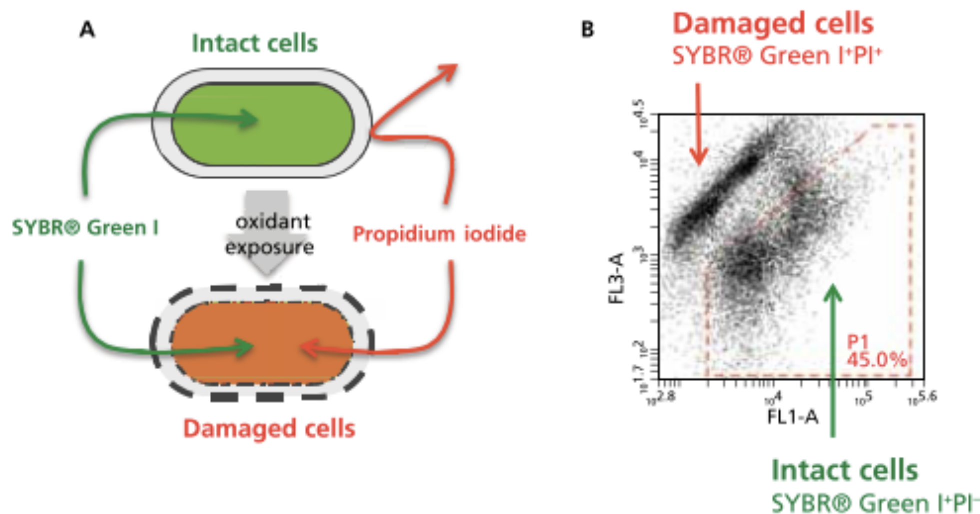

Figure 2. Description of staining protocol with SYBR® Green and Propidium Iodide (from Gatza, Hammes and Prest, 2013).

Besides getting an accurate count of intact and total number of cells it is also possible to analyze the community composition with flow cytometry using DNA staining. Bacteria in aquatic environment, such as drinking water, are often observed to be divided into bacteria with low nucleic acid content (LNA) or high nucleic acid content (HNA). This separates the bacteria cells from bacteria that have higher or lower content of nucleic acids in them and can be a factor when looking at differences in the bacterial communities between samples (Gatza, Hammes and Prest, 2013). Using the fact that higher % HNA gives of higher intensity levels in the flow cytometer and count of intact cells. Plotting histograms with the count of cells per each intensity level in green fluorescence gives a specific "fingerprint" for a sample, see Figure 3. Fingerprints and the % HNA of different samples can be compared to evaluate changes and differences of the bacterial community composition (Prest *et al.*, 2013). The ability to analyze a larger abundance of different bacteria, the speed, reproducibility, flexibility and equal or lower cost to compared to HPC has made researchers regard the method a competitor to HPC in routine drinking water analysis (Van Nevel *et al.*, 2017).

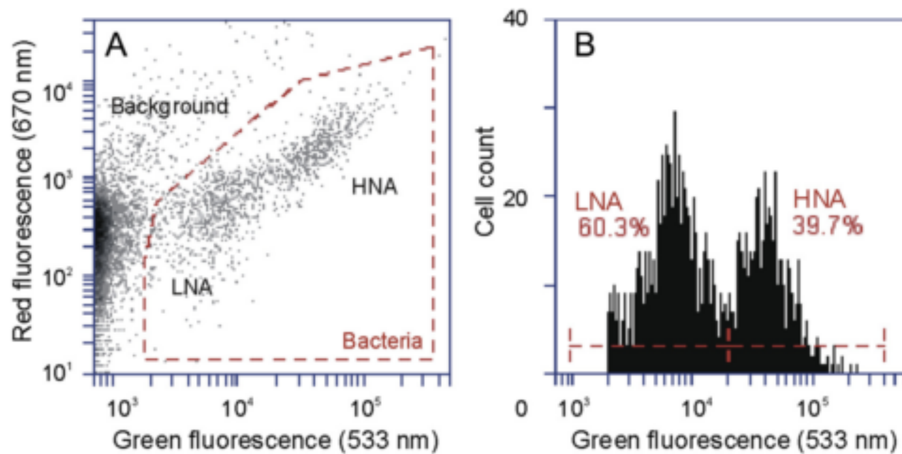

Figure 3. Flow cytometry concepts (A). Fingerprint analysis with flow cytometry (B) (from Prest *et al.*, 2013)

2.4 qPCR

Real-time PCR, or qPCR, is a method that molecularly analyses the DNA or RNA and amplifies it so it can later be sequenced. The PCR reaction is a cycle of three reaction steps. Denaturation of DNA followed by annealing of primers that bind into specific sites and elongation of the related segment of the DNA (amplicon). The product is an increased amount of the targeted amplicon that can be used in gene sequencing. In qPCR the elongation step gives of fluorescence that can be detected. Depending on how much DNA is present in the beginning higher or lower number of cycles is needed in order to reach a certain threshold value. This number of cycles is called the Cq-value, Figure 4 is an example of qPCR amplification curve that shows how Cq-value is determined.

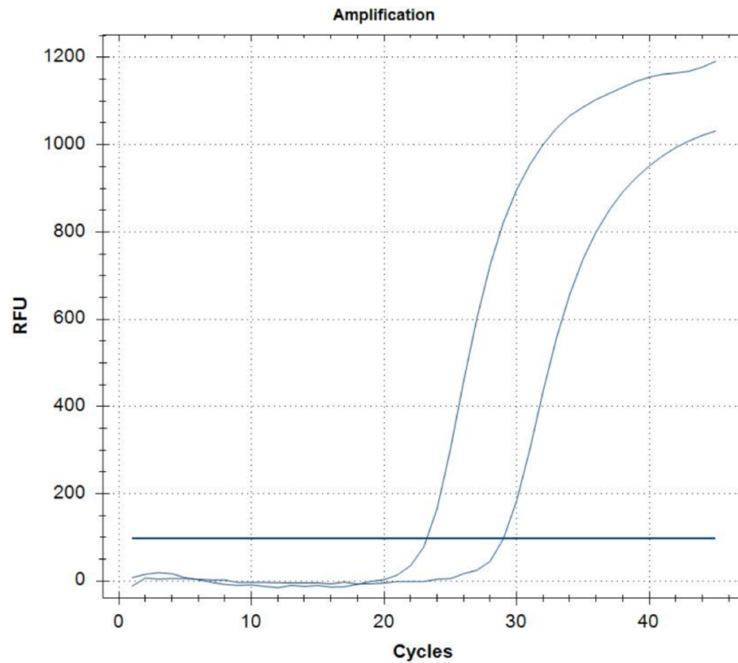

Figure 4. Example of qPCR amplification curve, y-axis is the fluorescence signal and x-axis is the cycles of the qPCR reaction. The vertical line Is the threshold value, the point where fluorescence signal crosses the threshold value is where the Cq-value is.

There are some theories of how UV damage to the DNA of bacteria cells could be detected. It is possible that the qPCR reaction will be obstructed either because that the primers cannot bind in to the amplicons or that the strand elongation will be hindered before completed PCR reaction. See

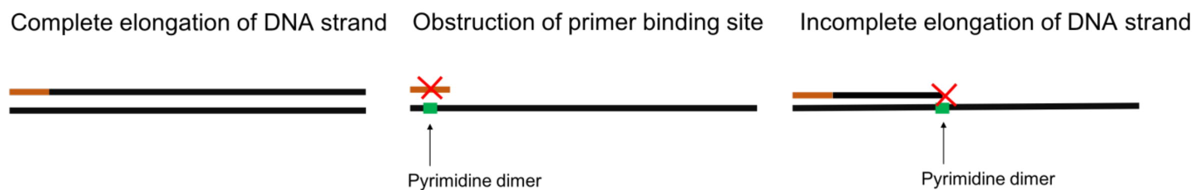

Figure 5. Amplification of damaged DNA in qPCR would then require more cycles in order to reach the same detection levels. This would mean that UV irradiated samples have a lower Cq value than a reference sample.

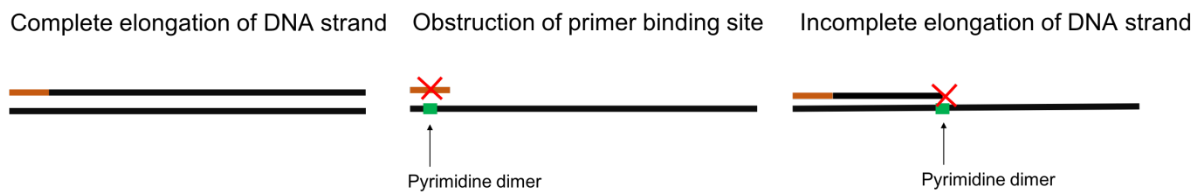

Figure 5. Illustration of how pyrimidine dimers could affect qPCR reaction. Either the primer can´t bind in to a separated DNA strand or the strand elongation will be hindered.

The 16S rRNA gene is a region that is included in most bacteria and that have conserved regions that has remained unchanged in the course of evolution, making it a good target for analyzing bacterial communities (Janda and Abbott, 2007). Primers that bind into the conserved regions will then amplify the DNA of all bacteria present. The 16S rRNA gene of bacteria in drinking water has been targeted in previous studies regarding next generation sequencing analysis (Lührig *et al.*, 2015).

3 Material and methods

3 experiments were performed. In the first experiment, TMB 7 °C, water from the division of applied microbiology (TMB) at Kemicentrum was taken. Water from Ringsjöverket was taken for the experiments Ringsjöverket 7 °C, and Ringsjöverket 22 °C. The samples were analyzed with the methods HPC, FCM and qPCR as a function of days after UV irradiation. Process schemes of the experiments can be seen in Figure 6 and

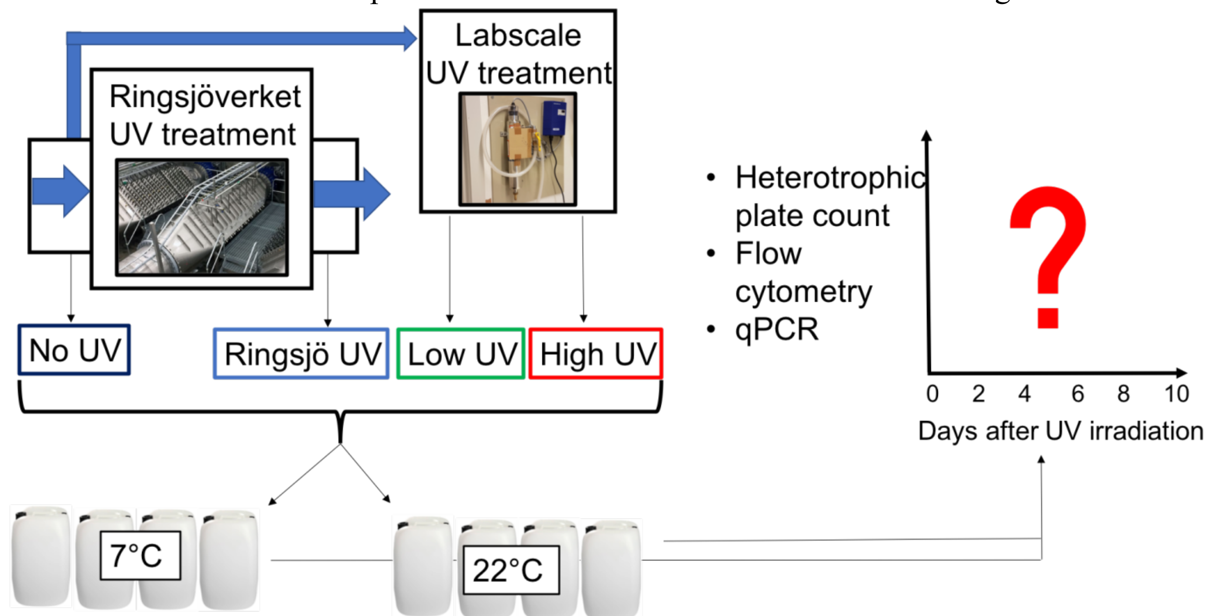

Figure 7.

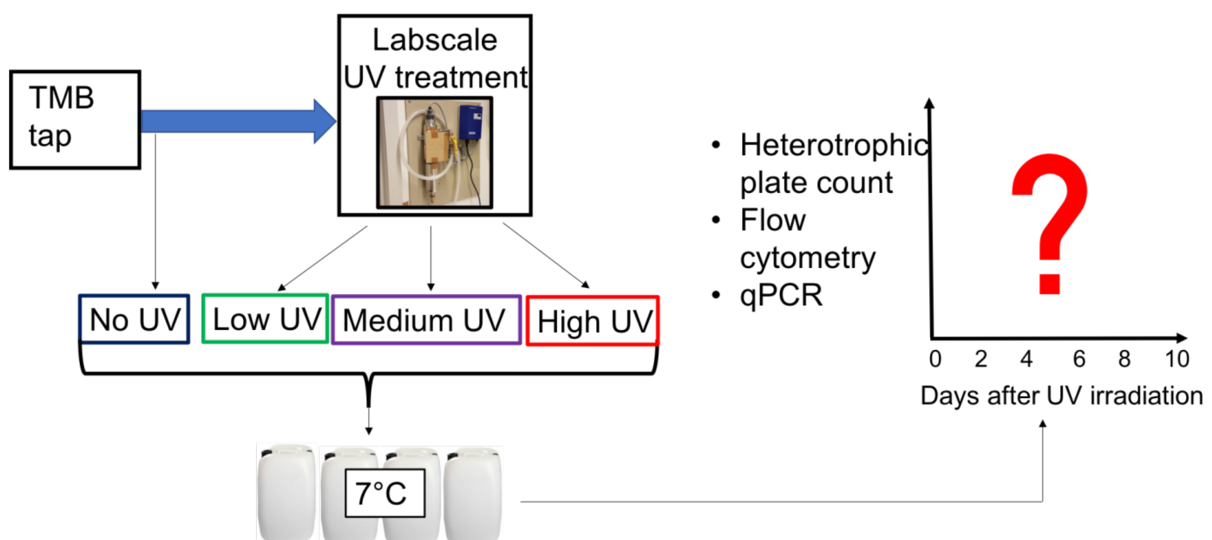

Figure 6. Process scheme of the "TMB 7 °C" experiment. Water from TMB with no UV irradiation (No UV) and after labscale UV irradiation at three different doses (High UV, Medium UV and Low UV) stored at 7 °C.

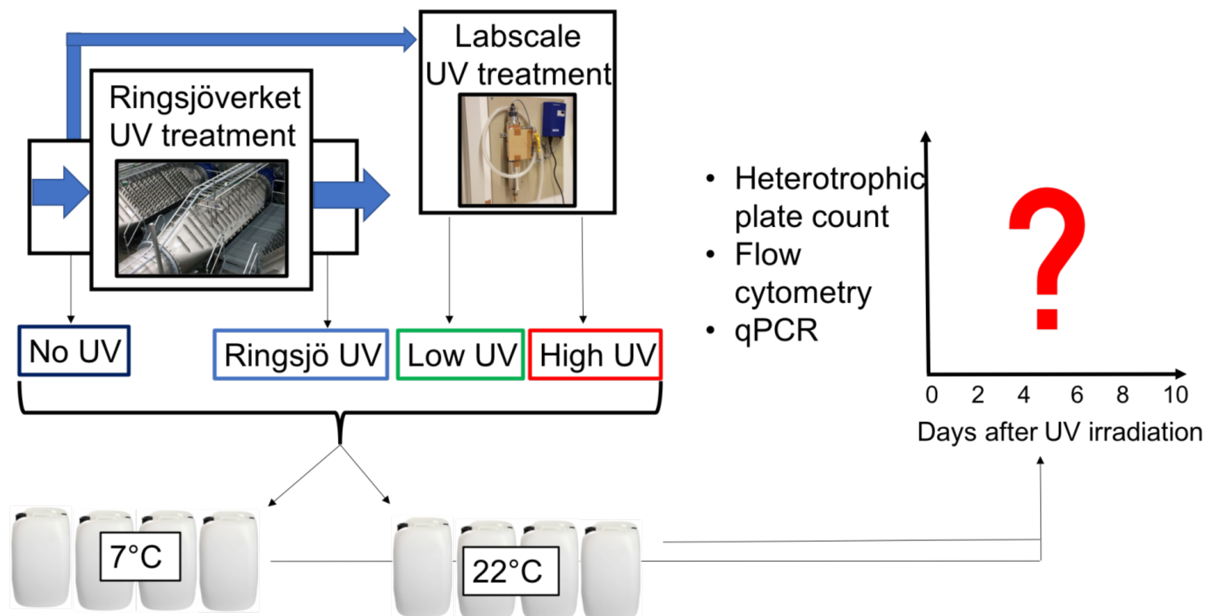

Figure 7. Process scheme of the "Ringsjöverket 7 °C" and "Ringsjöverket 22 °C" experiments. Water from Ringsjöverket before UV irradiation (No UV), after Ringsjöverket UV irradiation (Ringsjö UV) and after labscale UV irradiation at two different doses (High UV and Low UV) stored at 7 °C and 22 °C.

3.1 Experiment setup

The water from TMB was taken from the tap in the common room in the division Applied Microbiology at Kemicentrum. Water was run a couple of minutes before sample collection to ensure that the bacteria flora would represent the water in the DWDS and not that of bacteria that may have been growing at the end of the tap. 20 l per dose setup was taken and stored into 20 l plastic water containers enclosed in light-impermeable foil.

The labscale samples where run through an UV aggregate of the type Wedeco Auquada-UV, which utilizes UV-light at 254 nm (AB, 2017), see left picture of Figure 8. Buckets and a "Dränkbar pump DP 252" pump (Biltema, 2017) from Biltema were used. All equipment in contact with sample water was bleached with 10 % bleach and rinsed with ultrapure water prior to using the aggregate. The UV-dose was estimated through measuring the flow of water through the UV-aggregate manually. This was done through filling a measurement cylinder while taking the time of filling and then calculating the volume per second.

