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Evaluation of The Viral Reduction Potential using Ultrafiltration Membranes in the Drinking Water Treatment Process at Norrvatten

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Utvärdering av virusreduktion över ultrafiltermembran inom reningsprocessen av dricksvatten på Norrvatten

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Abstract

The present study was investigating the effectiveness of the ultrafiltration membrane as third biological barrier in Norrvattens drinking water treatment process, using a pilot scale model. This project aims to test the viral reduction capability of the membrane but also to remove other microbiological and chemical contaminants. To find suitable candidates for measuring the reduction capability, literature research has been performed as well as experimental testing of the raw water coming into the treatment plant and the backwash water from the membrane. Bacterial growth analysis using optical density (OD) measurements and plaque forming unit (PFU) has been performed to investigate the presence of bacteriophages. Approximately 9000 L of incoming and outgoing water from the ultrafiltration membrane has been concentrated using an electropositive membrane which then was eluted and ultracentrifuged. The pellet from ultracentrifugation has been tested for viral detection with PCR, qPCR and plating. TOC and absorbance measurement was also performed on the ingoing and outgoing water from the ultrafiltration pilot plant. Finally, a bench-scale experiment was performed using MS2-spiked water to investigate how well the filter reduced MS2 phages in the outgoing water.

The initial testing of the raw and backwash water showed that the plant virus Pepper Mild Mottle Virus (PMMoV) and *Pseudomonas* phages may be good candidates to use when evaluating the ultrafiltration membrane. When testing the eluate from the ultrafiltration pilot plant a reduction was seen in the starting DNA concentration when comparing the inlet and outlet water to the ultrafiltration pilot plant. The testing gave indications of a reduction of PMMoV and presence of *Pseudomonas* phages. The bench-scale experiment was hypothesized to stop all viral particles since according to theory the virus should be stopped by the membrane due to its pore size, but experimental testing indicated viruses in the outgoing water from the membrane as well. TOC and absorbance measurements showed a constant reduction over the membrane. The result of the study indicates that microbiological and chemical contaminants are removed by the filter, however, to determine the exact viral reduction potential of the filter and if all contaminant over the size of 20 nm is removed further testing is required.

No indications were seen for *Escherichia coli (E. coli)* phages in the water throughout the study, which in Livsmedelverket's (The National Food Agency) new regulations is used for determining the microbiological risks in water treatment processes. It may be of interest to investigate the possibility to also look for other type of phages to determine the microbiological risks, for example *Pseudomonas* phages which has been seen in this study.

Keywords

Microbiological barrier, Ultrafiltration, Source, Permeate, Backwash, NanoCeram filter, Drinking Water, Raw Water.

Sammanfattning

En pilotanläggning för ultrafiltering testas nu i Norrvattens reningsprocess för att undersöka ifall den kan användas som en tredje mikrobiologisk barriär i reningsprocessen. Målet med detta projekt är att testa membranets kapacitet att filtrera bort viruspartiklar men även membranet generella reduktionsförmåga för andra mikrobiologiska och kemiska kontamineringar. För att hitta lämpliga kandidater att använda sig av för att mäta reduktionskapaciteten av membranet har en litteraturstudie samt experimentell testning av råvattnet genomförts. OD mätningar på bakteriekulturer samt plackbildandeenheter (PBE) har undersökt för att se om bakteriofager kan finnas i proven. Ungefär 9000 L av ingående och utgående vatten från ultrafilteringen har koncentrerats med hjälp av ett elektropositivt filter som senare har eluerats och ultracentrifugerats. Pellet från ultracentrifugeringen har testat för virusdetektion med hjälp av PCR, qPCR samt PBE. TOC och absorbansmätningar har också genomförts på ingående och utgående vatten från ultrafiltermembranet. Slutligen utfördes ett bänkskaleexperiment för att undersöka hur väl filtret reducerade MS2 fager i utgående vatten.

Den inledande testningen visade att plantviruset PMMoV och *Pseudomonas* fager kan vara bra kandidater att använda sig av för att mäta virusreduktionen över ultrafiltermembranet. När elueringen från ultrafiltreringen testades indikerades en minskad DNA koncentrationen över ultrafiltermembranet med hjälp av Qubit-mätningar. Testningen visade även indikation på att PMMoV reduceras över membranet samt att *Pseudomonas* fager kan finnas i vattnet. TOC och absorbansmätningarna visade en konstant reduktion över membranet. I bänkskaleexperiment borde enlig teori alla fager stoppas av membranet eftersom viruset är större än porstorleken 20 nm, dock visade experimentell testning på att fager även fanns i utgående vatten från filteringen. Resultat av studien indikerar att mikrobiologiska och kemiska kontamineringar tas bort av membranet, dock för att bestämma den exakta virusreduktionen över membranet och ifall alla kontamineringar större än filters porstorlek (20 nm) tas bort kräver vidare testning.

E. coli fager, som i Livsmedelverket nya restriktioner används för att undersöka mikrobiologiska risker i vattenreningsprocesser, har också testats under studien på vattnet utan positiva utslag. Det kan därför vara av intresse att även undersöka andra fager, så som *Pseudomonas* fager för att kontrollera dem mikrobiologiska riskerna med vattenrening.

Nyckelord

Mikrobiologisk barriär, Ultrafiltrering, Källa, Permeat, Backspolning, NanoCeram filter, Dricksvatten, Råvatten

Abbreviation

CEB – Chemical Enhanced Backwash

LoD – Limit of Detection

LoQ – Limit of Quantification

PCR – Polymerase Chain Reaction

qPCR – Quantitative Polymerase Chain Reaction

PFU – Plaque Forming Unit

RNA – Ribonucleic Acid

DNA – Deoxyribnucleic Acid

UV – Ultraviolet

TOC – Total Organic Carbon

NaPP – Sodium Polyphosphates

WHO – World health Organization

PMMoV – Pepper Mild Mottle Virus

DH – Dorcoceras Hygrometricum

UMP – Uncultured Mediterranean Phage

NV – Norovirus

PP – Pseudomonas Phage

MBP – Mycobacterium Phage

1. Introduction

According to Statistikmyndigheten (The Statistical Authority) the population around Stockholm is expected to increase by 257 000 people until 2030. The expected population increase will put higher demands on the treatment facility to produce and deliver drinking water safe for human consumption, meeting the required standards for chemical and microbiological removal [1]. Norrvatten is a municipal drinking water treatment facility providing 14 municipalities with drinking water daily. The population increase puts pressure on Norrvatten to implement new treatment options to be able to continue delivering safe drinking water. A new treatment option in form of ultrafiltration is now tested at their treatment plant [2].