For the Ringsjöverket 7 °C, and Ringsjöverket 22 °C, water was collected at Sydvatten´s drinking water treatment facility Ringsjöverket, see right pitcture of Figure 8. Water for the No UV sample and water that was to be run through the labscale aggregate were taken from an outlet right before the treated water entered the full scale UV aggregate of Ringsjöverket. The labscale samples were run through the UV aggregate in the same way as the TMB tap water. Water for the Ringsjö UV samples were taken from an outlet right after the full scale UV aggregate. The taps for both outlets were sterilized with a burner. The water was also let to run a couple of minutes before sampling. 20 l per dose setup was taken and stored into 20 l plastic water containers enclosed in ligh-impermeable foil.

Figure 8. Labscale UV aggregate at TMB (left) and full scale UV aggregate of Ringsjöverket (right)

3.1.1 UV doses

The dose for UV doses for the labscale aggregate was calculated using information from the manual for the aggregate (AB, 2017), table for dose calculating from the manual of the UV aggregate (Wedeco, 2017) and formulas that were provided from the manufacturer of the UV aggregate. The doses for labscale aggregate was calculated from values given for UV lamps at the end of its life cycle, the actual dose might be higher. Also, it was estimated that the dose was linear with the flow rate in the aggregate, meaning that if a given value was 400 J m^2 for one specific flow rate the dose would be 400 J m^2 in the case of twice as high flow rate. For the full scale UV aggregate at Ringsjöverket, doses were estimated to be 400 J/m^2 continuously based on personal communication with Ringsjöverket staff. This dose was however estimated at the end of the light bulbs life cycle in the same way as the labscale aggregate (Wedeco, 2016).

3.1.1.1 TMB

For UV transmittance at 99 the table states that flow should be 400 J/m^2 at 0,73 m^3/h or about 200 ml/s.
No UV – water was run through the labscale UV aggregate while turned off.
High UV – water was run through the labscale UV aggregate at 86 ml/s, making the theoretical UV dose 800 J/m^2.
Medium UV - water was run through the labscale UV aggregate at 206 ml/s, making the theoretical UV dose 400 J/m^2.
Low UV - water was run through the labscale UV aggregate at 246 ml/s, making the theoretical UV dose about 300 J/m^2.

3.1.1.2 Ringsjöverket 7 °C

UV transmittance was 96.54, again flow of around 200 ml/s was estimated.
High UV – water was run through the labscale UV aggregate at 96 ml/s, making the theoretical UV dose 800 J/m^2.
Low UV - water was run through the labscale UV aggregate at 198 ml/s, making the theoretical UV dose 400 J/m^2.

Ringsjö UV – dose was estimated to 400 J/m^2 from talking with personnel at Ringsjöverket.

3.1.1.3 Ringsjöverket 22 °C

UV transmittance was 96.89, again flow of around 200 ml/s was estimated.
UV doses labscale
High UV – water was run through the UV aggregate at 94 m/s, making the theoretical UV dose 800 J/m^2.

Low UV - water was run through the UV aggregate at 200 m/s, making the theoretical UV dose 400 J/m^2.

3.1.2 Storage conditions

All water was stored in 20 l plastic water containers, one container for each respective dose. All water containers had been sterilized with 10 % bleach and rinsed with ultrapure water and then with the sample water before storage. In the TMB tap water experiment, the sample water was stored at 7 °C and in the main experiment with water from Ringsjöverket, the sample water was stored at 7 °C and 22 °C respectively. All water containers were covered with aluminum foil during storage to simulate conditions in DWDS and to make sure that the water was not further exposed to light that could aid in repair mechanisms of the microorganisms. Prior to all sample extraction for analysis the barrels were shaken to ensure homogeneity. Water was then extracted to fill up 2x 1 l water bottles for qPCR analysis and 5 ml falcon tube for HPC and FCM analysis.

3.2 Heterotrophic plate count

R2a agar was used for all the HPC experiments. The HPC was conducted with two different conditions with different amounts of water to make the observed spectrum of culturable bacteria concentration wider.

3.2.1 HPC of 100 µl

100 µl of sample water was spread out on plates of R2a agar in triplicates and then stored in an incubator at 22 °C, see Figure 9. Growth was noted after 7 days.

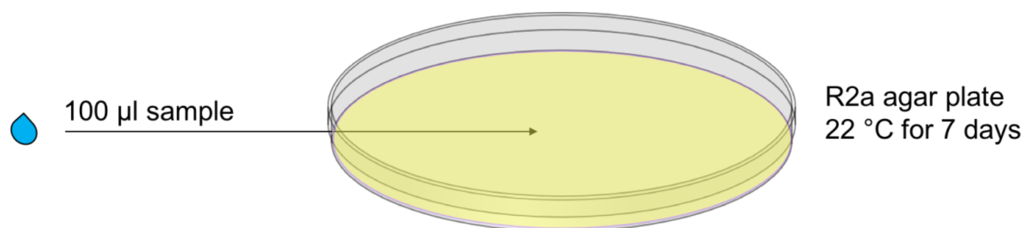

Figure 9. 100 µl R2a agar experiment setup.

3.2.2 HPC of 5 ml through filter paper

5 ml of sample water was filtered through Isopore Membrane Filters (Merck Millipore) with 0.22 µm pore size. The water was filtered with a filtering device made from aspirator, tubes, shake flask, metal filter, filter funnel and beaker. All filtering equipment in contact with sample water was sterilized using 10 % bleach before use in between different samples and also all work was performed in close proximity of a flame to ensure sterility. The filters were put on R2a agar plates and stored in 22 °C, see Figure 10. Growth was noted after 7 days.

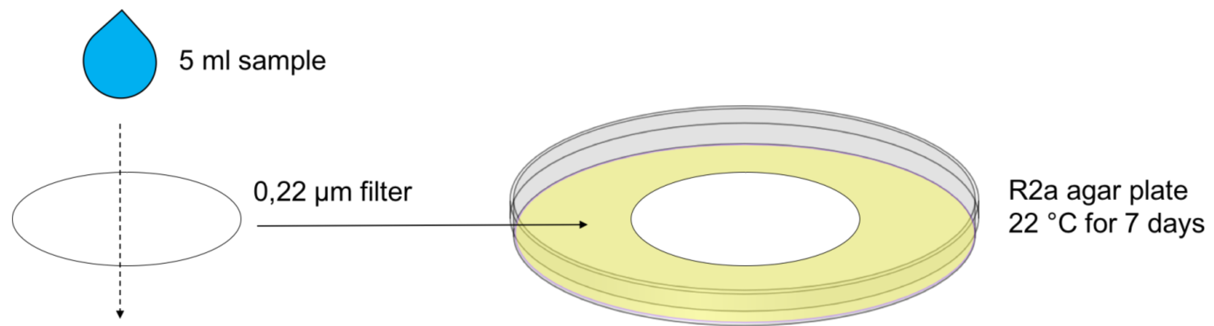

Figure 10.5 ml through filter paper on R2a agar experiment setup

3.3 Flow cytometry

BD Accuri C6 Flow Cytometer was used for flow cytometry analysis.

3.3.1 Staining protocol

As described in the background, staining protocol with SYBR® Green and SYBR® Green with Propidium Iodide can be used to get an accurate count of both total and intact cells in a water sample. In this study, protocol from Gatza et al report using SYBR® Green and Propidium Iodide was used (Gatza, Hammes and Prest, 2013).

Protocol also included one sample of dead *E. coli* to test the efficiency of the dyes as they have an expiration date. The *E. coli* was taken from a petri dish with strain DSM1116 and grown in LB medium for more than 12 hours. The *E. coli*/media solution was diluted to have an OD of about 0,2 in spectrophotometry at 600 nm. The solution was centrifuged and the supernatant removed. 500 µl of ethanol was added before centrifugation and removal of supernatant again. Finally, 1ml of PBS was added. All dead *E. coli* samples were stored in fridge at 7 °C.

500 µl of samples were taken from each dose in triplicates. No pretreatment of samples was made, only shaking the 20 l plastic water containers before sample extraction to ensure homogeneity. The 500 µl volume of samples and 500 µl dead *E. coli* were stained with SYBR® Green 1x concentration by 500 µl and then incubated at 37 °C for 15 min. SG + PI samples were in the same conditions but with 1x concentration of SG and 0.3mM concentration of PI (Gatza, Hammes and Prest, 2013). To prevent cross contamination in the flow cytometer equipment a sample of ultrapure water was run between each triplicate.

3.3.2 Flow cytometry settings

Quality check of the flow cytometer was made on each day before running it using 6-peak and 8-peak beads.
Following settings was used in the BD Accuri for respective type of sample.

MQ: 2 min, fast
Samples: Limit of 50 µl volume, medium, FL1>500, 1 cycle agitation
ECD: Limit of 100 000 event or 50 µl volume, medium, FL1>500, 1 cycle agitation

3.3.3 FlowJo

The software FlowJo (FlowJo, LCC) was used to analyze the flow cytometry data. Gating of the intact bacteria events can be seen in the left picture of Figure 11. Limit of HNA/LNA was set to 10^4 relative fluorescence intensity following Prest´s report (Prest *et al.*, 2013). For the

fingerprints, a window of 500 to 50 000 in relative fluorescence intensity was set, see the right picture of Figure 11.

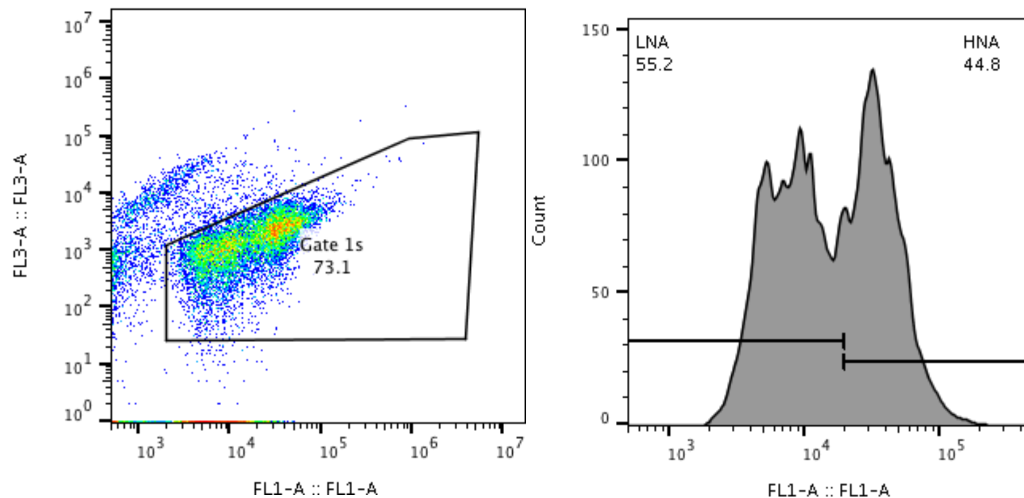

Figure 11. Gating of intact cells in a plot of Red fluorescence (FL3A) vs Green fluorescence (FL1A) (LEFT PICTURE) and limit of HNA in fingerprint analysis in a histogram plot of bacteria count vs Green fluorescence (FL1A) (RIGHT PICTURE).

3.3.4 Inducing damage experiment

In order to simulate conditions in the DWDS an extra experiment was conducted with only flow cytometric analyses. In this experiment, called "Inducing damage" sample water from each of the different UV doses was transferred to two 50 ml falcon tubes. The falcon tubes were put in an 22 °C incubator where falcon tubes containing one each respective dose lied still and the other falcon tubes taped to an rocking platform shaker, see Figure 12.

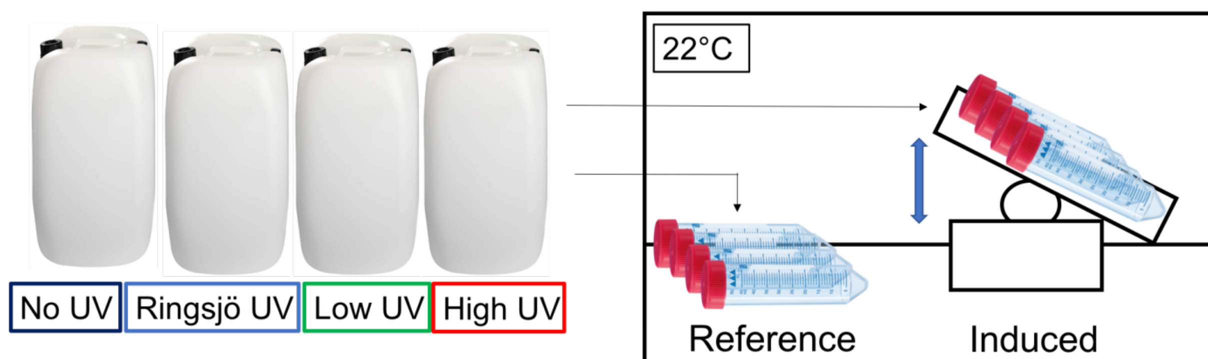

Figure 12. Inducing damage experiment setup.

3.4 qPCR

3.4.1 Concentrating of cells

In order to give high enough yields of DNA, 1 l of sample water was filtered through Isopore Membrane Filters with 0.22 µm pore size (Merck Millipore). The water was filtered with a filtering device made from aspirator, tubes, shake flask, metal filter and beaker. All filtering equipment in contact with sample water was sterilized using 10 % bleach before use in between different samples. Directly after filtration the filters were stored in petri dishes sealed with parafilm that were put in -20 °C freezer until DNA extraction. Replicates for each dose was produced.

3.4.2 DNA extraction

FastDNA Spin Kit for Soil was used on the 0.22 µm filters with 1 l of sample water filtered through since Lührig´s report on biofilms stated that this method had been suitable for extracting DNA from bacteria in biofilms in the DWDS (Lührig *et al.*, 2015, p9). Protocol from FastDNA Spin Kit for Soil was followed (MP Biomedicals).

In order to make a standard curve for calibration *Escherichia coli* J53was used. The reason behind this was that obtaining enough of DNA for calibration using the same filter method on water mentioned above would be too time consuming. Also, the J53 strain was available in the laboratory that was used. The *E. coli* DNA was extracted using GeneJet genomic DNA purification kit. Protocol from GeneJET genomic DNA purification kit was followed.

3.4.3 qPCR with Biorad

The Biorad CFX96 Touch Real-Time PCR Detection System (Bio-Rad) was used for qPCR. All samples, including filter duplicates of the samples, were analyzed in technical duplicates. The same polymerase that was used on most of Lührig´s experiments, ExTaq, was used in this study.

The 16S rRNA gene is a potential good target when looking at whole bacterial communities as mentioned in the background, and thus the 16S rRNA gene was chosen in this study. Following sequences were used for primers and probe. Forward primer (Bact F): 5´-TCCTACGGGAGGCAGCAGT-3´ (melting temperature at 59,4 °C). Reverse primer (Bact R), 5´-GGACTAC- CAGGGTATCTAATCCTGTT-3´ (melting temperature at 58,1 °C). Bacterial probe: (6-FAM)-5´-CGTATTACCGCGGCTGCTGGCAC-3´-(TAMRA) (melting temperature at 69,9 °C). Amplicon length was at 466 bp, calculated through that the residues 331 and 797 on *E. coli* 16S rRNA gene were starting and end points (Nadkarni *et al.*, 2002).

Master mix with final concentrations of 2mM Extaq Buffer, 2.4 mM dNTP, 25mM $MgCl_2$, 0.3 µM of Bact F and Bact R primer respectively, 0.4 µg/ µl BSA, 0.5 µM bacterial probe and 0.05 U/ µl Extaq HS. In each well 5 µl sample DNA and 15 µl master mix was used.

The protocol in the qPCR reaction started with 2 min initial denaturation at 95 °C. This was followed by 45 cycles of denaturation for 20 s at 95 °C, annealing for 20 s at 60 °C and elongation for 30 s at 72 °C repeated for 45 cycles. The protocol and master mix recipe was devised after testing with protocol from Nadkarni´s report regarding bacterial load analysis through a universal probe and primer set (Nadkarni *et al.*, 2002).

The dyes EVA Green and bacterial probe was tested and also different dilution series to confirm qPCR efficiency. Also, comparisons with and without BSA was made. In order to test the protocol, standard curves with qPCR data from dilution series of 1x, 10x and 100x were constructed. The efficiency was calculated using equation 1 where the *k* is the slope of standard

curves made when plotting Cq against log(concentration) with a dilution series. It is mentioned in that at least 5-fold dilution series should be made but since only a three-fold dilution was possible due to low starting concentrations the standard curve was made as mentioned above. When the efficiency was 90-100 % the protocol was deemed to work. (Svec *et al.*, 2015).

Equation 1 $$E = 1^{(-\frac{1}{k})} - 1$$

4 Results

4.1 Sampling

The UV transmission was measured for each of the water samples on day 0 after UV irradiation to calculate the theoretical UV dose. Results of the UV transmission is given in 3.1.1 UV doses.

4.2 Heterotrophic plate count

Results for the triplicates of 100 µl sample water or 5ml sample water through filter papers on R2a agar plates are described below. Note that growth on the agar plates was only observed on the Ringsjöverket 22 °C experiment.

4.2.1 HPC of 100 µl

4.2.1.1 TMB 7 °C

No growth on any of the plates occurred for the TMB tap water stored at 7 °C.

4.2.1.2 Ringsjöverket 7 °C

No growth on any of the plates occurred for the Ringsjöverket water stored at 7 °C.

4.2.1.3 Ringsjöverket 22 °C

Growth was observed for the Ringsjöverket water stored at 22 °C. Estimated CFU count can be seen in Figure 13 and photos of one of the triplicates for each respective dose at day 7 after UV irradiation can be seen in Figure 14, photos from the rest of the experiment days can be seen in Appendix: "HPC, 100 µl " The results show that No UV samples show little increase in CFU over time whereas labscale UV and Ringsjö UV grows exponentially on 2-4 days after UV irradiation.

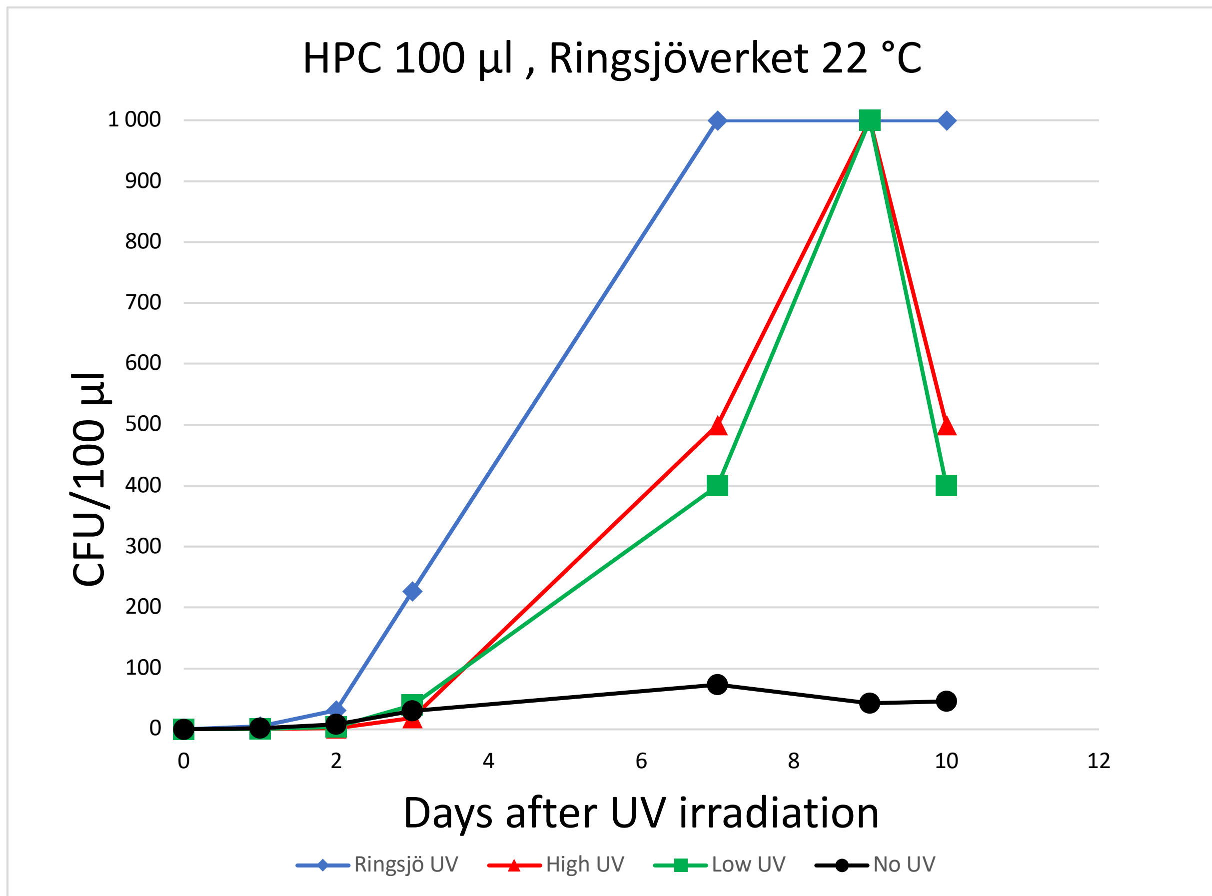

Figure 13. Estimated CFU count of 100 µl sample water from Ringsjöverket stored at 22 °C on R2a agar at 22 °C for 7 days as a function of days after UV irradiation.

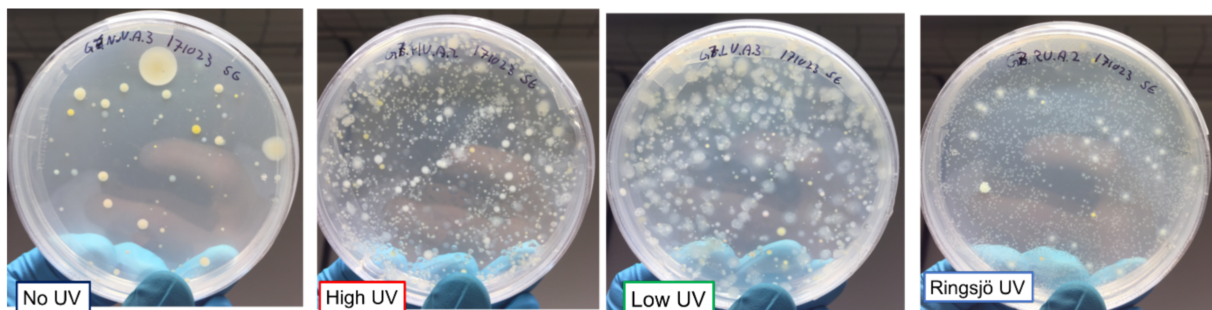

Figure 14. 100 µl sample water stored at 22 °C day 7 after UV irradiation on R2a agar at 22 °C for 7 days.

4.2.2 HPC of 5 ml on filter paper

4.2.2.1 TMB 7 °C

No growth on any of the plates occurred for the TMB tap water stored at 7 °C.

4.2.2.2 Ringsjöverket 7 °C

No growth on any of the plates occurred for the Ringsjöverket water stored at 7 °C.

4.2.2.3 Ringsjöverket 22 °C

The Ringsjöverket water stored at 22 °C had growth similar as in the 100 µl experiment. Estimated CFU count for the samples can be seen in Figure 15. Photos of the agar plates from each respective dose at day 7 after UV irradiation can be seen in Figure 16, photos from the rest of the experiment days can be seen in Appendix: "HPC photos, 5 ml through filter paper

“. The non UV treated water showed very little growth compared to the UV treated samples similar to the 100 µl setup. Even with the increased volume CFU count does not get in such a high number that they cannot be counted by eye. Furthermore, Ringsjö UV yielded significantly higher count of CFUs compared to labscale UV. Also, the non UV treated samples had a higher variation of how CFU morphology (eg yellow, white, orange, purple and varying size whereas the UV treated had exclusively beige CFU´s). The exception was Ringsjö UV that had some occurrences of larger black colonies.

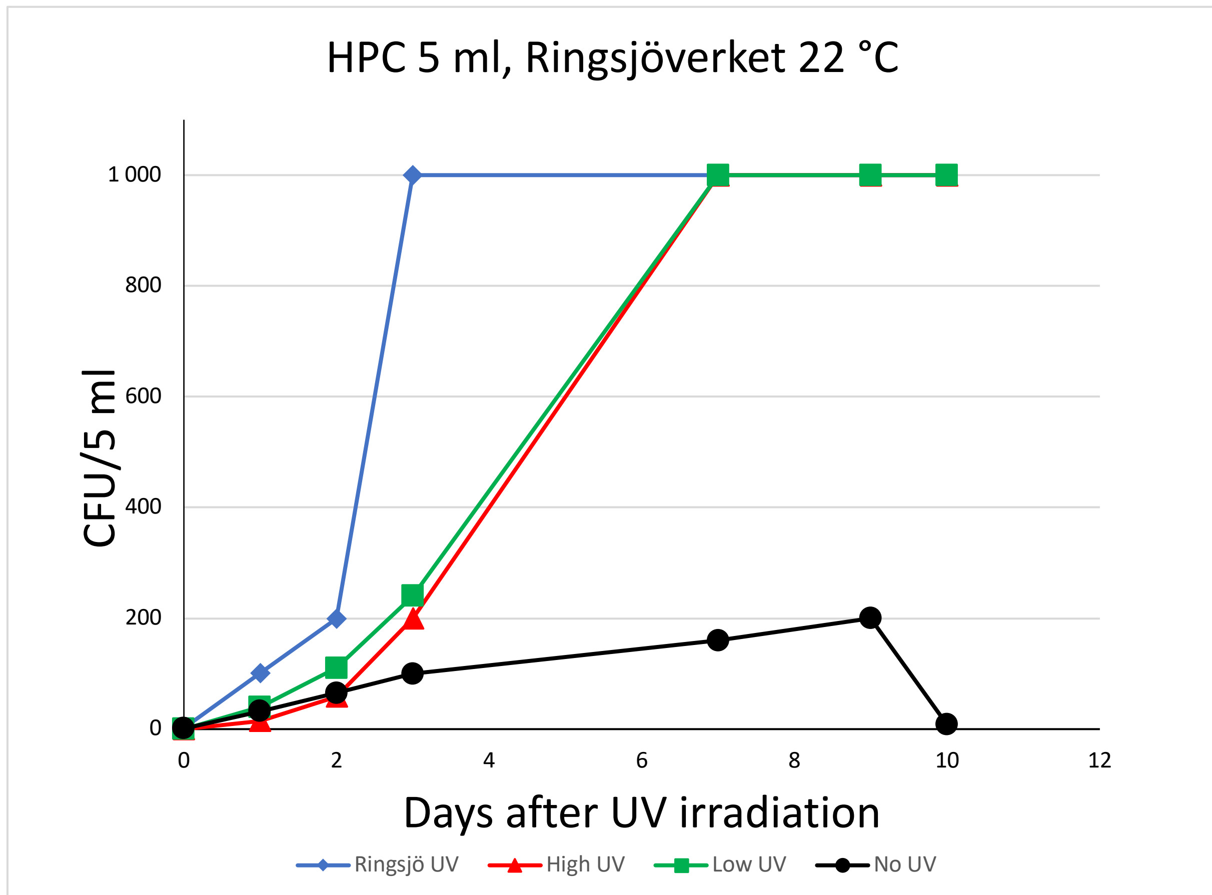

Figure 15. Estimated CFU count of 5ml sample water stored at 22 °C through 0,22 µl filter paper on R2a agar at 22 °C for 7 days as a function of days after UV irradiation.

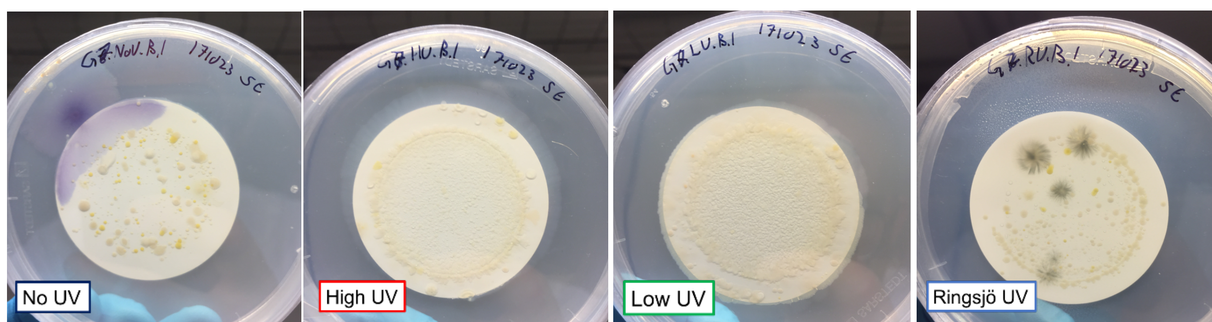

Figure 16. 5ml sample water stored at 22 °C day 7 after UV irradiation through 0,22 µl filter paper on R2a agar at 22 °C for 7 days. (From left to right: No UV - High UV - Low UV - Ringsjö UV)

4.3 Flow cytometry

Data for intact cells and fingerprint analysis with % HNA is shown below. Data from total cell number was not included due to not contributing to the discussion of this report.

4.3.1 Intact cells

4.3.1.1 TMB 7 °C

Results from the TMB 7 °C water points towards that water left untreated shows higher levels of intact bacteria after a couple of days, see Figure 17. The trend was not always clear though, since the data point of day 7 in the No UV could not be statistically differentiated from the other samples.

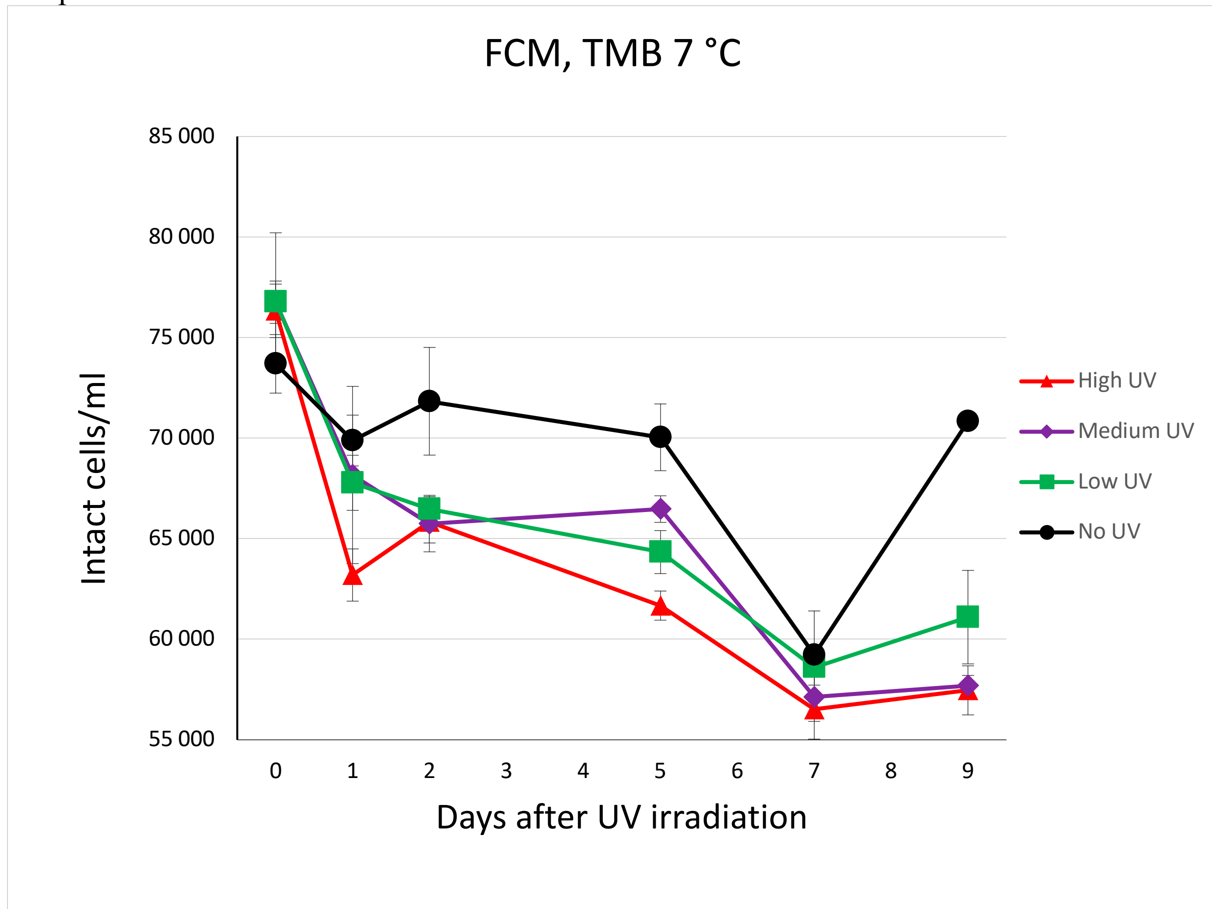

Figure 17. Flow cytometry data of intact cells, tap water experiment. Error bars shows statistical difference between triplicates of the samples..

4.3.1.2 Ringsjöverket 7 °C and 22 °C, labscale UV

The results from labscale UV for 7 °C and 22 °C storage can be seen in Figure 18. In the beginning there´s no big difference in bacteria count but as the days go it seems that the reference sample, No UV, has much higher count of intact bacteria cells. Furthermore, higher storage temperature makes the trends even clearer, both the regrowth of the and the decline in growth for the labscale samples.

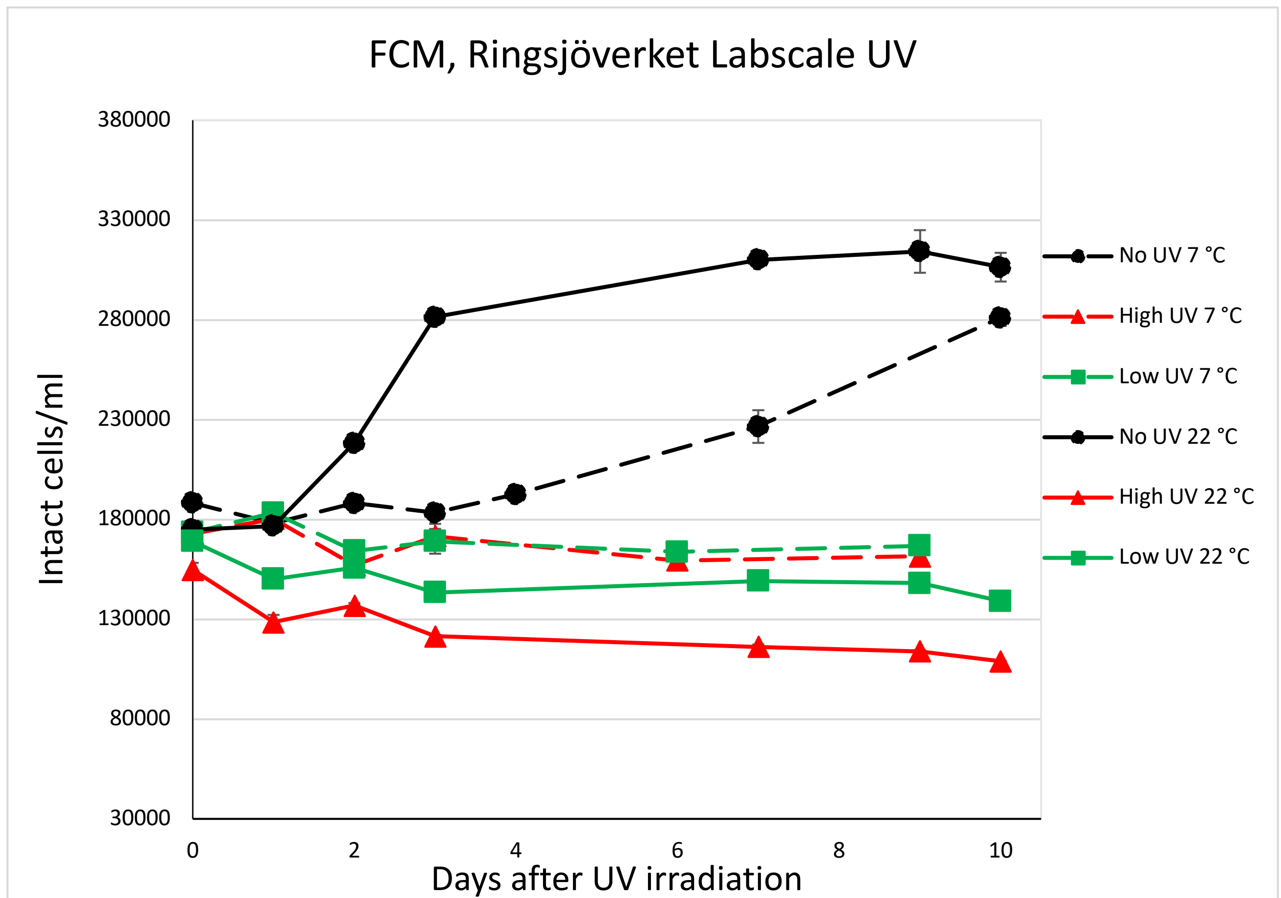

Figure 18. Flow cytometry data of intact cells, Ringsjöverket 7 °C and Ringsjöverket 22 °C labscale UV. Error bars shows statistical difference between triplicates of the samples..