Today over 2 billion people live in water-stressed countries. Being able to deliver safe drinking water is essential for maintaining public health. Sustainable Development Goal 6 “Clean Water and Sanitation”, presented by the UN targets this specifically. Contaminated water leads to spread of diseases causing for example 485 000 diarrheal deaths each year. It has been observed that clean water increases a country's economic growth and reduce poverty greatly [3]. Countries welfare is dependent on clean water since it is essential for maintaining for example industry and agricultural practices. The drinking-water services in Sweden hold a high standard but still multiple outbreaks have occurred due to pathogenic microbiological contaminants. Folkhälsomyndigheten (the Public Health Authority) has between 1992 and 2011 recorded a total of 78 accidents [4]. The new restrictions from Livsmedelverket (The National Food Agency) requires treatment plants to test for *E. coli* phages in the raw water. If concentrations above 50 PFU/100 mL are found, the analysis has to be performed later in the cleaning process as well, to make sure pathogenic viruses are removed in the process [5].

1.1 Risk of Infection Spread from Drinking Water

The source of drinking water (surface water) carries a biological risk since pathogenic viruses and bacteria may be present in the ingoing water to the treatment plant. One common group of viruses transmitted in the drinking water is enteric viruses. Enteric viruses are commonly found in the feces from human or animals infected by the virus. Viral contaminants in the feces, may be excreted into different water sources causing contamination. Enteric viruses are also the smallest group of microorganisms, with a size range from 20 to 350 nm. Therefore, there is a possibility that the smallest viruses may escape the ultrafiltration membrane depending on its morphology [6].

Norovirus (35-40nm) is the virus that most frequently has caused outbreaks in Sweden in the past [7]. Furthermore, other viruses such as the adenovirus (90-100 nm) [8], hepatitis (27-34nm) [9], parvovirus (18-26nm) [10], rotavirus (70 nm) [11], poliovirus (25-30 nm) [12] and so on has been classified by the

world health organization (WHO) to be water-transmitted viral pathogens with moderate to high health impact on humans [13]. Therefore, those viruses may be significant tracking to ensure safe drinking water when implementing new biological barriers. Daily testing may not be feasible since tracking the contaminants by laboratory analysis can be difficult [14]. The composition of the water also changes depending on multiple factors such as season, temperature and weather [15].

Most virulent waterborne viruses are icosahedral or have a helical capsid structure. The figure 1, illustrates some of the most common waterborne viruses causing illness in humans and an RNA bacteriophage. The RNA bacteriophage is commonly found in the surface water and can be used as indicator organisms to see if the viral removal is efficient or not [16].

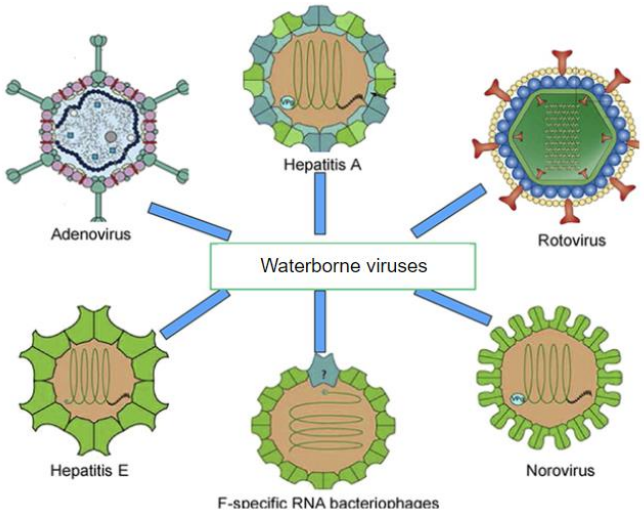

Figure 1: Structure of 6 different commonly found waterborne viruses. Original figure was retrieved and modified from "Biosensor for Waterborne viruses: Detection and removal" Altintas et al [16].

1.2 Viral Detection in Water Samples

A challenge when analyzing samples from drinking water treatment processes is that it often contains non detectable viral concentration by using conventional methods. To be able to detect viruses in these samples concentrating steps must be performed to get concentrations above the limit of detection (LoD) and the limit of quantification (LoQ) [17]. If possible, the ingoing water into the treatment plant may also be spiked using different types of viruses, this makes it easier to get viable starting concentrations [18].

The concentration can be performed in multiple steps where the volume is decreased based on different properties. It is also possible to add steps which can remove cytotoxic and PCR-inhibitory compounds.

Viral particles in the drinking water can be concentrated using properties such as ionic charge, particle size, density and sedimentation coefficient. The techniques that are applicable for performing viral concentration using ionic charge is adsorption/elution techniques with electropositive or electronegative membranes, glass wool or glass powder. When concentrating based on particle size ultrafiltration can be performed and for density and sedimentation coefficient ultracentrifugation may be used. Other techniques include iron oxide flocculation, immunoaffinity and magnetic beads [14]. It important when using these methods to take into account that the viral particles may be in free-living form or attached to other particles [19].

After concentration viruses may be detected using molecular methods such as PCR. This method has a high sensitivity allowing for detection of down to a single copy of DNA template. On the other hand, the technique can only be used to amplify specific DNA target which gives limited amounts of information. The techniques does not differentiate between contagious and non-contagious virus particles, it only gives information whether the DNA or RNA sequence investigate was present in the sample [20].

1.3 Norrvatten's Overall Treatment Process

To be able to produce drinking water, the raw water at Norrvatten is processed through multiple steps. An overview of the process can be seen in figure 2. Raw water is taken into the treatment facility at Görväln from Mälaren at two different depths (usually at 22 meters depths if the lake is not frozen). First, the raw water is treated by using a micro strainer removing fishes and algae. The water gets pumped into a flocculation chamber, which uses aluminum sulfate and a small amount of sodium silicate to create flocks with microorganisms, humus substances and suspended particles which sediments to the bottom of the chamber. After the flock sedimentation, the water is led into sand filters (last flocks are removed) followed by biofilters containing granulated carbon to increase the quality of the water in regards to smell and taste. Finally, the water is passed by UV-reactors that disinfects the water using ultraviolet light. The ultraviolet light decreases the microbiological growth by damaging DNA and blocking the replication. UV-radiation is very efficient against bacteria and parasites. The qualitative efficacy against viruses is good in most cases but inadequate to for example adenoviruses, which can resist higher doses of UV radiation than most microorganisms [21]. Lastly, the pH is adjusted to decrease the risk of corrosion in the distribution network and monochloramine is added mainly to decrease the risk of bacterial growth. The drinking water is let into a reservoir and from there pumped into the distribution network [22]. Norrvatten's distribution network is used to deliver the water to the municipalities. The municipalities are then responsible for the local distribution network, providing the households with water [23].

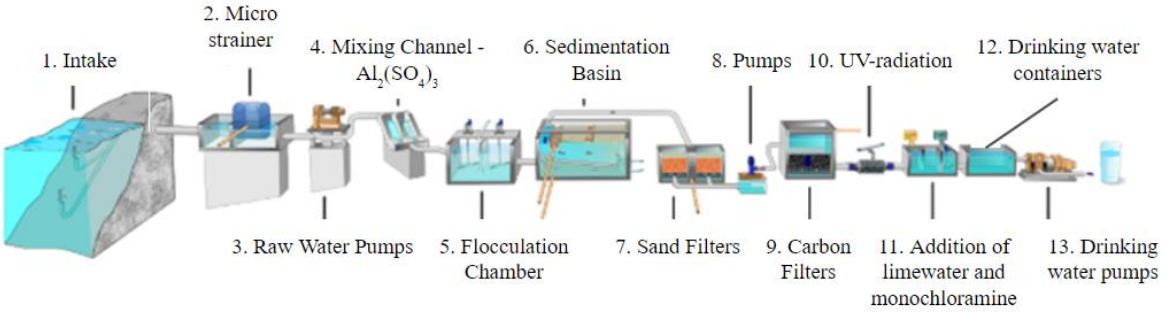

Figure 2: Norrvattens overall treatment process. The figure was retrieved and modified from https://www.norrvatten.se/dricksvatten/dricksvattenproduktion/reningsprocessen/ [22].

From the general treatment process at Görväln an ultrafiltration pilot plant is connected via the carbon filters. Implementing ultrafiltration would give a third microbiological barrier to the treatment system decreasing the risk of microbiological contaminants spreading in the drinking water since it improves the water quality based on other properties than UV-radiation and chemical flocculation. According to theory, Norrvattens ultrafiltration membrane should remove all contaminants larger than 20 nm (personal communication).

1.4 Ultrafiltration

Ultrafiltration removes particles based on a size-exclusion principle, therefore both microbiological contaminants such as bacteria and viruses can be removed meanwhile also suspended solids and other particles can be extracted. Implementing a filtration system into the drinking water treatment has multiple advantages. Filtration has a low construction cost and simple installation. The operation and maintenance are also simple which decreases the demands on trained personnel [21].

The ultrafiltration membrane used in the pilot plan at Görvälnverket contains a pre-filter with a pore size of 200 micrometers and two PES hollow fiber membranes having a pore size of 20 nm. According to the membrane manufacturers, a 4-log reduction is expected to be seen over the membrane (personal communication). Viruses with a size larger than 20 nm should get stopped by the membrane. This means that the filter can remove common viruses such as covid-19 which has an approximate size of 100 nanometer and adenovirus with an approximate size of 90-100 nm although smaller viruses such as the parvoviruses (18-26 nm) may escape the filter [24].

An overview of the pilot plant can be seen in figure 3. The ultrafiltration membrane used in this project is connected to one of Görvälverkets carbon filters. The water is first passed by a prefilter and is then collected in a feed tank. The water is pumped from the feed tank and the water is pH adjusted and

coagulant is added. The water is then passed by the ultrafiltration membrane and collected in the permeate tank. Water going through the semipermeable membrane is called the permeate meanwhile what is retained in the membrane is called the retentate. Chemical backwash is performed daily on the membrane were chlorine, base and acid are added in order to clean the filter which then is collected in the chemical enhance backwash (CEB) tank [25].

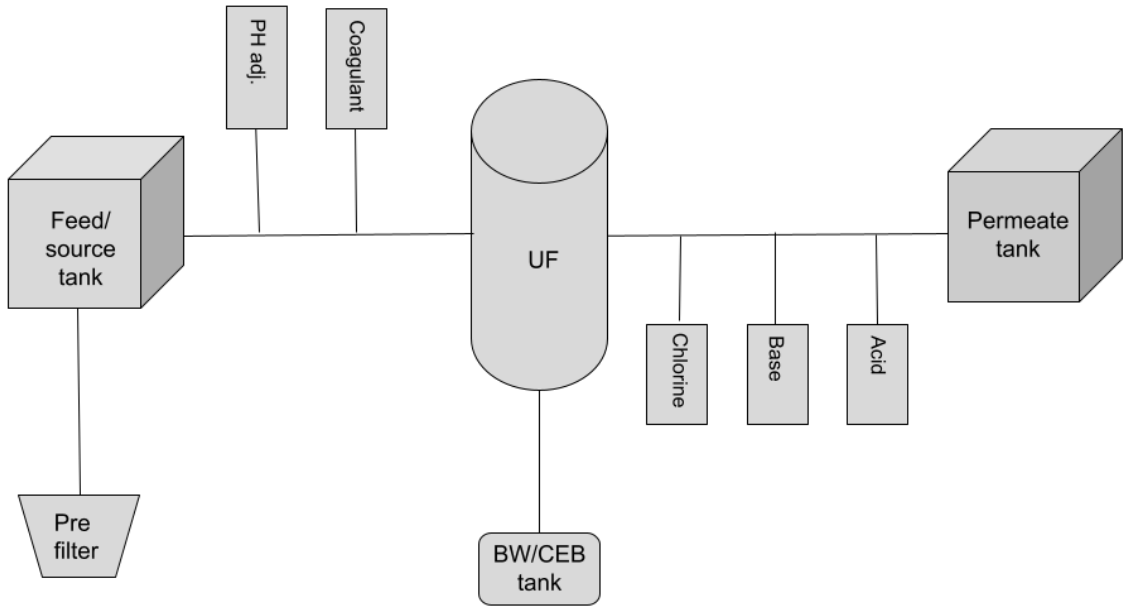

Figure 3: Overview of ultrafiltration pilot plant at Görvälnverket.

1.4 Project Aim and Specific Objectives

Today Norrvattens treatment plant in Järfälla consists of two microbiological barriers, chemical flocculation and UV-radiation. To be able to continue delivering safe drinking water Norrvatten is now looking into implementing a third biological barrier, ultrafiltration through polyethersulfone (PES) membranes with a pore size of 20 nm. This project's main aim is to test the viral reduction capability of the membrane to help assess the risk of infection spread by waterborne viruses. Traditional chemical- and bacterial analyses will be performed to indicate the general reduction over the membrane [26].