4.3.1.3 Ringsjöverket 7 °C and 22 °C, Ringsjö UV

The results for the Ringsjö UV can be seen in Figure 19. The Ringsjö UV seems to behave in similar ways as labscale only slightly less effective.

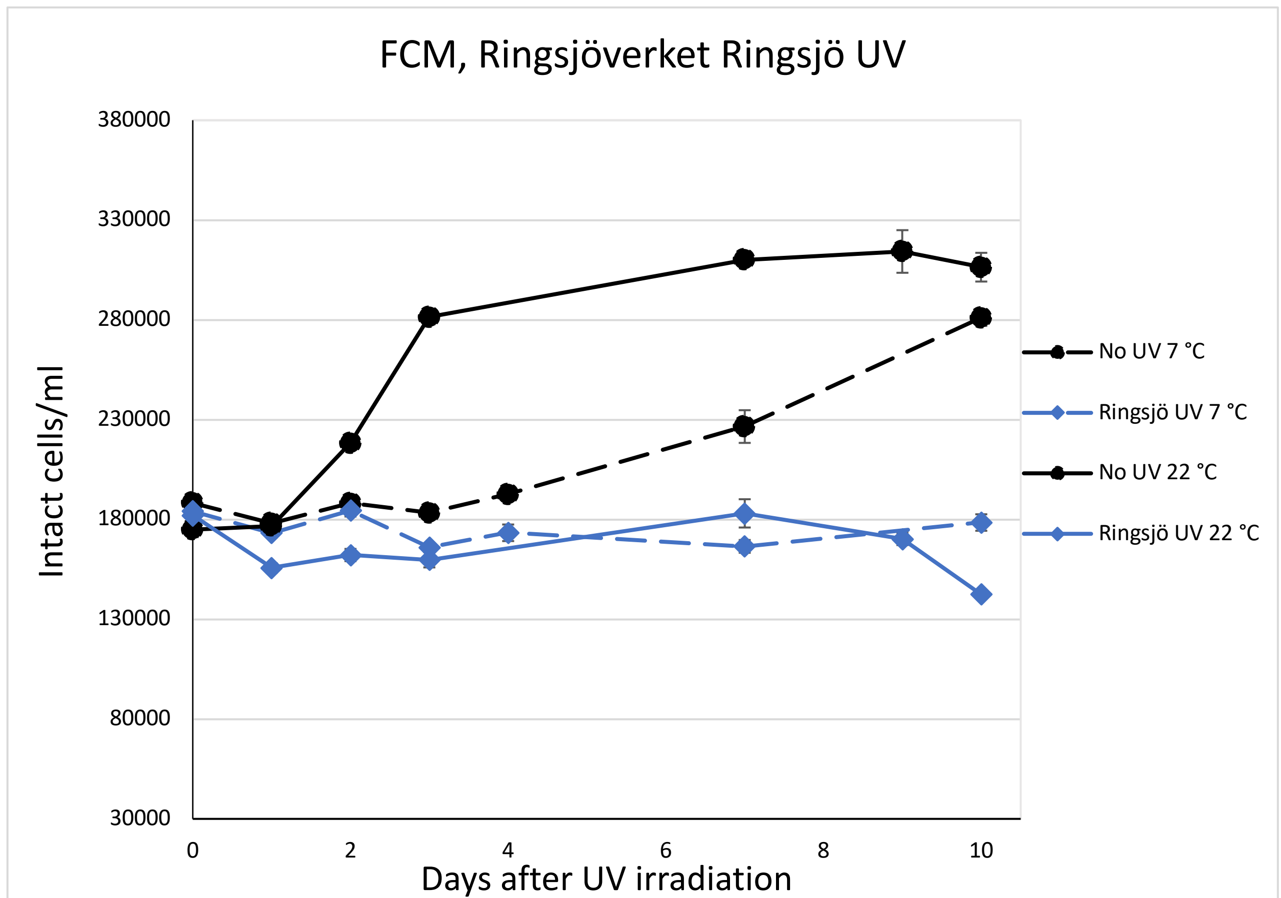

Figure 19. Flow cytometry data of intact cells, Ringsjöverket 7 °C and 22 °C Ringsjöverket UV. Error bars shows statistical difference between triplicates of the samples..

4.3.1.4 Inducing damage experiment

The "Inducing damage"-experiment results can be seen in Figure 20 for labscale UV and Figure 21 for Ringsjö UV. The resulting trends were similar to that of the other experiments. However, in the induced setup an instant cell kill-off was observed and also the curves of different UV doses seem to be even less different from each other. The same trend appeared with Ringsjö UV except that it was less efficient compared to labscale as before. It might be possible to induce UV-related damage if more optimization is done with tuning the type of damage and dose of damage. This would be beneficial as it could yield faster results when finding out the grade of UV-disinfection. The results might also imply that conditions and also that even differences in handling samples could have impact on the outcome.

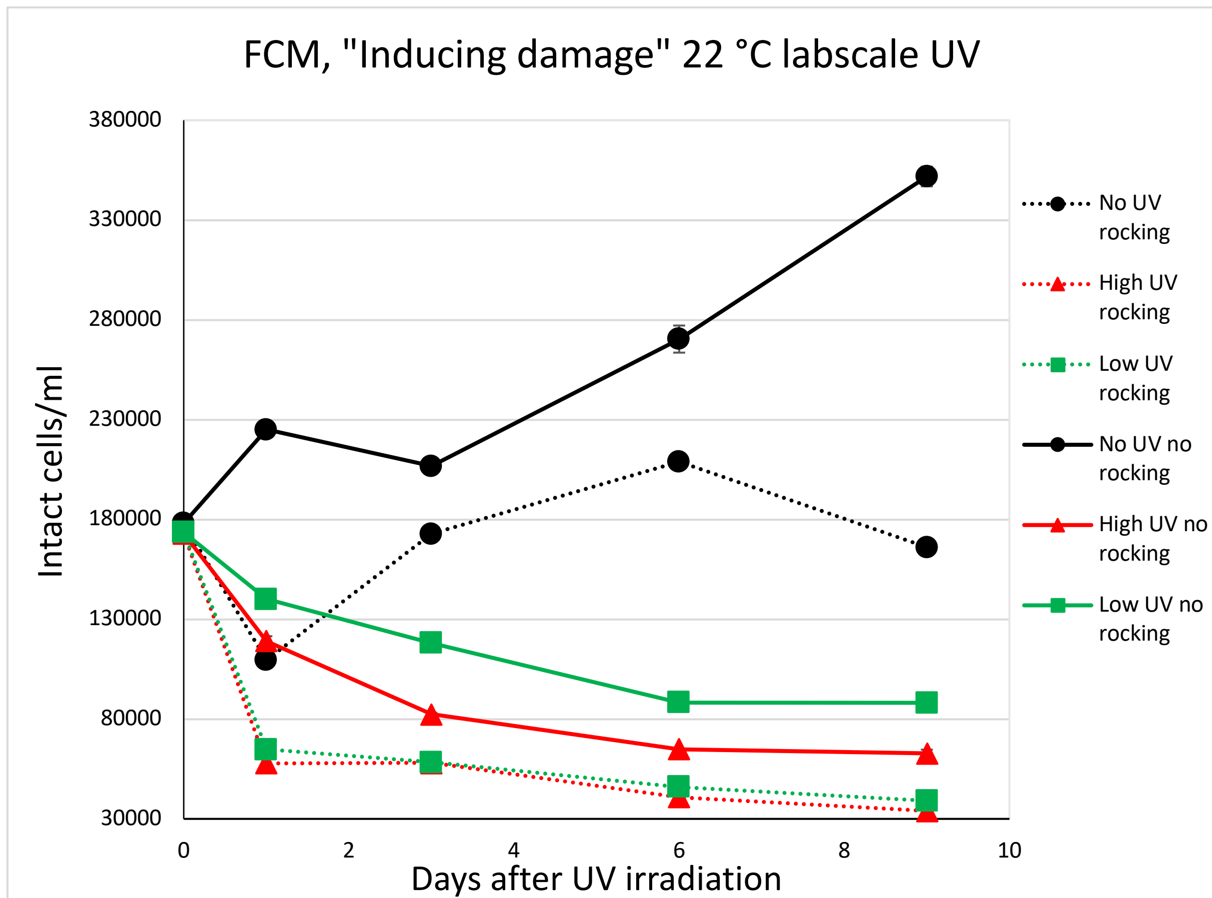

Figure 20. Flow cytometry data of intact cells Inducing damage, labscale. Error bars shows statistical difference between triplicates of the samples.

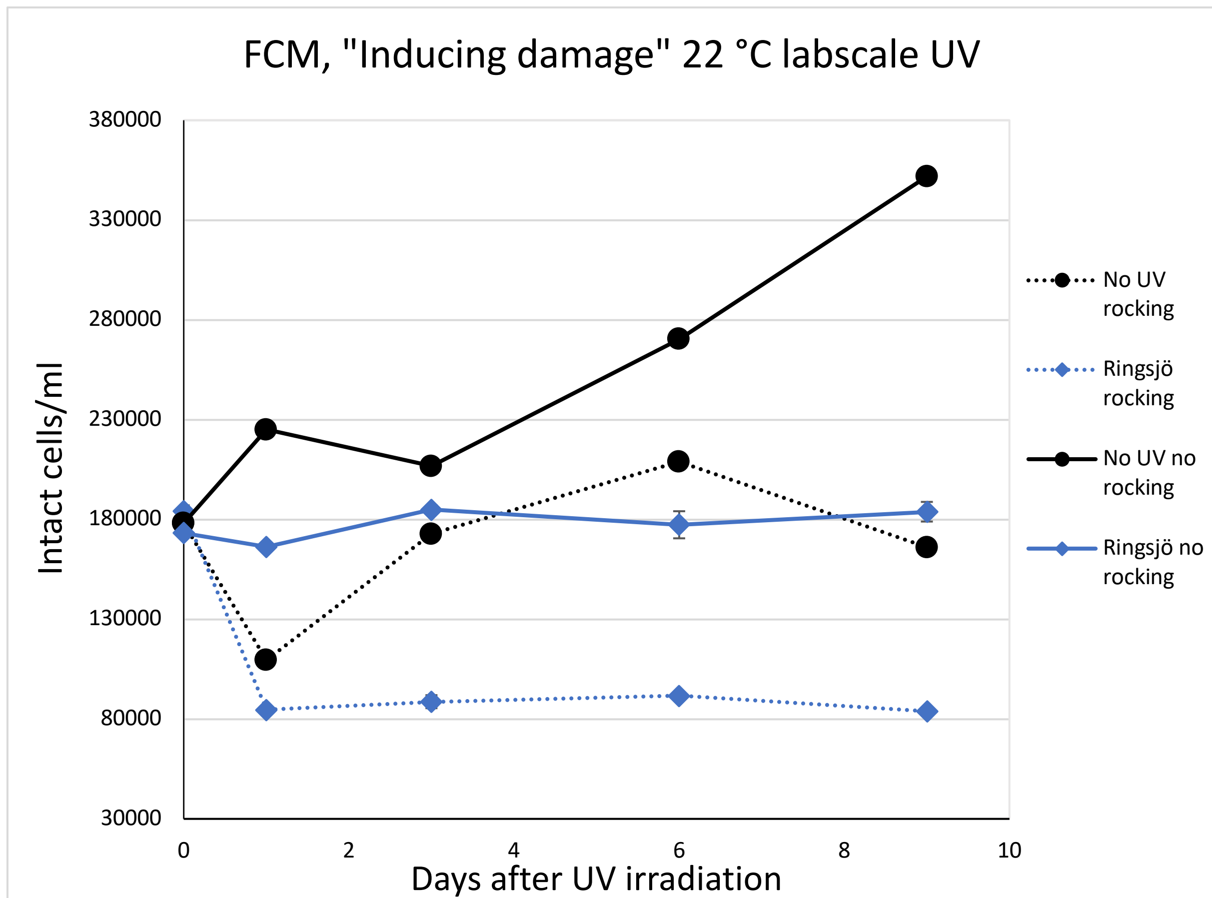

Figure 21. Flow cytometry data of intact cells Inducing damage, Ringsjö. Error bars shows statistical difference between triplicates of the samples..

4.3.2 Fingerprints

No clear limit on how to gate the HNA/LNA is seen. The relative fluorescence intensity of 11 000 from Prest´s report is used (Prest *et al.*, 2013).

4.3.2.1 TMB 7 °C

The line in the middle refers to at which point of fluorescence intensity the events were divided into LNA (left) and HNA (right). As can be seen the still follows the same trend with No UV still having significant more regrowth than all UV treated samples but also that the labscale UV yielded lesser regrowth than the Ringsjöverket UV. In the "Inducing damage" experiment the % HNA was not affected much by the induced effect. Results are not shown in this report. The TMB tap water does not seem to become much different after UV irradiation, regardless of dose, when looking at the fingerprints seen in Figure 22.

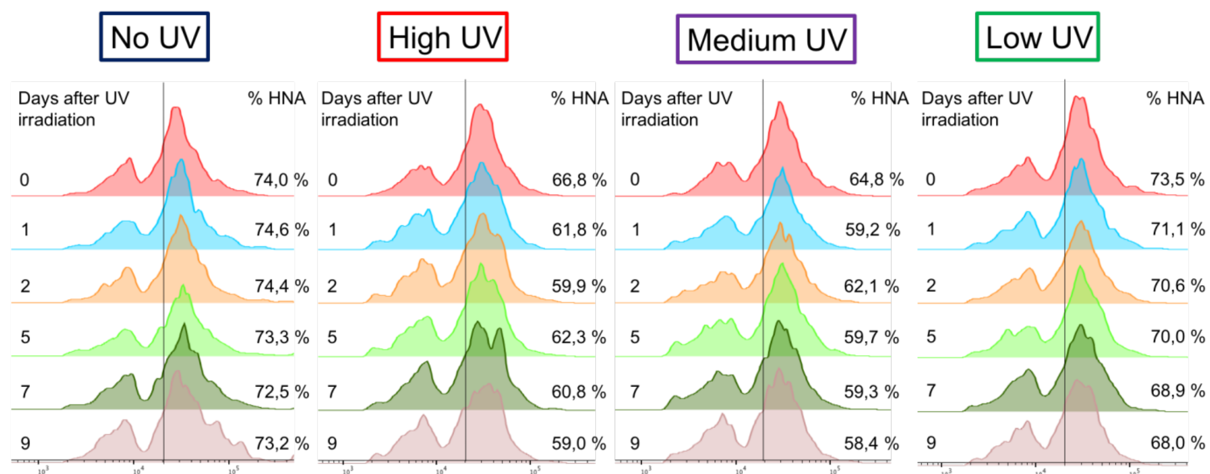

Figure 22. Fingerprints from flow cytometry on TMB tap water stored in 7 °C.

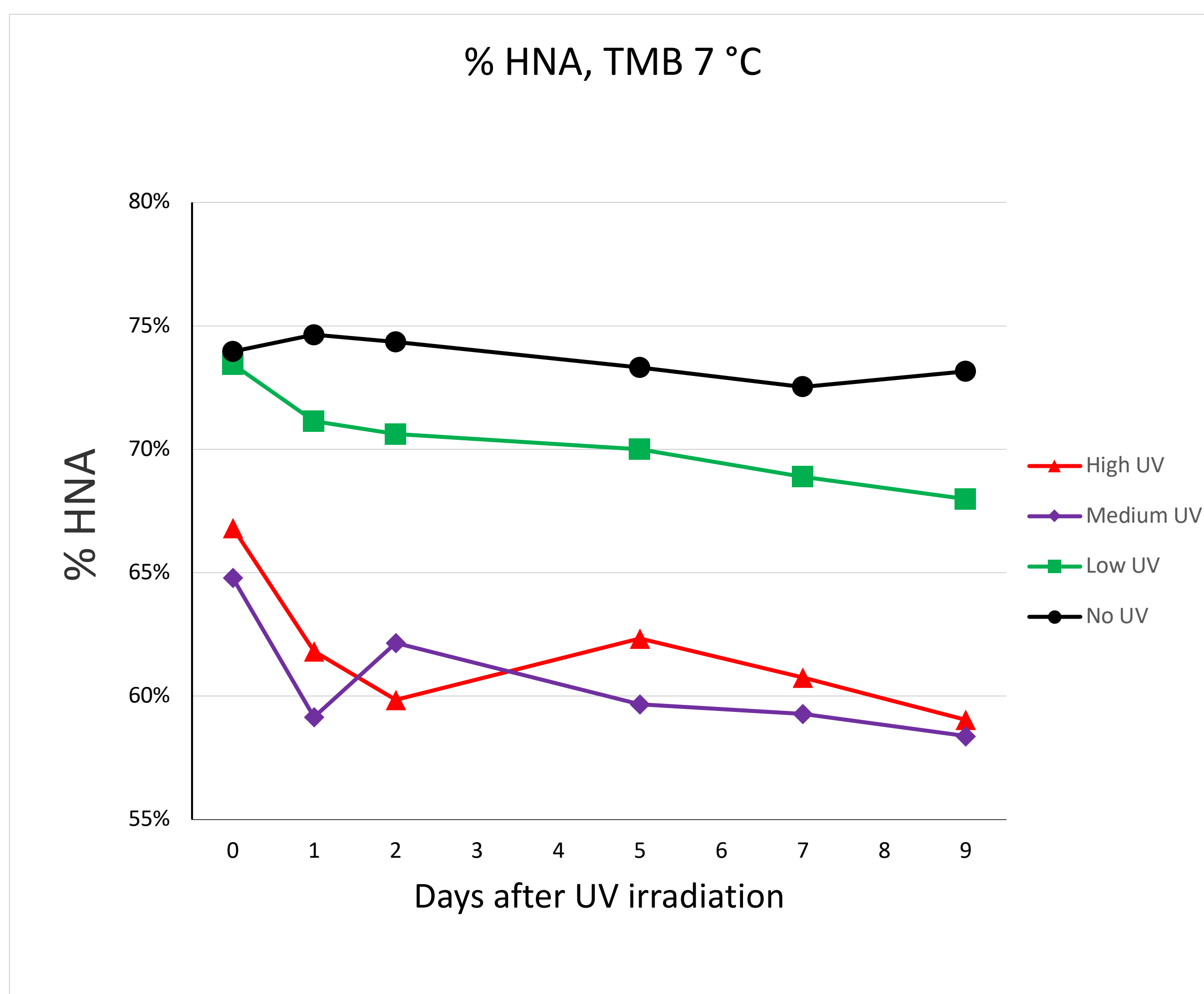

Figure 23. % HNA for TMB 7 °C. Error bars shows statistical difference between triplicates of the samples..