To be able to detect viruses concentration measures has been performed using an electropostive filter followed by ultracentrifugation. The electropositive filter is called NanoCeram and was used concentrate the particles in the ingoing and outgoing water to the ultrafiltration pilot plant as well as the raw water coming into the treatment plant, followed by an ultracentrifugation step. NanoCeram filters absorbs particles in a broad size spectrum with an absolute rating of 0,2 microns [27]. Figure 4 shows two

pictures of Nanoceram filters, one being placed in the filter house at the ultrafiltration pilot plant and one before before the filters has been processed. Using 1% NaPP (sodium polyphosphates), phosphate buffer and glycine has an 86% ± 9% elution efficiency when measuring the recovery rate of MS2 coliphages, meanwhile eluting with 3% beef extract only allows for 34% ± 18% elution efficiency [28].

Figure 4: NanoCeram filters. The figure to the left shows the filter before being placed in the filter house and the figure to the right shows the filter meanwhile filtering water through the filter house.

The pellet from ultracentrifugation of the eluate was then tested for viruses of different sizes and shapes. To determine the efficiency of the ultrafiltration filter. Table 1 shows the size, shape and nucleic acid of some of the viruses investigated during the project. Norovirus is the most common pathogenic virus associated with waterborne outbreaks in Sweden and was therefore used in the project [4]. Covid-19 has been seen in many wastewater samples and was therefore also of interest for the project [29]. The plant virus PMMoV was analyzed since it is commonly found in water sources [30]. The virus is also very volatile [31] which may causes cross contamination during PCR experiments if not carefully handled. An external project which has been performed in collaboration with Norrvatten showed that the inlet water into their treatment plant contains phages such as *Pseudomonas* phage (personal communication). Specific and degenerative primers for bacteriophages was used throughout the project. MS2 phage was used during a spiking experiment. MS2 phages are frequently utilized as a model organisms to study the efficacy of water treatment systems such as ultrafiltration [32]. Using a spiking experiment enables having a set starting concentration which can be evaluated against the concentration after the membrane filtration. This also enables having concentration above the LoQ and LoD for the method [33].

Table 1: Example of viruses investigated during project.

Virus Name	**Size**	**Shape/Morphology**	**DNA/RNA**
Norovirus [34]	27-38 nm	Icosahedral	RNA
COVID-19 [35]	70-90 nm	Icosahedral	RNA
PMMoV [36]	18 nm in diameter, 300-310 in length	Rod-shaped	RNA
Pseudomonas Phage [37]	6 nm in diameter and 3,700 in length	Filamentous	DNA
MS2 Phage [38]	23-28 nm	Icosahedral	RNA

Doceras Hydrometricum and uncultured Mediterranean phage are non-pathogenic DNA viruses that also has been used throughout the project since they have been detected at high concentration in various water samples, $1.8*10^8$ gene copies/L and $1.0*10^8$ gene copies/L respectively. This can be compared to PMMoV which is a commonly used indicator organism for viruses which vary between $3.0*10^3$ to $2.9*10^6$ gene copies/L in various water samples [39].

2. Material and Methods

Material and Methods include all methods performed to concentrate the inlet, outlet and backwash water from the ultrafiltration membrane as well as the raw water. The section also includes analyzing methods performed on the concentrated water as well as absorbance and TOC measurements and a bench-scale experiment.

2.1 Water Sampling

Water sampling was performed either by collecting the backwash water in 1L sterilized bottles or by processing water through NanoCeram filters which was later eluted. The collection bottles for the backwash water had been sterilized with sodium hypochlorite and rinsed with milliQ. Sampling of backwash water has been performed 31/1/2023, 14/2/2023 and 20/2/2023. Water sampling using NanoCeram filters was conducted twice on the raw water coming into the treatment plant at Norrvatten (calendar week 10 and 14, 2023) and twice for source and permeate water to the ultrafiltration membrane (calendar week 7 and 14, 2023). During the first filtration, approximately 400L of raw water was processed through the membrane and 7000L of the source and permeate water (fig 5). The second filtration was performed to send the filter for sequencing, see section 9.1 for further details.

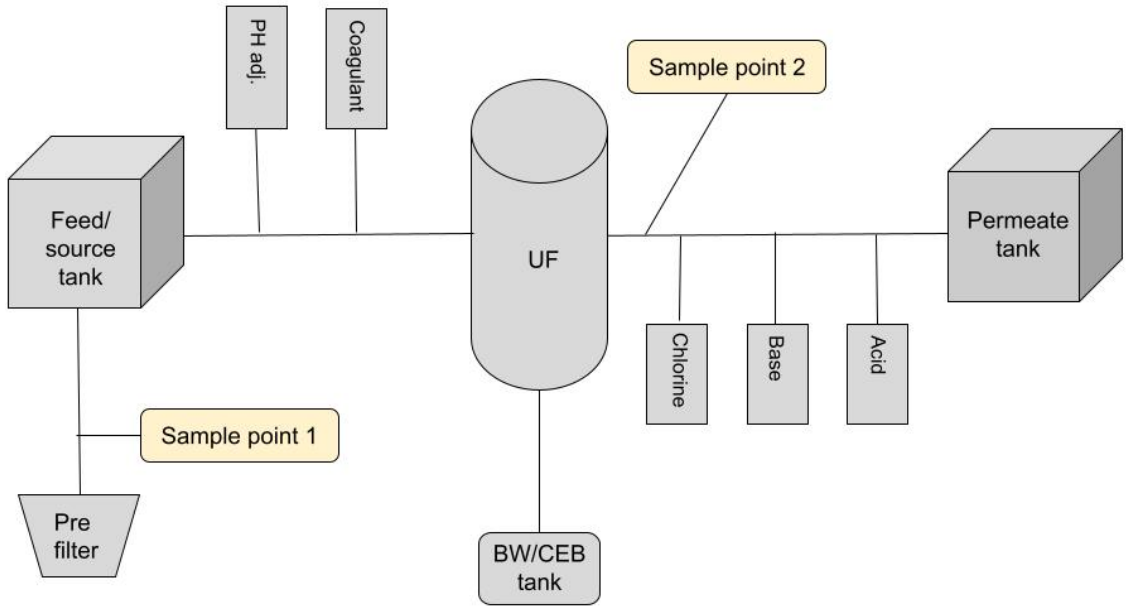

Figure 5: Sampling point in the ultrafiltration pilot plant used for NanoCeram filtration on source and permeate water.

2.2 Concentration of Backwash Water

The backwash samples were concentrated using Maxwell RSC Enviro TNA Promega KIT by following instructions from the manufacturer [40]. In summary, 40 mL samples were treated with a protease

solution and centrifuged to eliminate any solids and proteins present in the sample. The supernatant was then filtered in a column-based system followed by an elution using 500 µL of pre-heated (60°C) nuclease-free water.

2.3 Concentration of Raw Water and Ultrafiltration Samples

The raw water and ultrafiltration samples (permeate and source water) was concentrated using NanoCeram filters and ultracentrifugation. Two different protocols for filter elution were tested in the project. During the first concentration of source and permeate water (fig 5) the viral particle bound to the NanoCeram filter was eluted by using a solution of 1.0% NaPP (sodium polyphosphate), phosphate buffer containing 3.8mM Na_2HPO_4 and 6.5 mM KH_2PO_4 and 0,05M glycine, pH 9,3 [28].

The filter was immersed in 420 ml of the buffer solution in a beaker. The beaker was tightly closed with parafilm and inverted 10 times, followed by a 15 min incubation at room temperature in shaker (120 RPM). After the incubation, the beaker was again inverted 10 times, followed by another 15 min incubation at room temperature in the shaking incubator. The eluting solution was then passed into a sterile bottle under positive pressure in forms of N_2 gas [41].

The eluted solution was then passed by a 0,8 µm syringe filter and ultracentrifuged for 6h at 50,000 RPM and 4°C. The pellet was collected and stored in -20°C, before proceeding to DNA and RNA extraction.

The NanoCeram filter that was used to process the raw water was eluted using a buffer containing 3% beef extract, 0,05M glycine and 0.2M phosphate buffer, pH 9,5. The elution buffer (420 mL) was added to beaker to immerse the filter, the beaker was inverted 10 times followed by a 15 min incubation at room temperature using a shaker at 120 RPM. The beaker was then again inverted 10 times, followed by another 15 min incubation at room temperature in the shaker. The eluting solution was then passed into a sterile bottle under positive pressure in forms of N_2 gas. After elution, the solution was ultracentrifuged for 4 hours at 50,000 RPM and 4°C. The pellet was resuspended in 10 mM Tris-HCl, pH 8 and stored at -20 before proceeding to DNA and RNA extraction [41].

2.4 Molecular Analysis

Molecular analysis has been performed using qPCR and PCR after DNA and RNA has been extracted from the samples.

2.4.1 DNA/RNA extraction

DNA and RNA extraction for the samples from the backwash and the NanoCeram filter elution was performed by using the Maxwell RSC Instrument. The Maxwell RSC Pure Food GMO program was

chosen from the instrument software, followed by an automated extraction protocol where the nucleic acids are eluted in 80 µL of nuclease free water.

2.4.2 PCR and qPCR Primers, Probes and Programs

Literature research was performed in order to find suitable primers to test the viral particles, also PCR/qPCR conditions used for the different primers/viruses was investigated. Both consensus and degenerative primers were used in the project. Degenerative primers (T4 to Vi1 in table 2) were used for different types of bacteriophages. MS2 phages was used for a bench-scale experiment (see section 2.7). All potential viruses from the literature research can be seen in the table 2.

Table 2: Virus Name, Forward/Reverse Primer, Probe sequence (if applicable), suggested PCR conditions by the article and nucleic acid for each of the viruses are present.

Name, reference	**Forward Primer**	**Reverse Primer**	**Probe**	**Suggested PCR conditions by articles**	**RNA/DNA**
PMMoV [29]	GAGTGG TTTGACCTTA ACGTTTGA	TTGTCGGTTGC-AATGCAAGT	-	50 °C 10 min, 95 °C 30 s, followed by 45 cycles of 95 °C 10 s, 60 °C for 30 s	RNA
COVID-19 [29]	GGGAGCCTTGA-ATACACCAAAA	TGTAGCACGA-TTGCAGCATTG	-	50 °C 10 min, 95 °C 30 s, followed by 45 cycles of 95 °C 10 s, 60 °C for 30 s	RNA
Dorcoceras hygrometricum (DH) [42]	AAGCCTGAAC-GTGTTCCGAT	CTGCCCGCAG-GATTGTTAGA	-	3 min, 95 °C; followed by 39 cycles, 10 s, 95 °C; 45 s, 60°C	DNA
Uncultured Mediterranean phage (UMP) [42]	GCATCTTCG-TCAATGCGTCC	GAGGTCGTGG-TGTGGCTATC	-	3 min, 95 °C; followed by 39 cycles, 10 s, 95 °C; 45 s, 60°C	DNA
Norovirus (NV) [43]	ATGTTCAGRTGGATGA-GRTTCTCWGA	TCGACGCCAT-CTTCATTCACA	-	RT was carried out for 30 min at 50°C, and denaturation was carried out for 5 min at 95°C, followed by 45 cycles of PCR amplification (denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min).	RNA
Mycobacterium Phage (MBP) [44]	AGCCGATCA-GAAGCACGGGC	AGCGGCTCTT-AGGAGGGGCC	-	Initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 70°C for 30 s, and extension at 72°C for 30 s.	DNA
Pseudomonas Phage (PP) [45]	AGCGATGGG-TATCGGCAAAG	TGGGCATTA-CCGAGGTTGAC	-	Annealing temp: 55	DNA
T4 [46]	CCC TGC TGT TCC AGA TCG ANA ARG ARG C	CTG CCT GGC GTA CTG GTC DAT RWA NAC	-	Annealing temp: 50	DNA
T7 [46]	GAC AAG CGG AAG GAC ATC AAN CAY ACN GAR A	CGC GTA GTT GGC GGC RTT NGG CAT NA	-	Annealing temp: 55	DNA

N4 [46]	GGA TGA TCG TAA TAT TAA TGA TCA GGG NAT HRA YGC	GAC ATA AAG CCC ATT TCG CCR WAN GGR TC	-	Annealing temp: 55	DNA
GJ1 [46]	GGC TGC GCG TAT GAT TAG GAY ATH GAY GA	CCA ATG CAT CAC CGG CAD CCA DAT YTC	-	Annealing temp: 50	DNA
SP6 [46]	CAC CGT GAT TGC GCG TAA YAC NGT NGC	TTC CCA ACG ATC CGG AAT NGC NCC YTC	-	Annealing temp: 55	DNA
F01 [46]	CGC CAT TGA AGA ACT GCG TRW RCA YAT GGA	GGC ATC ATA TAG GAA TGC GCY TCR AAR TC	-	Annealing temp: 50	DNA
Vi1 [46]	GCC GAT TAA TAT TGC GAT GGA YTT YTT	CCA GCA TAA AGG TCA TAA ATT TCC AYT TYT C	-	Annealing temp: 50	DNA
MS2 Phage [47]	TGCTCGC-GGATACCCG	AACTTGCGTTC-TCGAGCGAT	ACCTCGG-GTTTCC-GTCTTGC-TCGT-BHQ1	55°C for 30 min, 95°C for 2 min, followed by 45 cycles of 95 °C for 15 sec and 58°C for 30°C	RNA

For the experimental set-up similar PCR/qPCR programs was combined to be able to run experiments for multiple primers at the same time. PCR/qPCR experiments performed throughout the project is summarized in section 9.9.