4.3.2.2 Ringsjöverket 7 °C and 22 °C

For the Ringsjöverket samples from 7 °C storage seen in Figure 24, the fingerprints look similar to each other on day 0. This can be excepted since UV irradiation doesn´t affect the amount of DNA directly. As the days progress the No UV samples has an increase in % HNA. The UV affected samples does however get a decrease in HNA, particularly the labscale samples although there is little difference between High UV and Low UV.

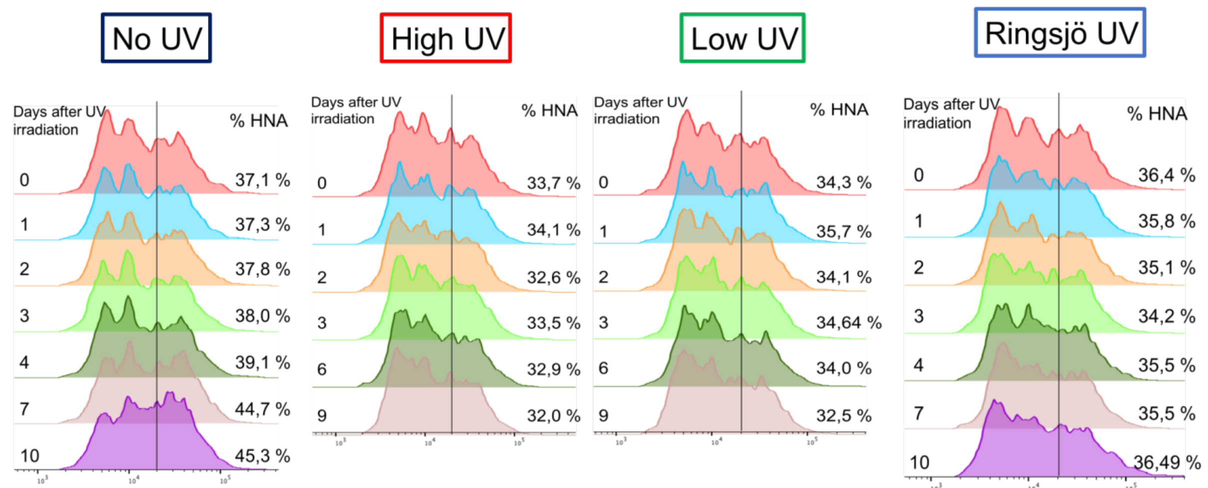

Figure 24. Fingerprints from flow cytometry on Ringsjöverket water stored in 7 °C.

The samples from Ringsjöverket that was stored in 22 °C, seen in Figure 25, shows similar trends, but stronger.

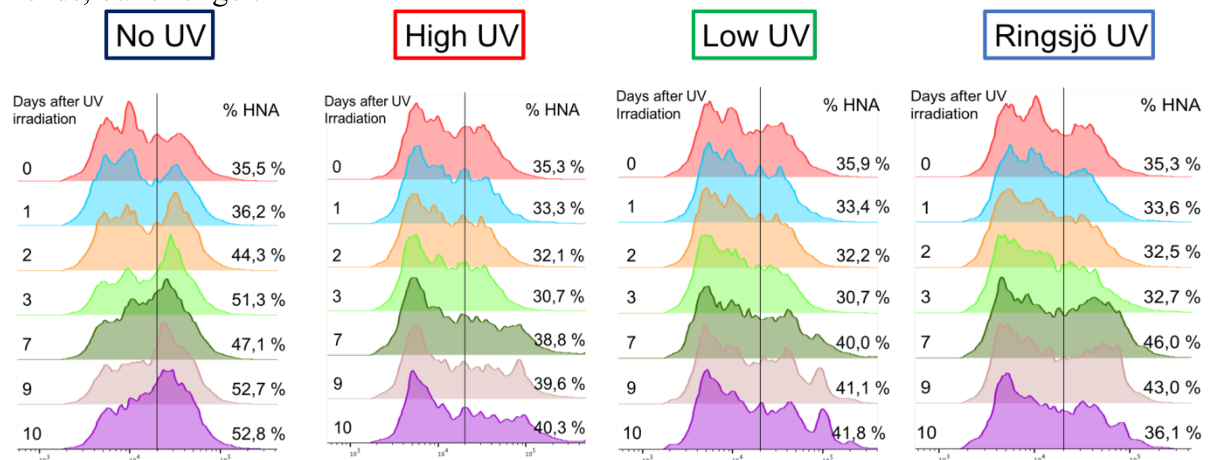

Figure 25. Fingerprints from flow cytometry on Ringsjöverket water stored in 22 °C.

From the fingerprint analysis, it is evident that all samples show similar bacterial community composition even directly after UV irradiation. However, as time progresses the non UV treated samples shows difference with a higher HNA bacteria cells in its bacterial community. Between high and low UV dose in the labscale aggregate there doesn´t seem to be a large difference, implying that there is a threshold dose after which additional UV irradiation has less effect. Ringsjö UV has similar effect as the labscale aggregate but seems slightly less effective. Graphs of the Ringsjöverket water can be seen in Figure 26 and Figure 27.

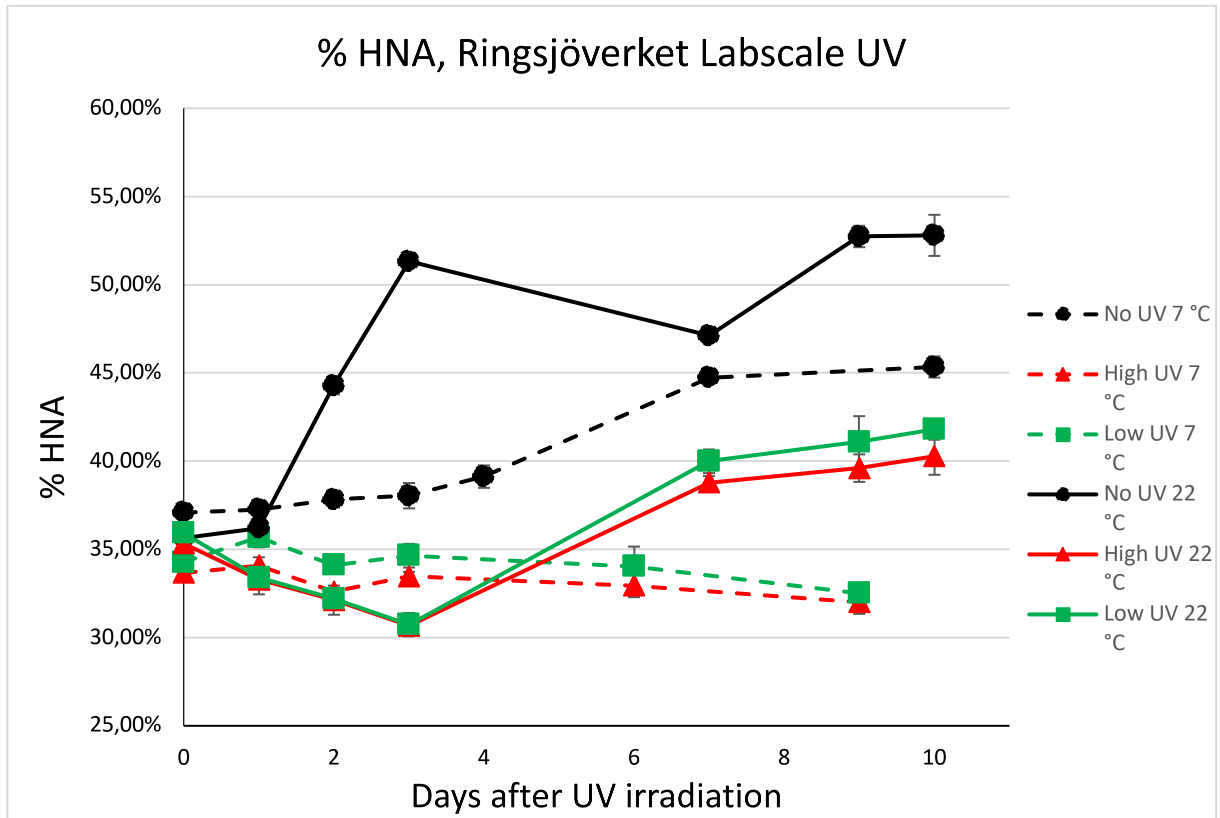

Figure 26. % HNA for Ringsjöverket 7 °C and Ringsjöverket 22 °C, No UV, High and Low UV. Error bars shows statistical difference between triplicates of the samples..

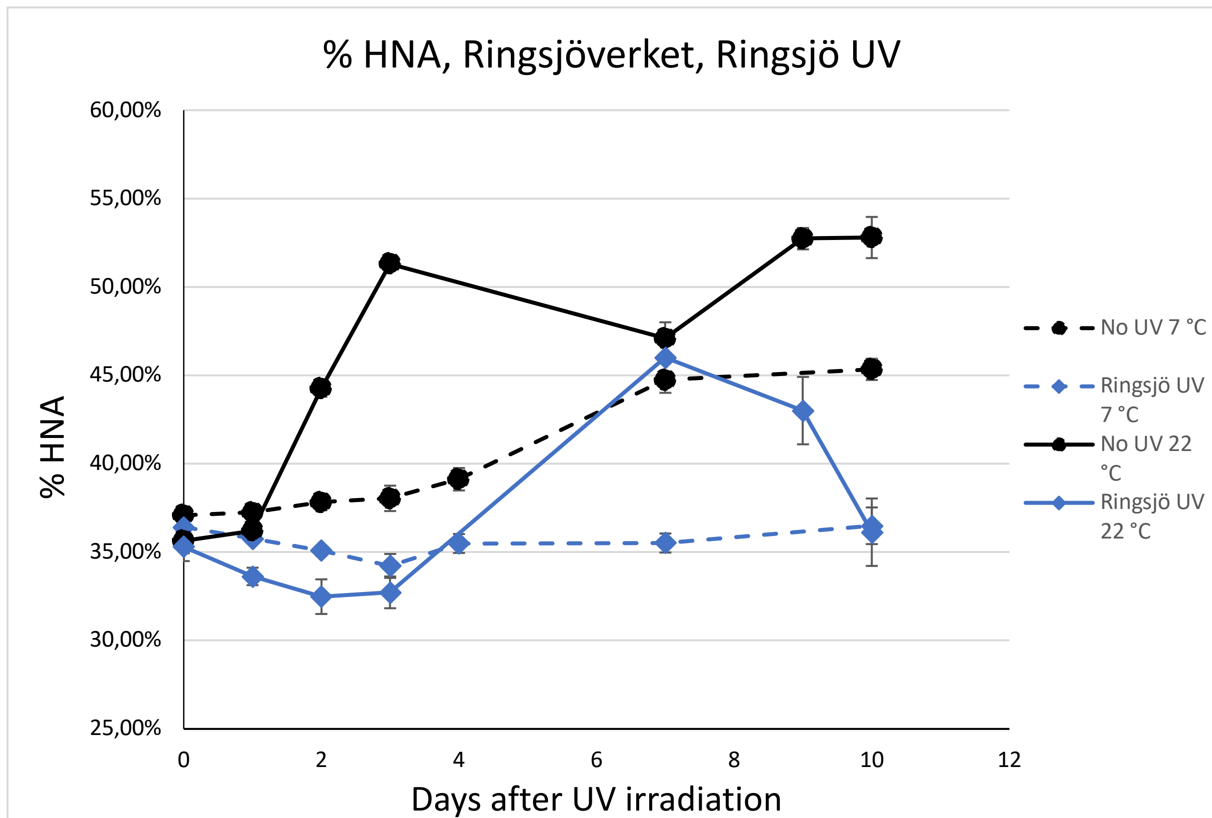

Figure 27. % HNA for Ringsjöverket 7 °C and Ringsjöverket 22 °C, No UV and Ringsjö UV. Error bars shows statistical difference between triplicates of the samples..

4.4 qPCR

4.4.1 TMB 7 °C

The TMB 7 °C qPCR results can be seen in Figure 28. Data points for No UV 1 on day 6 and days 7 after UV irradiation, No UV 2 on day 6 and all samples of Low UV were left out in qPCR analysis due to insufficient amount of sampling water since enough water loss in running the labscale aggregate had not been taken account. The water that hadn´t been affected by UV showed regrowth of DNA after a couple of days whereas the UV-affected was rather steady. The High UV dose samples also had a higher Cq than Medium UV. The standard deviation was calculated on Cq values without taken log transformation into account.

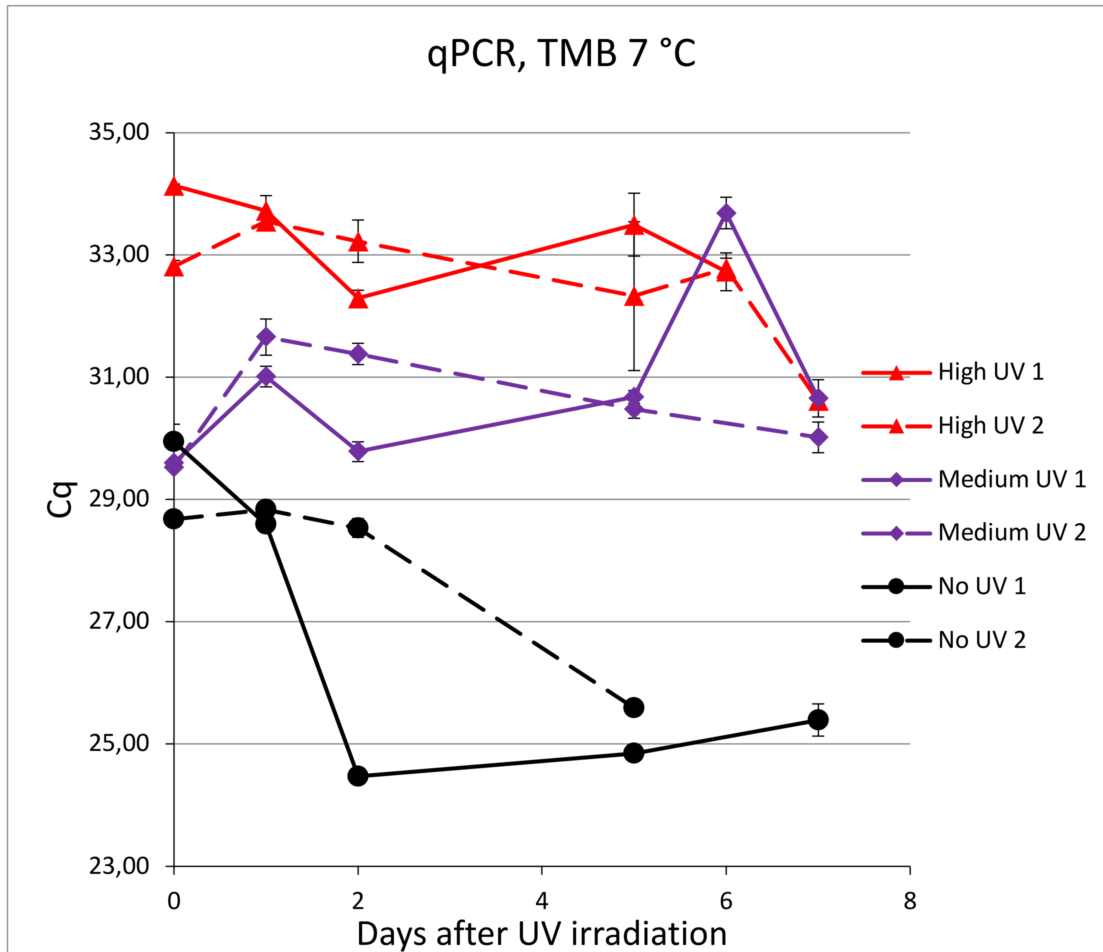

Figure 28. qPCR results of TMB 7 °C. Error bars shows statistical difference between duplicates of the samples run in the qPCR.

4.4.2 Ringsjöverket 7 °C

Results of qPCR from Ringsjöverket 7 °C can be seen in Figure 29. The water that hadn´t been affected by UV showed regrowth of DNA after a couple of days whereas the UV-affected only had slight regrowth. Although the trend was not as prominent it also seemed that the higher the UV dose, lesser regrowth occurred. Also, the water treated from Ringsjö UV seemed to have less consistent results. Furthermore, the No UV had more consistent results between the filter replicates than the UV affected samples. The standard deviation was calculated on Cq values without taken log transformation into account.