2.4.3 qPCR analysis

The RT-qPCR and qPCR analysis was performed using Thermo Fisher Scientifics Real-Time PCR machine QuantStudio3 and BIO-RAD CFX96^{TM} Real-Time System. The reagent mixture used for RT-qPCR and qPCR consisted of 10 µL iTaq, 0,25 µL of iScript, 0,6 µL of forward and reverse primer for respective virus, 2 µL of BSA per reaction. To each well 12 µL of the reagent mix and 8 µL of sample was added to get a final volume of 20 µL. Samples were performed in duplicates on the PCR plate. Both negative (nuclease free water and reagent mix) and positive controls (PMMoV 2e10^5 copies/mL /2e10^4copies/mL and reagent mix) were included on the plate.

2.4.4 PCR analysis and Qubit detection

The PCR analysis was performed on the MiniAmp^{TM} Plus Thermal Cycler (Thermo Fisher Scientific). The reaction mix used to perform the PCR analysis consisted of 4 µL of 5x PCR buffer, 2 µL of forward and reverse primer each, 0,4 µL dNTP mix, 0,2 µL of Taq polymerase and a sample volume of 13,4 µL to get a final volume of 20 µL. For RNA viruses 0,25 µL of iScript was also added to the reaction mix.

The DNA concentration was quantified using a Qubit 4 Fluorometer (Thermo Fisher Scientific) to assess possible amplification of the starting material. DNA concentration was measured by adding 2 µL of starting material or amplified material to 198 µL of Qubit reagent.

2.5 Investigation of Bacteriophages

2.5.1 Bacteriophage Detection using Bacterial Growth Experiments and OD measurements

The presence of *Pseudomonas* and *Escherichia. coli* phages were investigated using bacterial cultivation. Five mL of agar broth was inoculated with *P. Flourescens* and *E. coli* and left overnight in an incubator at 30ºC and 37ºC respectively. Backwash water from the ultrafiltration membrane was concentrated using centrifugation for 10 min at 10 000 RPM and 4°C. The pellet was collected after the centrifugation and resuspended in the backwash water (1-6 mL). The resuspended water (100-300 µL) was then added to the overnight culture and left in the incubator; sterile water was used as a control. The OD was measured at different timepoint over a period of 48 hours to see if the bacterial culture continued to grow or if there was a drop in OD, indicating the presence bacteriophages. Details about the separate experiments can be found in table 3.

Table 3: Experimental details from Bacterial Growth Experiment

EXP #	Cultures	Sample	Volume centrifuged	Final pellet volume	Pellet volume added to culture	Time points for OD measurement
1	*Pseudomonas, E. coli*	Backwash 31/1	40 mL	4 mL	200 and 100 µl	0h, 3h, 3 days
2	*Pseudomonas, E. coli*	Backwash 31/1	80 mL	5 mL	200 and 100 µl	0h, 2h, 1 day
3	*Pseudomonas*	Backwash 31/1	80 mL	1,5 mL	200 and 300 µl	0h, 2h, 19h, 1 day

2.5.2 Bacteriophage Detection using Plating and Plaque Forming Units (PFU)

Two hundred µL of overnight culture of *P. Flourescens* and *E. coli* was added on to agar plates and left for 30 min in an incubator of 30ºC and 37ºC respectively. After the incubation backwash or sterile water was added to the plate (50-200 µL). The plates were incubated for 24h before inspection of the plate. Any round clearing zones was expected to be due to bacteriophages. The same type of experiment was performed for eluted buffer from the NanoCeram filters. After ultracentrifugation 50 µL of pellet from the permeate and the source sample (fig 5) was added to the plates as well as 100 µl of supernatant. The phosphate buffer used for elution was used as a negative control in the experiment. Picture was taken either using a phone camera or using the gel imagining of stain-free gel in Gel DOC EZ Imager (BIO-RAD).

2.6 Analysis of Chemical Parameter from the Ultrafiltration Membrane

Sampling of the permeate and source water was performed at Norrvatten 6 times during the time period 2023-01-12 to 2023-02-09. The samples total organic carbon (TOC) and absorbance 254 nm was measured for the sample. The absorbance was measured in a 5 cm cuvette and TOC was measured in mg/L.

2.7 Spiking Experiment using Bench-Scale Ultrafiltration Membrane

Two mL of 2,9x 10^10 PFU MS2 phages/mL stock solution was added 500 mL of milliQ water giving a final concentration of 1.16x10^8 PFU MS2 phages/mL. 400 mL of the diluted stock solution was filtered through the ultrafiltration at a pumping speed of 0.1 L/min. 100 mL of the diluted stock solution was kept in a sterile bottle for further qPCR analysis and 400 mL of the permeate water was collected in a sterile bottle. The filtration was performed in Pentair X-FLOW RX300 0.83UFC ultrafiltration membrane, the data sheet is found in section 9.8. 80 mL respectively of inlet water, permeate water and backwash water from the filter membrane was concentrated using Maxwell RSC Enviro TNA Promega KIT as described in section 2.2. RNA and DNA was then extracted with Maxwell RSC Instrument by running the Maxwell RSC Pure Food GMO program in the instrument software.

Three µL of extracted RNA was then added to 17 µL of master mix containing in total 100 µL of 5x reaction mix, 10 µL of ROX dye, 10 µL of forward and reverse primer, 5 µL of probe, 10 µL of DNTPs, 12,5 µL iTaq and 12,5 µL of reverse transcriptase. The backwash, inlet and outlet water were tested as well as three positive controls containing 2,9 x 10^10 PFU MS2 phages/mL, 2,9 x 10^8 PFU MS2 phages/mL and 2,9 x 10^6 PFU MS2 phages/mL respectively.

Optimization of a qPCR protocol to use for determining a standard curve was performed, the optimization process and result is described in detail in section 9.7.

3. Results

The results section includes testing of the backwash and raw water to find suitable candidates to determine the viral reduction potential of the membrane. Results from PCR and qPCR experiment on the filter eluate from the source and permeate water (fig 5) is also presented. Furthermore, the section includes bacteriophage detection, results of the bench-scale experiment and chemical parameters (TOC and Absorbance). On overview of the different parts of the result section can be seen in figure 6.

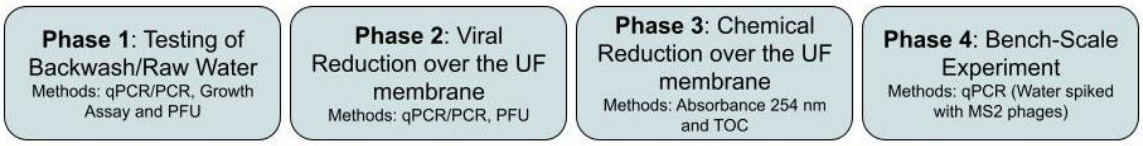

Figure 6: The figure illustrates the different phases of the result section.

3.1 Phase 1: Testing of Backwash and Raw Water

3.1.1 qPCR Backwash

Two replicated qPCR experiments were performed on the backwash water taken from the pilot plant 31/1 to test for the plant virus PMMoV and COVID-19. Sample 1 and 2 is from two separate extraction samples. No amplification was seen of COVID-19 during the experiments, this suggests that it was likely not present in the sample. Some amplification was seen of PMMoV in both experiments. The first experiment had PMMoV contaminations in 2 out of the 4 negative controls that is supposed to only have sterile water and reagent mixture, therefore the experiment was repeated. Positive controls containing PMMoV worked as expected for both experiments. The Ct-values for PMMoV can be found in table 4. A low Ct value corresponds to a larger amount of starting genetic material meanwhile a high Ct value corresponds to a smaller amount of starting genetic material. NaN stands for Not a Number and is used when no amplification has been detected.

Table 4: qPCR results from testing for the plant virus PMMoV.

Experiment	Sample	Ct value 1 (Replicate 1)	Ct value 2 (Replicate 2)	Mean Ct	Standard Deviation Ct
Backwash Experiment 1	PMMoV sample 1	37,53	NaN	37,53	NaN
	PMMoV sample 2	38,09	35,8	36,95	1,62
	Negative control 1	37,69	NaN	37,69	NaN
	Negative control 2	37,73	NaN	37,73	NaN
	Positive control 1 (2e10^5)	17.43	17,78	17,605	0,24
	Positive control 2 (2e10^4)	21,44	21,34	21,39	0,07
Backwash Experiment 2	PMMoV sample 1	45,46	NaN	45,46	NaN
	PMMoV sample 2	38,43	NaN	38,43	NaN
	Negative control 1	NaN	NaN	NaN	NaN
	Negative control 2	NaN	NaN	NaN	NaN
	Positive control 1 (2e10^5)	17,95	17,95	17,95	0
	Positive control 2 (2e10^4)	21,58	21,53	21,55	0,035

3.1.2 Bacteriophage Detection using Plating and Plaque Forming Units with Backwash Water

Plating of backwash water or sterile water on the bacterial culture was checked by visual inspection. Some indications were seen of *Pseudomonas* phages as can be seen in figure 7. Plating of backwash water on *E. coli* cultures did not show any indication of phages forming clearing zones. The clearing zone are indicated in red circles in the figure.

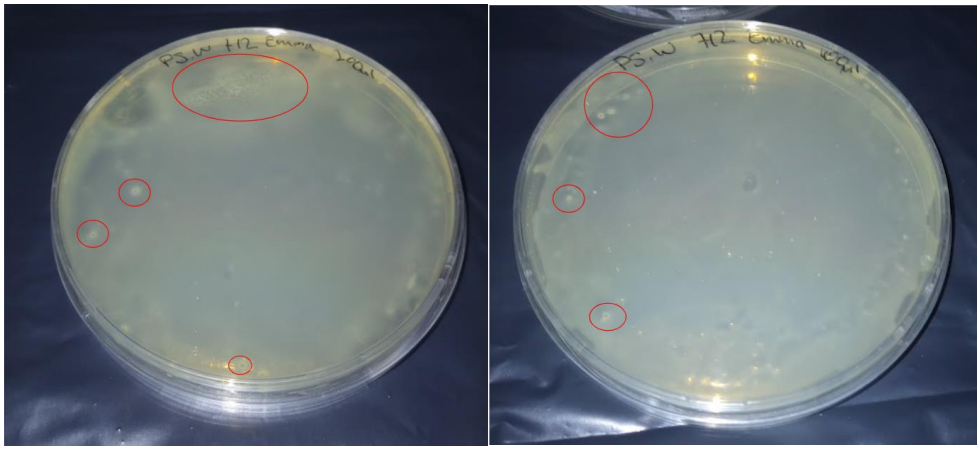

Figure 7: In the left picture 200 µL of concentrated backwash water has been added to the plate. In the picture to the right 100 µL of concentrated backwash water has been added to the plate.

3.1.3 Bacteriophage Detection by Growth Assay using OD measurement on the Backwash Water

Three experiments were conducted by adding backwash water to *Pseudomonas* cultures to see if the OD dropped in comparison to adding sterile water as a control (described in section 2.5.1). Two experiment was also performed adding backwash or sterile water to *E. coli* cultures. No indications were seen for *E. coli* phages being present in the water as the OD measurements was similar or higher for the backwash water compared to the control. Experiment 3 gave some indications of *Pseudomonas* phages being present in the backwash water. 19 hours after 200 µL of backwash water had been added to the culture, a 0,61-log reduction was recorded when compared to adding the same amount of sterile water. At 24 hours, a 0,36-log reduction was observed, meanwhile the addition of 300 µL resulted in a 0.11 log reduction during the same time period. The result of the third experiment can be seen in table 5 and the results from first and second experiment is found in section 9.2.

Table 5: Results from Experiment 3. W300, W200 represent backwash water sample with 300 and 200 µL volume added to the culture respectively. C300, C200 represents control (sterile water) with 300 and 200 µL volume added to the culture respectively.

Sample	**OD 2h**	**OD 19h**	**OD 24h**
PS W300	21,94	106	370
PS C300	11,02	87	483
PS W200	21,96	22	233
PS C200	12,88	89	537

3.1.4 PCR analysis of Raw Water

NanoCeram filter used to concentrate the viral particles from the raw water and was eluted using 3% beef extract [28]. The elution buffer was then supposed to be filtered through a 0,8 µm filter but due to clogging this was not possible. Therefore, the sample was taken directly to ultracentrifugation followed by DNA and RNA extraction. After DNA and RNA extraction the concentration was measured using Qubit. The sample had an average starting DNA concentration of 23,8 ng/µL (23,3 ng/µL and 24,3 ng/µL). The raw water was tested for *Pseudomonas* phage (PP) and PMMoV as well as for uncultured Mediterranean phage (UMP) and degenerative phage primers SP6, N4 and T7 (see section 2.4.2). The experiment did not show any amplification when measuring the final DNA concentration after PCR, detailed results can be found in section 9.4.