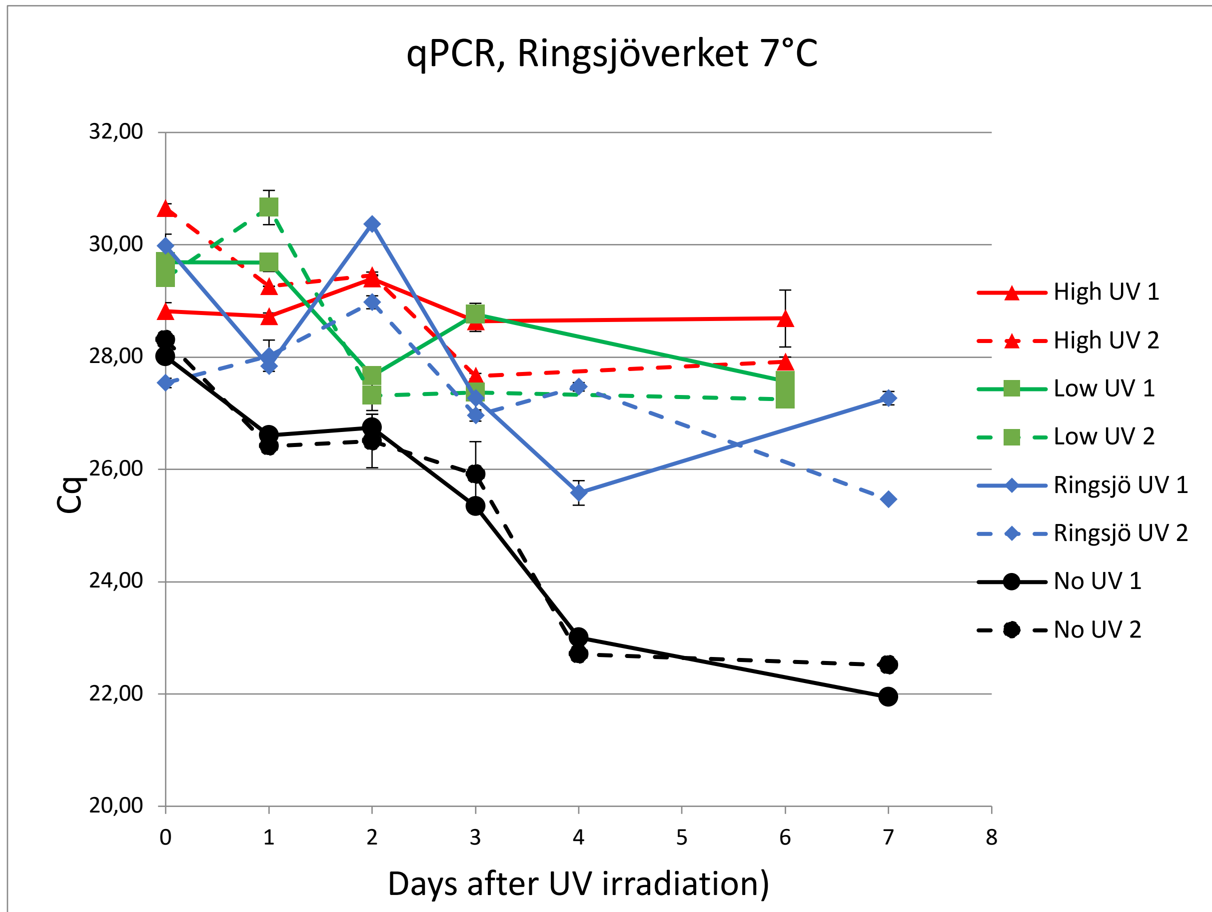

Figure 29. qPCR results of Ringsjöverket 7 °C. Error bars shows statistical difference between duplicates of the samples run in the qPCR.

4.4.3 Ringsjöverket 22 °C

As can be seen in Figure 30 the qPCR of the Ringsjöverket 22 °C did not show a clear trend in contrast to the previous experiments where the water was stored in fridge temperature. The standard deviation was calculated on Cq values without taken log transformation into account.

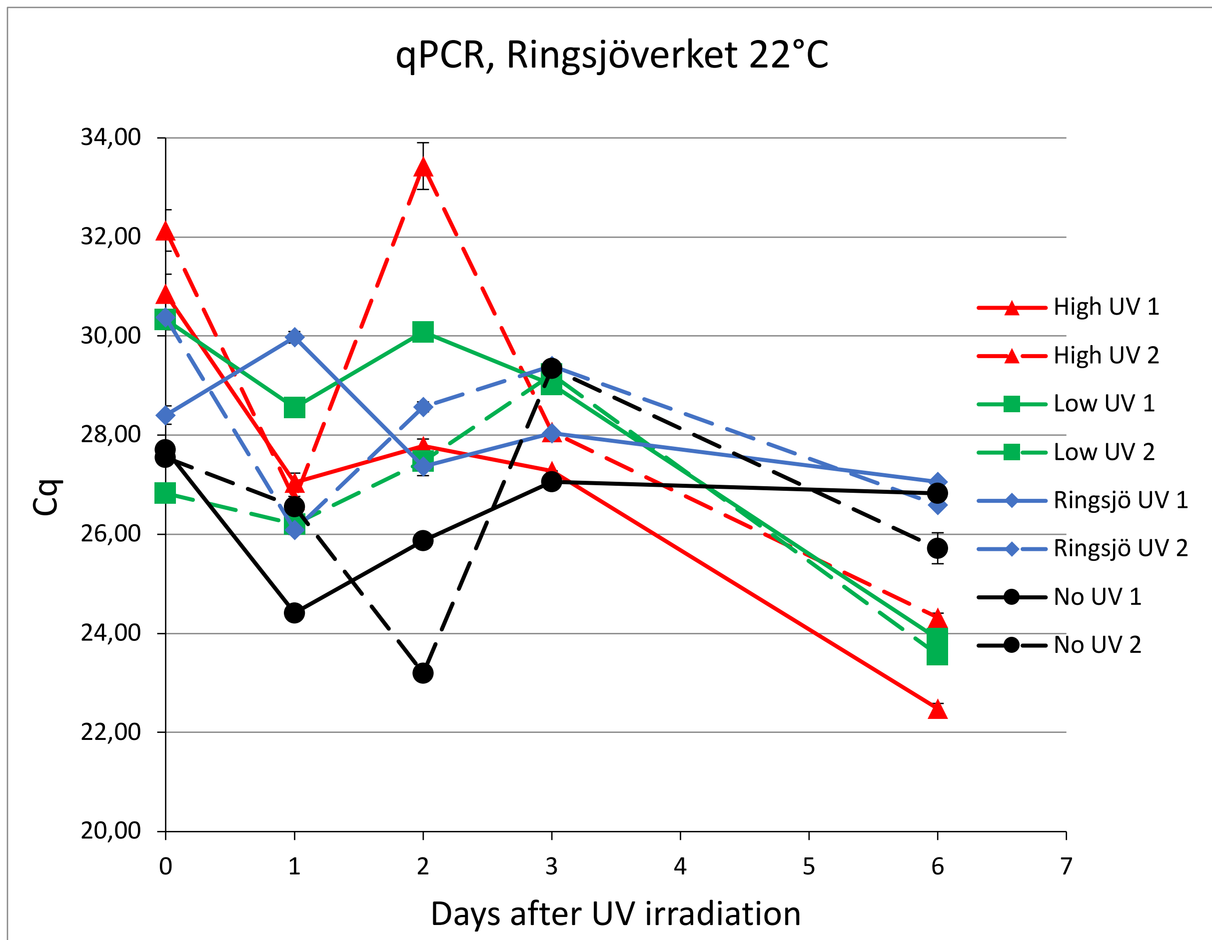

Figure 30. qPCR results of Ringsjöverket 22 °C. Error bars shows statistical difference between duplicates of the samples run in the qPCR.

Overall the different qPCR tests yielded similar results for day 0 after UV irradiation with Cq values ranging between 28-32.

5 Discussion

The project resulted in detectable effects of UV irradiation on the bacterial community of drinking water. Regrowth patterns could mostly be detected in FCM but also in qPCR. Regrowth was also observed in the 22 °C storage setup for HPC. In FCM it could be seen that the community of untreated samples changed with an increase in % HNA, approximately from 35 % to 50 %, while the treated samples had a relatively unchanged % HNA. Also, to some extent diversity decrease could be detected in HPC through a more similar morphology of the different colonies. qPCR alone could give estimates of the instant effect of UV. The project gave further insight in how the methods can be applied and how future research could be directed.

First a short summary of the different methods ability to detect the different aspects of UV irradiation in terms of instant effect, regrowth effect and community composition effect.
HPC was not accurate when it came to count the number of viable bacteria, which has already been confirmed by theory which points towards a plate count anomaly. However, it is still a result that the bacteria left after UV irradiation seems to have a stronger ability to grow on heterotrophic plate count agar. Also, HPC showed less variation in CFU color and size after UV treatment of sample.

FCM with SYBR Green and Propidium Iodide staining protocol gives an accurate count of total cells and cells with intact membrane integrity. This method does not show any significant difference between UV affected samples and untreated reference, which can be explained by that UV only affects the DNA and not the cell membrane directly. Regrowth analysis can however be done with good accuracy. Though the UV affected samples did not regrow the numbers did not decrease drastically, implying that even UV affected samples could remain somewhat biologically active after treatment. Also, changes in community composition showed changes in the number of bacteria with low or high amounts of DNA. It was evident that % HNA increased in the untreated samples whilst % HNA slightly decreased for the treated samples. Higher temperatures had larger effect on both regrowth and community changes. Given that flow cytometry is a fast method that is easy to use further investigation on how to optimize detections of UV disinfections is recommended.

In the flow cytometric analysis of intact cells it could be observed that the samples that have not been affected by UV show significantly more intact bacteria as time progress. These results were consistent between the different storage temperatures of water samples. It did however seem that for the TMB 7 °C experiment, the difference between treated and untreated water was not as clear as in the Ringsjöverket water experiments. This could be explained by the fact that the water taken from TMB has already had UV treatment and is not as susceptible to it any longer. For example the initial UV exposure could select for bacteria that are more resistant to UV irradiation that then take a larger part of the bacteria community. Compared to the Ringsjöverket 7 °C the Ringsjöverket 22 °C samples showed an earlier difference between the UV treated samples, where High labscale UV favored regrowth of bacteria the least and Low UV the most. This is not to surprising since higher temperatures is favorable for growth conditions. The results from flow cytometric analysis of intact cells in labscale UV for 7 °C and 22 °C storage can be seen in Figure 18. In the beginning there´s no big difference in bacteria count, which is not surprising since the staining method tells the difference of intact or damaged cell membrane that UV has no effect on.

As the days go it seems that the reference sample, No UV, has much higher count of intact bacteria cells. This correlates with theory, and furthermore shows that the CFU count in HPC does not necessarily give an accurate number of viable cells from a sample. Also, there is a difference between High and Low UV dose, but not a big difference, implying that the dose does not have increased effect after a certain level. Furthermore, higher storage temperature makes the trends even clearer, both the regrowth of the and the decline in growth for the labscale samples.

qPCR theoretically good way to measure since the process of getting results is highly dependent on the quality of DNA in a sample, which UV is the part where UV has impact on. Compared to the other methods qPCR is a bit more labour intensive and might not be the perfect option for fast measurement on site. As can be seen in Figure 30 the qPCR of the Ringsjöverket 22 °C did not show a clear trend in contrast to the previous experiments where the water was stored in fridge temperature. Reasons behind this can be speculated on, the bacteria might form sediments in the barrels at 22 °C and then require more homogenization than simply shaking it by hand. Further optimization of filtration protocol and testing is needed in order to start drawing conclusions.

The storage setup of 20 l plastic water containers in 7 °C and 22 °C was a limiting factor in the study. Firstly, the process of collecting samples and running it through the UV aggregate would be very impractical with only one person doing the experiments. Also, the amounts of water made temperature control difficult as the 20 l containers cannot fit into conventional incubators. Hence only the 7 °C fridge room and air-conditioned rooms with 22 °C were available. In the book *"Heterotrophic plate counts and drinking-water safety"* on can read that the "bottle effect" is a factor to be taken into consideration when investigating how the water changes over time. Leclerc 2003 mentions that the activity and number of bacteria is related to the size of container. The nutrients in water are often low in concentration and can get adsorbed to the surface of containers. Higher surface to volume ratio will then give lower concentration in the bulk water (Leclerc, 2003). This could explain why the UV-affected samples are in the non-induced samples in the "induced experiment" as 50 ml Falcon tubes were used for storage instead of 20 l plastic water containers. Furthermore, it is a drawback in the experiment setup that needs to be taken into consideration when making conclusions. This bottle effect might make it more desirable to have larger containers for sample analysis, making the drawback of large storage requirements and more difficult handling somewhat less.

Both the labscale aggregate and the Ringsjöverket aggregate could not give exact measurements of UV doses so this can only be speculated on. Not only intensity and time of exposure but also reactor structure and hydraulic conditions play a role in the effect. Therefore, the comparisons between the doses and the aggregates (Ringsjö UV, Low UV and High UV) used in this study should not be directly translated into theoretical doses. This is described as a problem when it comes to validating if a UV treatment step is efficient enough. Measuring the actual intensity on-site in an operating water treatment step has been regarded as very difficult (Nizri *et al.*, 2017).

The HPC results are somewhat peculiar. There are some theories of why the CFU increases with UV-irradiation. Lehtola et al (2003) suggested that UV can actually enhance microbial growth by increasing the amount of total organic carbon and total phosphorous, compounds that may be used by heterotrophic organisms. A study by Lehtola et al (2003) did however conclude that the effect of UV on the chemical and microbial characteristics differ depending on waterworks. Note that this study used HPC to evaluate microbial activity (Lehtola *et al.*, 2003). Another study that looked at UV irradiated ballast water concluded that the growth rate of bacteria in UV irradiated water could increase after irradiation (Hess-Erga, Blomvå Gnes-Bakke and Vadstein, 2010). The author's point toward that surviving bacteria with high growth rate are favored since the disinfection process kills of predatory and competitive bacteria. Another factor stated by Hess-Erga et al (2010) is that the amount of dissolved organic carbon is potentially increased by UV irradiation. This could happen either through the release of biodegradable matter and enzymes with biodegradable functions from bacteria that has been killed by the disinfection or direct alterations to dissolved organic matter by the UV irradiation. Although this study was done on seawater the authors believed similar effect could occur in drinking water (Hess-Erga, Blomvå Gnes-Bakke and Vadstein, 2010).

It still does not explain why the results contradict results from FCM and qPCR. It has been reported that not only can HPC yield less numbers of bacteria, as discussed in the background. One study by Hoefel also points towards that the ratio between culturable bacteria and active bacteria, determined with flow cytometric methods, is inconsistent (Hoefel *et al.*, 2003). Van Nevel et al (2017) also states that and questions that HPC and FCM is used for the same purpose in drinking water monitoring. Van Nevel´s report discuss that the absence of correlation between HPC and FCM points towards that the two methods are not providing the same information (Van Nevel *et al.*, 2017). ATP measurement is another method that can be used when analyzing drinking water and that yields fast results and is affordable compared to HPC. The method determines activity through giving information of the amount of adenosine tri-phosphate in a sample (Siebel *et al.*, 2008). In Van Nevel´s study it was concluded that correlation with measurement of ATP was found between FCM but not HPC (Van Nevel *et al.*, 2017).

When evaluating effectiveness of UV disinfection with flow cytometry a different approach than a SYBR Green and Propidium Iiodide protocol, which only tells cells apart depending on cell membrane integrity and amount of DNA instead of the state of the DNA (Gatza, Hammes and Prest, 2013). There are alternatives of dyes for staining can be used to target other functions of the cells than the amount of DNA. For example, according to Diaz et al (2010) different dyes can bind at different rates depending on DNA, RNA, protein, lipids, pH, membrane energization, antibodies or oligonucleotides. Using the dyes could make it possible to differentiate cells by other means than just viability or cell membrane integrity. Level of metabolic activity, macromolecule synthesis or, as in the case of the dye CTC (5-Cyano-2,3 Ditolyl Tetrazolium Chloride), respiratory chain activity could be analyzed. (Díaz *et al.*, 2010). Studies using the Metachromic fluorochrome acridine orange has been used in medicine to specifically detect DNA damage in eukaryotic cells. Although it is not clear if the dye could be used for detecting DNA damage in drinking water bacteria but it opens up that there are many possible ways of using flow cytometry (Darzynkiewicz *et al.*, 2011).

When looking at the community composition in terms of how large fraction of the total cells are considered to have low (LNA) or high (HNA) amounts of DNA it might be a good idea to think about where the limit for HNA and LNA is. In this study the limit for was set according to the same limit that was found in the project plan literature (Prest *et al.*, 2013). There, everything above 11 000 in relative fluorescence was considered to be HNA. The limit is converging with a "dip" in bacteria count for both LNA and HNA in the report but not all samples has defined clusters of bacteria like this (Prest *et al.*, 2013). Also, different dyes could yield better differentiation of LNA and HNA. In Lebaron´s report a comparison between SYBR II, SYBR I and Syto 13 is conducted, where SYBR II was deemed to be better at separating HNA from LNA. It is not certain that the staining protocol used in this report with SYBR® Green and Propidium Iodide was the most efficient at differentiating. (Lebaron *et al.*, 2001). Example of how the % HNA changes depending on what limit is set can be seen when comparing Figure 31 and Figure 32 with Figure 33 and Figure 34 that shows fingerprints and graphs of % HNA with different limits for HNA. The fingerprints in Figure 31 and Figure 33 are the same, but with the red line splitting the profile into LNA and HNA at different places. Changing the limit does change the absolute values of % HNA, it is important to take this into consideration when comparing absolute values of different samples. When looking at the graphs it is clear that the small change in limit of HNA does not necessarily make a huge difference in how the trend of % HNA looks. However, the data points for No UV and Ringsjö UV day 7 after UV irradiation stored at 22°C shows different trends.

When the limit is set at 11 000 relative fluorescence the No UV and Ringsjö UV data points seems to converge and have a much more similar % HNA whereas in the case whereas fluorescence limit is set at 10 500 the trends are more consistent.

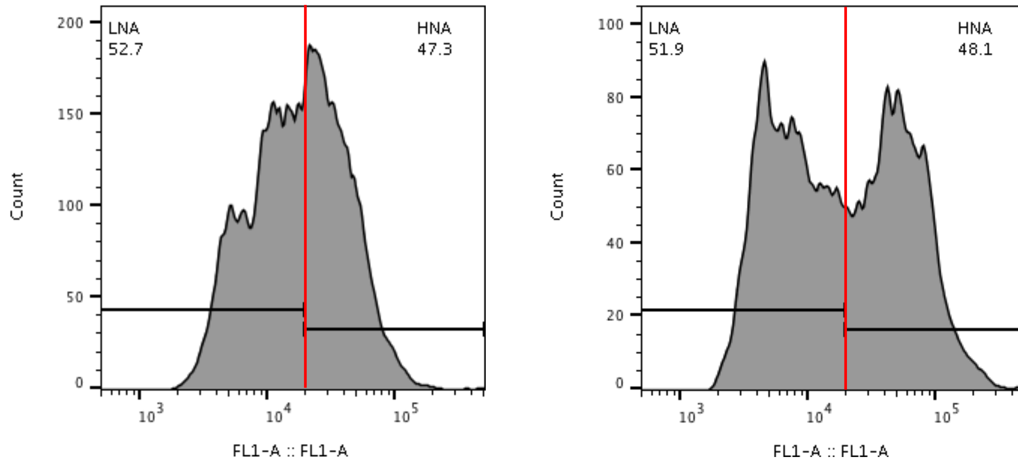

Figure 31. Fingerprints with HNA limit set at 11 000 relative fluoresence intensity No UV (left) and Ringsjö UV (right) of day 7, 22 22°C storage.

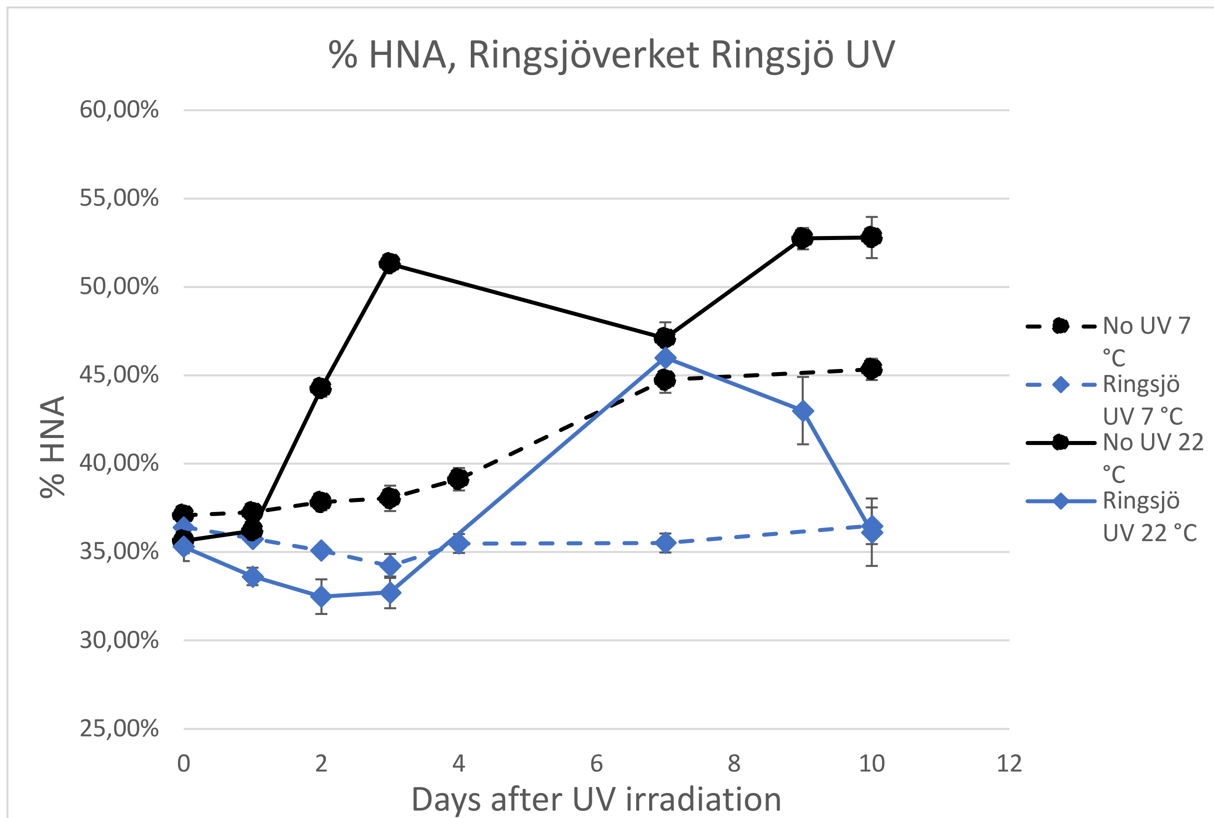

Figure 32. % HNA for Ringsjöverket 7 °C and Ringsjöverket 22 °C, No UV and Ringsjö UV with HNA limit set at 11 000 relative fluorescence intensity.

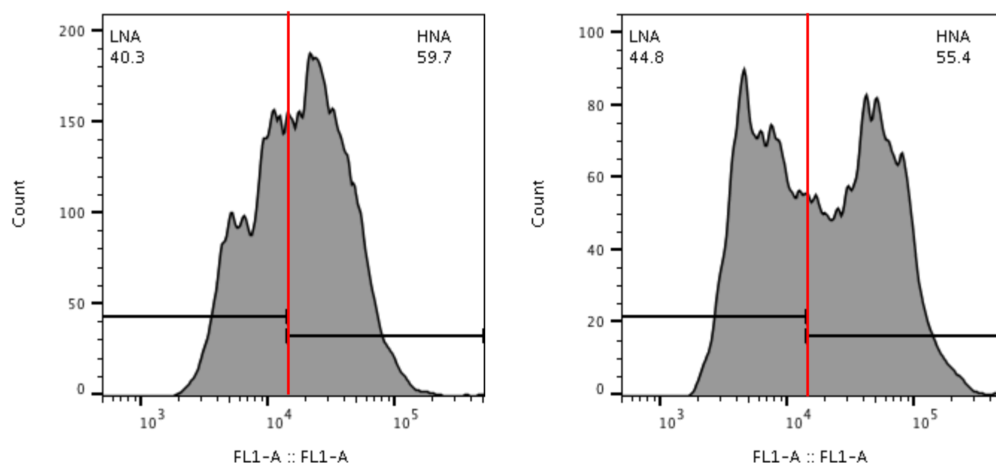

Figure 33. Fingerprints with HNA limit set at about 10 500 relative fluoresence intensity No UV (left) and Ringsjö UV (right) of day 7, 22 22°C storage.

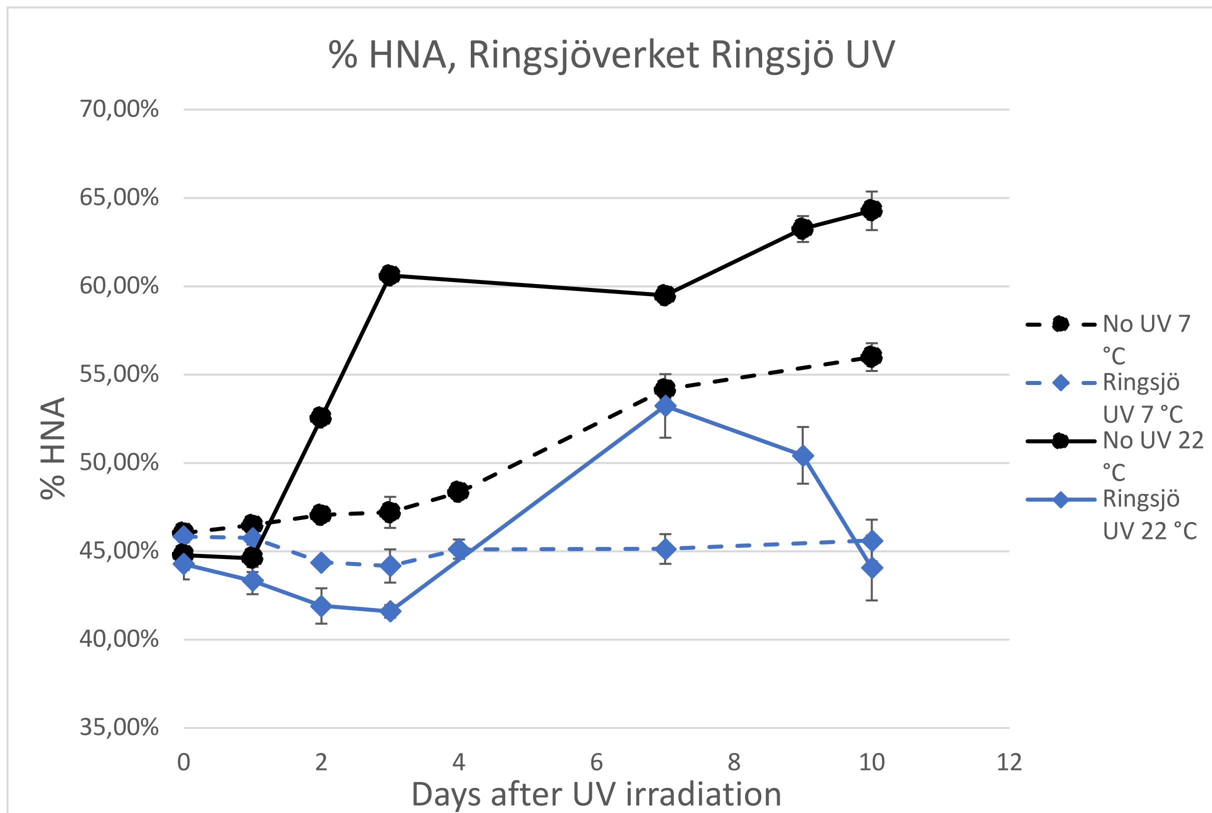

Figure 34. % HNA for Ringsjöverket 7 °C and Ringsjöverket 22 °C, No UV and Ringsjö UV with HNA limit set at 10 500 relative fluorescence intensity.

This also raises the question of how the intensity is measured in case one wants to repeat experiments between different flow cytometry equipment or different samples. Molecules of Equivalent Soluble Fluorochrome (MESF) is a unit that is used to compare measurements of quantitative fluorescence intensity across instruments (Schwartz *et al.*, 2004). If you want to compare different samples it is important to have a reference, beads of a certain size for example. Since flow cytometers use bandpass filter and only analyzes a narrow emission spectrum it is important that the reference and analytic has matching emission spectrums (Wang *et al.*, 2002). Since MESF is relative, the numbers of % HNA in regards are not an absolute measurement of how the community in a sample looks like. It is still a good tool to give ideas of trends and changes of different samples.

HNA has been believed to be more active than LNA cells according to Lebaron et al (2001). It is suggested that the content of nucleic acid is a better indicator for growing cells than the total number of cells. This correlates with the results that the No UV samples from all experiments has higher % HNA (Lebaron *et al.*, 2001). More recent studies does however show that LNA bacteria also can be active and grow (Harry *et al.*, 2016). Therefore one should not draw to strong conclusions on how the activity of the bacterial community is affected based on HNA/LNA ratio. Another study concluded that HNA cells could be separated from LNA cells through filtration with 0.45 µm, thus there might be a size difference which could be interesting to further explore. The size of bacteria could for example be a factor for UV irradiation sensitivity. Regardless of what properties the LNA and HNA bacteria has the ratio between them still serves as an indicator of change in the community though.

The theory behind that damage in bacteria affected by UV irradiation could be induced via a stress protocol could be further investigated. Sonication, rocking shaker tray, flask-shaker are potential means of inducing damage to cell membranes in UV irradiated samples, see Figure 35. The results from the "Inducing damage experiment" showed that this is not implausible. The results that all samples had decreased number of intact cells also suggests that experiment settings needs to take into account of how the sample is handled. Inducing cell damage like this could yield faster results pointing towards differences in UV-effect on bacteria cells.

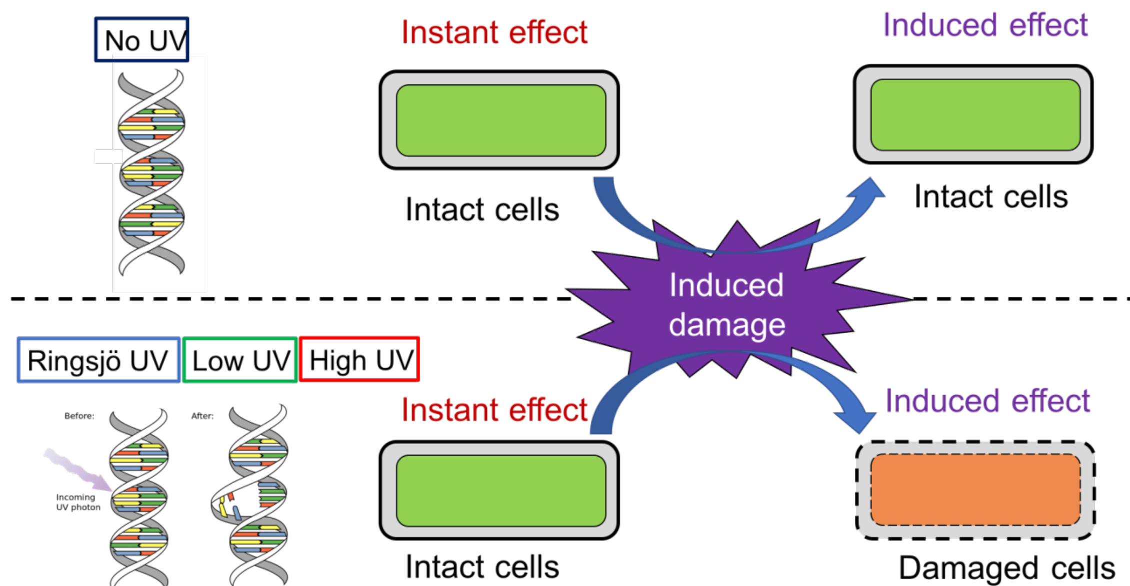

Figure 35. Description of how induced damage could affect UV irradiated samples. The UV irradiation does not instantly affect cell membrane integrity and can therefore not be detected with the SG + PI staining protocol. In this study it was suggested that damage to cell membrane in cells affected by UV irradiation could be induced.

qPCR as a method of detecting UV damage could be promising due to results that are dependent on the quality of DNA. A recent study by Nizri et al (2017), which only used single PCR reactions, concluded that PCR can efficiently be used to detect UV damage. It is suggested that the PCR should be run on larger fragments (900-1500 bp) than what is conventionally used (200 bp) since the DNA damage is more detectable. In this study the amplicon length was at 477 bp, suggesting that UV damage could not be detected as efficiently as desired (Nizri *et al.*, 2017). Another study with similar conditions as in this report had been conducted on wastewater, with the results that qPCR does correlate with UV damage (Süß *et al.*, 2009).
This study that was conducted on different *E. coli* strains only was specifically directed at investigating different lengths of amplicons as a means of getting a more sensitive method of analyzing UV damage on drinking water bacteria. The authors similarly concluded that the longer the amplicon, the more efficient UV detection since shorter wavelengths give lower likelihood of a damage encounter in the PCR reaction (Rudi *et al.*, 2010). This difference in damage encounters is illustrated in Figure 36. All studies mentioned in this paragraph used the 16S rRNa gene for amplicon elongation. However, Lührig (2015) mentions that 16S rRNA is not 100 % reliable for studies on community since different regions of the 16S rRNA gene can evolve at different rates, giving variable results of community composition. It is important to be aware of its drawbacks as well, especially since bacteria in drinking water vary from different locations (Lührig *et al.*, 2015). It does not mean that 16S rRNA gene should not be used but still gives an idea that is also is not 100 % accurate.

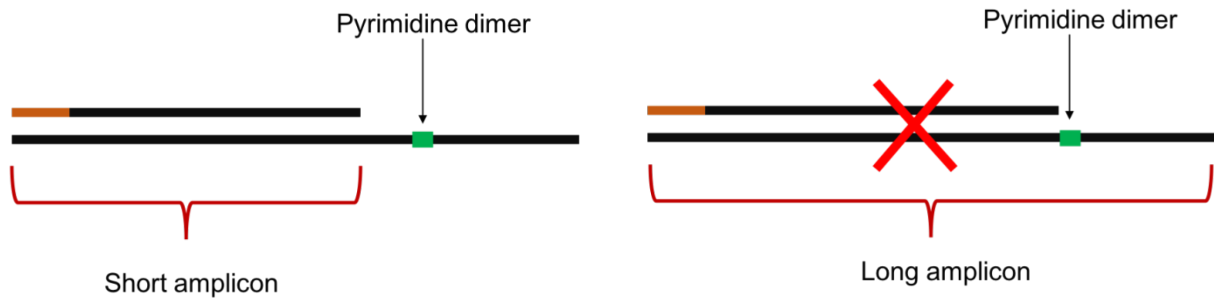

Figure 36. Illustration of damage encounter in short vs long amplicons in qPCR reaction.

6 Future

The dose of UV is hard to estimate accurately. If techniques could be developed to get a more exact measurement of UV-dose future research might be able to give a better dose-effect relationship. Especially when applying knowledge to full-scale aggregates.

Inducing damage in UV-affected bacteria cells could be a way to gain faster results in flow cytometry. Using dyes that stains into functions of the bacteria cells that are more closely related to DNA damage in flow cytometry could yield faster results when evaluating UV disinfection efficiency.

The sensitivity of qPCR as a method has potential of being enhanced through using larger amplicons, since they have a higher chance of getting pyrimidine dimers that could interfere with polymerase chain reaction. Next generation sequencing of the amplicons gained from qPCR is also an option to gain further insight into the community composition.

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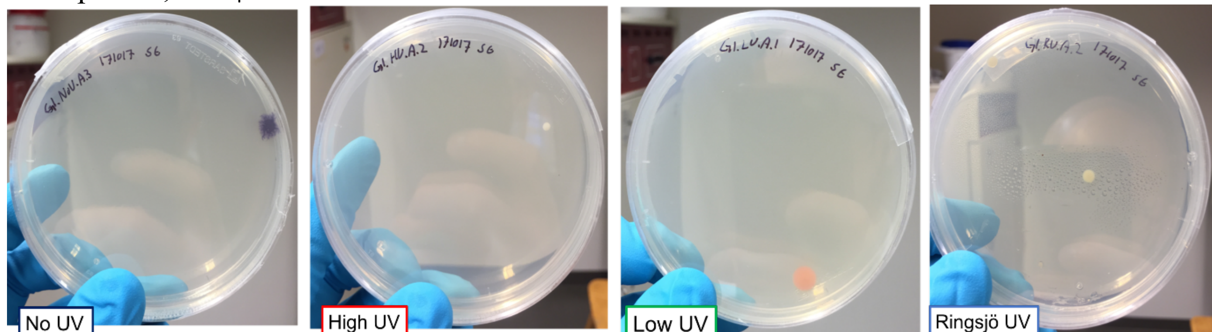
8 Appendix

HPC photos, 100 µl



.*Figure 37. 100 µl sample water stored at 22 °C day 1 after UV irradiation on R2a agar at 22 °C for 7 days.*

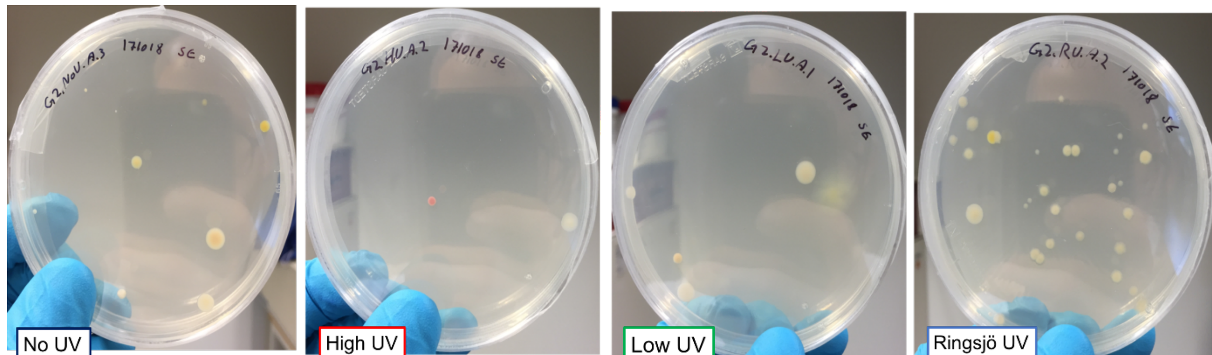


Figure 38. 100 µl sample water stored at 22 °C day 2 after UV irradiation on R2a agar at 22 °C for 7 days.

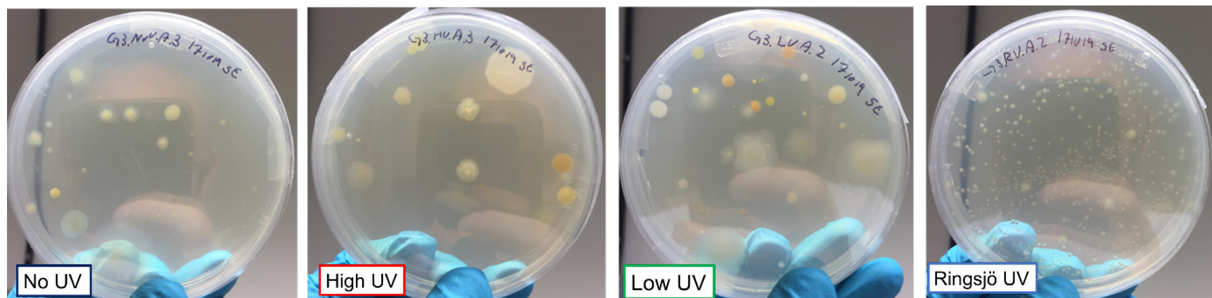


Figure 39. 100 µl sample water stored at 22 °C day 3 after UV irradiation on R2a agar at 22 °C for 7 days.

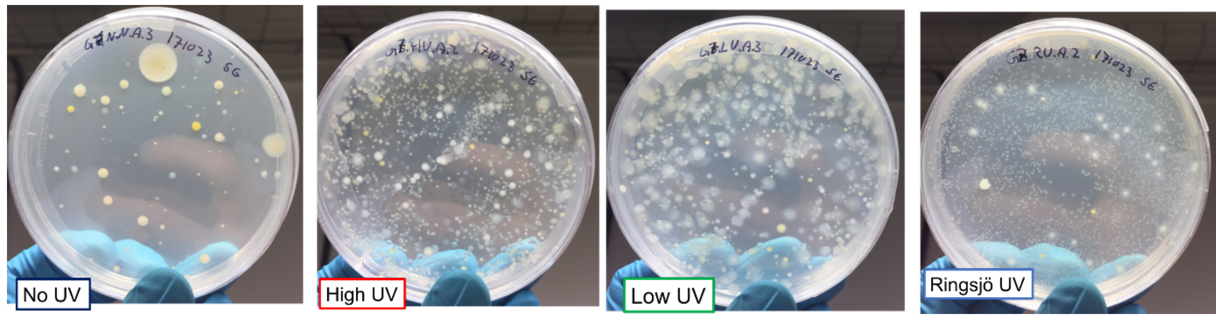


Figure 40. 100 µl sample water stored at 22 °C day 7 after UV irradiation on R2a agar at 22 °C for 7 days.

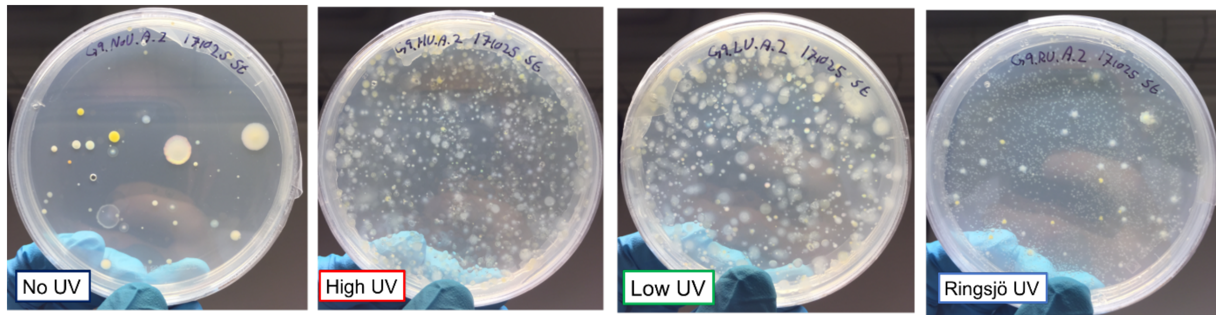


Figure 41. 100 µl sample water stored at 22 °C day 9 after UV irradiation on R2a agar at 22 °C for 7 days.

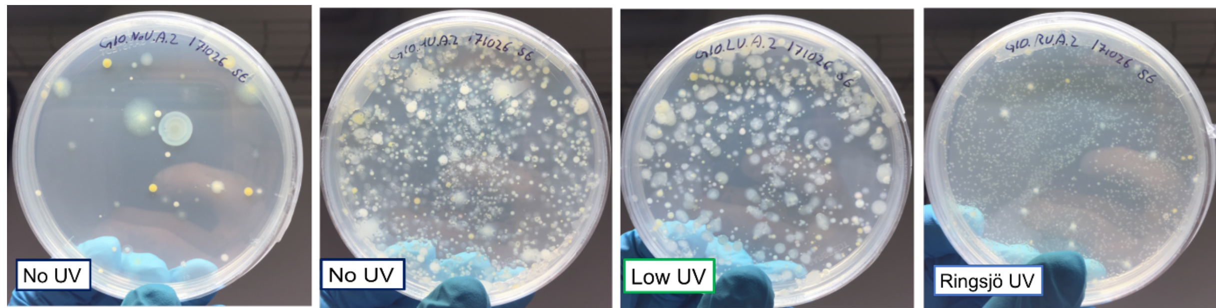


Figure 42. 100 µl sample water stored at 22 °C day 10 after UV irradiation on R2a agar at 22 °C for 7 days.

HPC photos, 5 ml through filter paper

Figure 43. 5ml sample water stored at 22 °C day 0 after UV irradiation through 0,22 µl filter paper on R2a agar at 22 °C for 7 days. (From left to right: No UV - Ringsjö UV)

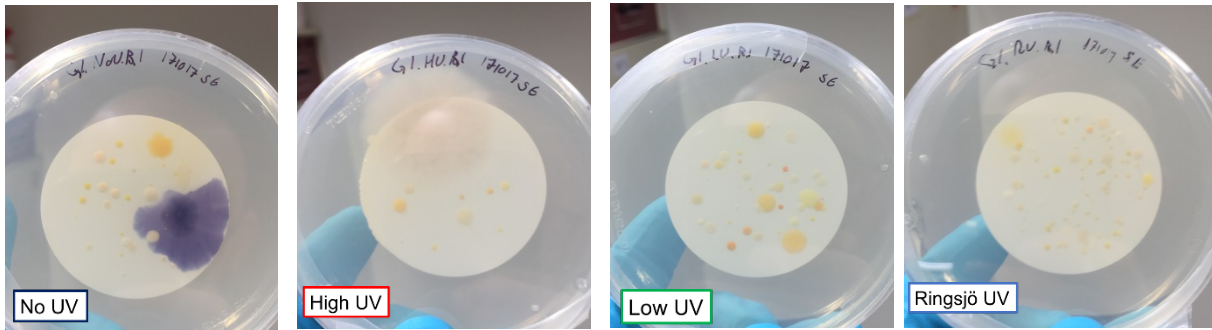


Figure 44. 5ml sample water stored at 22 °C day 1 after UV irradiation through 0,22 µl filter paper on R2a agar at 22 °C for 7 days. (From left to right: No UV - High UV - Low UV - Ringsjö UV)

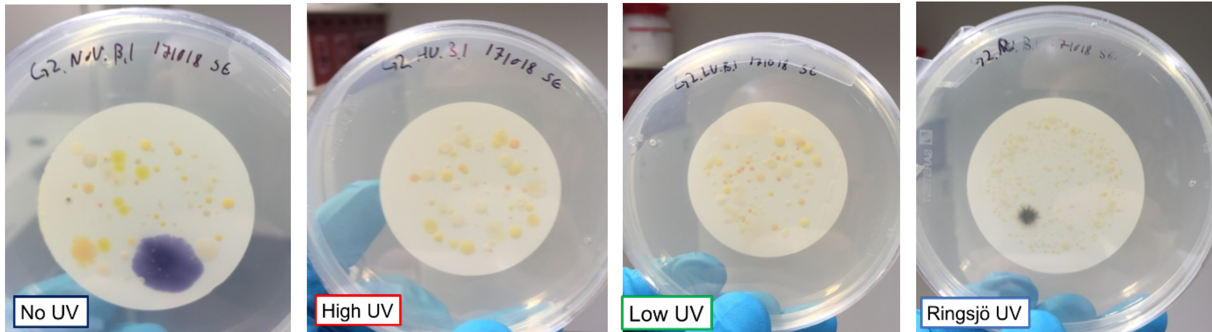


Figure 45. 5ml sample water stored at 22 °C day 2 after UV irradiation through 0,22 µl filter paper on R2a agar at 22 °C for 7 days. (From left to right: No UV - High UV - Low UV - Ringsjö UV)

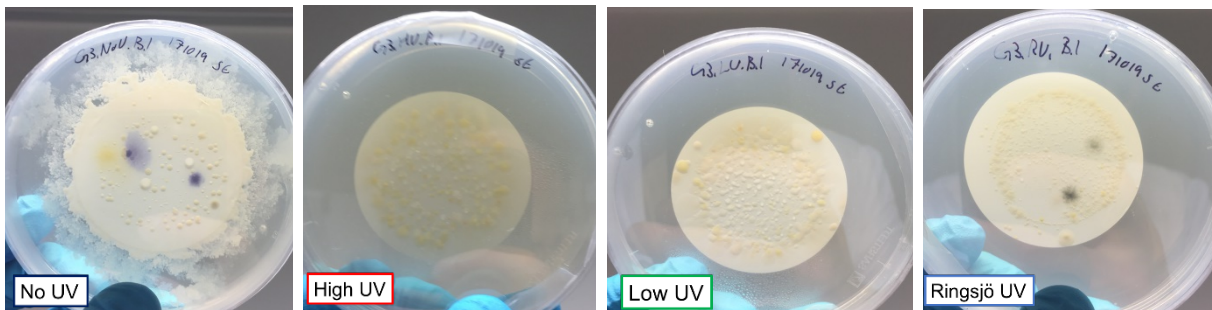


Figure 46. 5ml sample water stored at 22 °C day 3 after UV irradiation through 0,22 µl filter paper on R2a agar at 22 °C for 7 days. (From left to right: No UV - High UV - Low UV - Ringsjö UV)

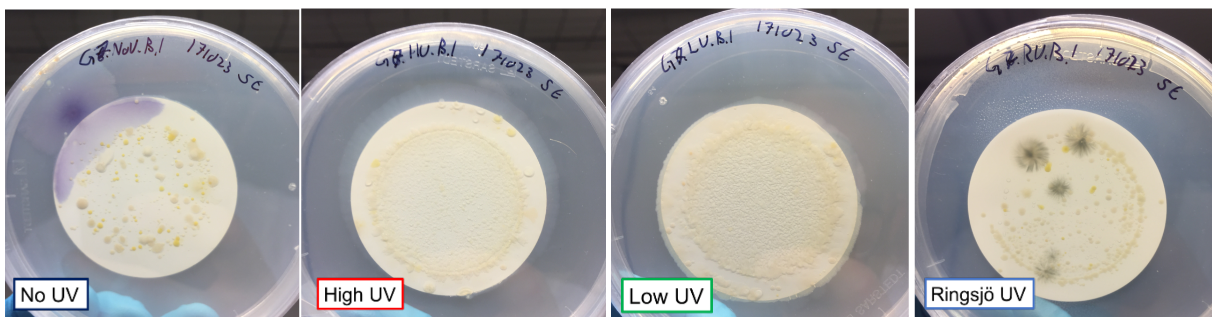


Figure 47. 5ml sample water stored at 22 °C day 7 after UV irradiation through 0,22 µl filter paper on R2a agar at 22 °C for 7 days. (From left to right: No UV - High UV - Low UV - Ringsjö UV)

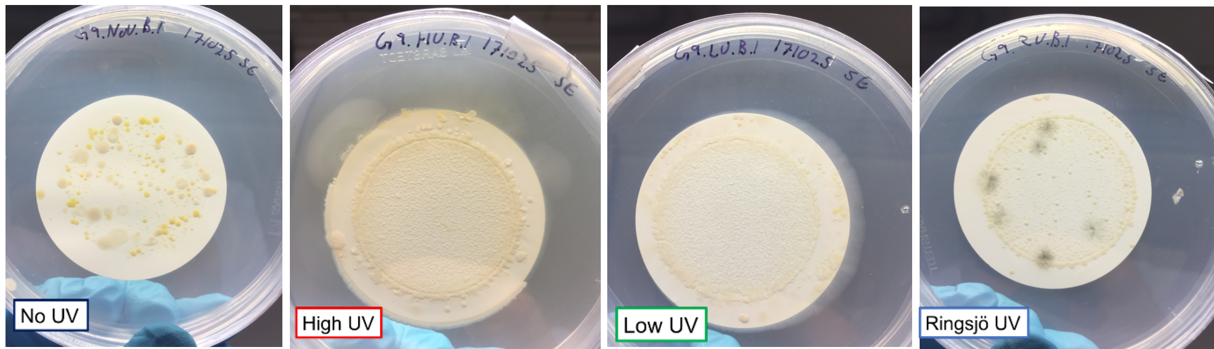


Figure 48. 5ml sample water stored at 22 °C day 9 after UV irradiation through 0,22 µl filter paper on R2a agar at 22 °C for 7 days. (From left to right: No UV - High UV - Low UV - Ringsjö UV)

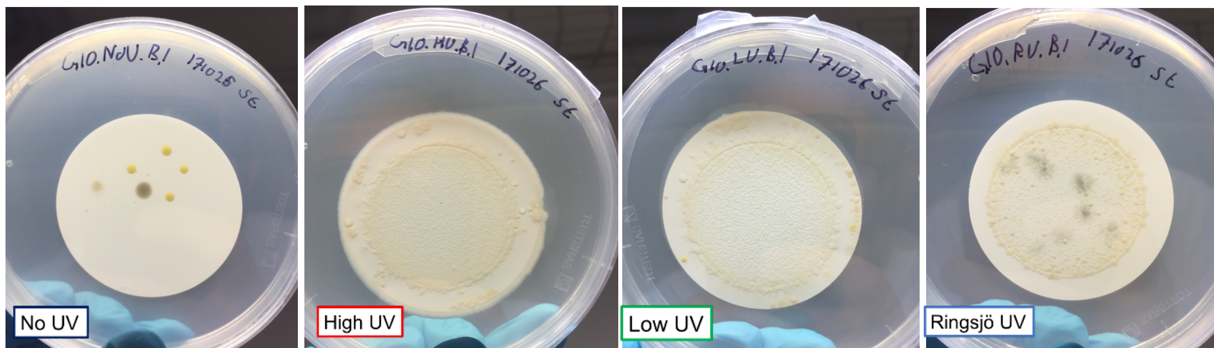


Figure 49. 5ml sample water stored at 22 °C day 10 after UV irradiation through 0,22 µl filter paper on R2a agar at 22 °C for 7 days. (From left to right: No UV - High UV - Low UV - Ringsjö UV)