3.2 Phase 2: Viral Reduction over the UF Membrane

3.2.1 Analysis of Filter elution from Source and Permeate Water in the Ultrafiltration Pilot Plant

The filter elution from the source, permeate and backwash water (BW) was then analyzed using PCR and Qubit measurement. Both Pseudomonas phages and PMMoV was tested for, and the result can be found in table 6.

Table 6: Amplified product of PCR measured using Qubit before and after PCR with primers targeting the *Pseudomonas* phage (PP) and PMMoV.

Sample Name	Starting concentration [ng/µL]	Average final concentration measurement [ng/µL]
PP:Concentrated BW	0,966	1,40
PP; BW	0,542	0,79
PP: Source	0,0710	1,21
PP: Permeate	Out of range/to low	0,82
PMMoV: Concentrated BW	0,966	28,5
PMMoV: BW	0,542	23,2
PMMoV: Source	0,0710	18,4
PMMoV: Permeate	Out of range/to low	9,5

As seen from table 6, some amplification of the *Pseudomonas* phage has taken place in the source, permeate and backwash water, indicating it being present in the elution. For PMMoV amplification has been seen for all samples. As expected, the final concentration is higher in the concentrated backwash compared to the backwash sample. A reduction can also be seen when comparing the source sample to the permeate, indicating that most of the viruses gets stuck in the membrane.

The experiment was repeated for the source and permeate sample to investigate the reliability of the results. Although a new ultracentrifugation and DNA/RNA extraction had to be performed to have enough starting material. The experiment indicates no amplification of *Pseudomonas* phage and a reduction of PMMoV over the membrane, although with a considerable difference between the two measurements of the source PMMoV sample as can be seen in table 7. The starting DNA concentration in this experiment is not comparable to the starting concentration from the raw water filter elute since the eluate had been left for 3 weeks before extraction enabling microbiological growth in the flask.

Table 7: Amplified product of PCR measured using Qubit before and after PCR with primers targeting PMMoV and *Pseudomonas* phages.

Sample Name	Starting concentration [ng/µL]	Final concentration measurement 1 [ng/µL]	Final concentration measurement 2 [ng/µL]
PP: Source PP	22,04	16,08	15,21
PP: Permeate PP	13,7	12,82	12,72
PMMoV: Source	22,04	183,9	139,2
PMMoV: Permeate	13,7	92,2	104,6

A PCR experiment testing the backwash, concentrated backwash, source and permeate water was performed for Doroceras Hygrometricum (DH) and T7, a degenerative phage primer, however the experiment did not show any amplification. The filter eluate from the source and permeate was also analyzed using qPCR experiments with primers for PMMoV, SP6, N4, DH, PP, T7 and NV giving no amplification. Further details about the experiments can be found in section 9.5.

3.2.2 Bacteriophage Detection using Plaque Forming Units on Source and Permeate Concentrate

Plating was performed to investigate if any *E. coli* phages or *Pseudomonas* phages were present in the elution sample from the NanoCeram filters. No indications of *E. coli* phages were found but some was seen for *Pseudomonas* phages. In the figure below clearing zones, which indicates that phages may have lysed the plated bacteria are circled with red.

Figure 8: To the left concentrated sample from the source water has been spread out on the bacterial culture. To the right NanoCeram elution buffer without NaPP has been added to the plate to act as a negative control.

3.3 Phase 3: Analysis of Chemical Parameter connected to the Ultrafiltration Membrane

A general reduction of absorbance can be seen in figure 9 when comparing before and after ultrafiltration. The permeate water had an average value of 0,34 meanwhile the source, meaning the incoming water, had an average value of 0,38. These values can be compared to an average absorbance value for the raw water during the same time period of 1,11. For TOC a general reduction is also seen when comparing the permeate and source water. The permeate water had a value of 3.82 mg/L and the source water had an average value of 4,2 mg/L. This value can be compared to an average TOC value for raw water during the same time period of 7,93 mg/L.

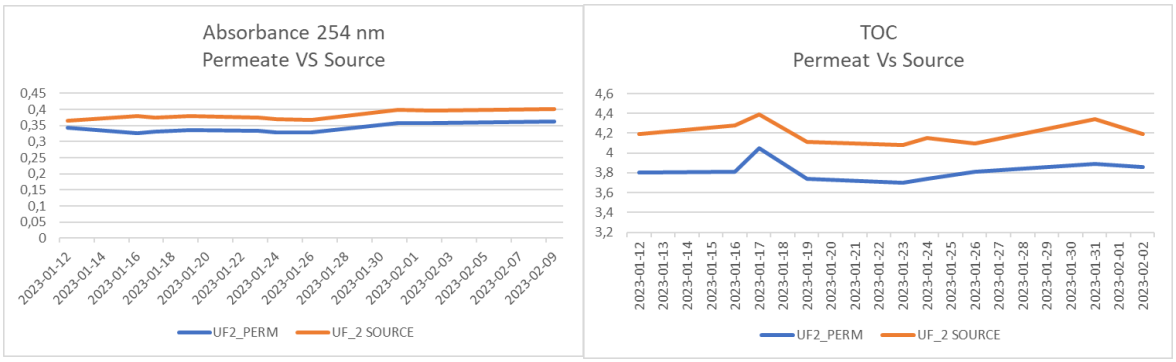

Figure 9: The figure to the left shows the absorbance measurements between 2023-01-12 to 2023-02-09 on source and permeate water from the ultrafiltration pilot plant. The figure to the right shows the TOC measurements between 2023-01-12 to 2023-02-02 on source and permeate water from the ultrafiltration pilot plant.

3.4 Phase 4: MS2 Reduction in Bench-Scale Ultrafiltration Membrane

The result of the qPCR experiment testing backwash, in and outlet water from the bench scale filtration is presented in table 8. Three positive controls containing 2,9 x 10^10 PFU MS2 phages/mL, 2,9 x 10^8 PFU MS2 phages/mL and 2,9 x 10^6 PFU MS2 phages/mL was used giving Ct values of 25.067, 30.339 and 30.679 respectively.

Table 8: Recorded Ct-values from Bench-Scale Experiment

Sample Name	Ct Replicate 1	Ct Replicate 2	Mean Ct	Standard Deviation Ct
Backwash	31,42	33,70	32,56	1,61
In	28,99	33,59	31,29	3,25
Out	38,50	35,93	37,21	1,81

4. Discussion

Experimental analysis on the backwash water is presented in the result section 3.1.1 to 3.1.3. qPCR experiment of the backwash water gave amplification of the PMMoV virus. The Ct values were relatively high ranging between 37,53 to 45,46, presented in table 4. This gives an indication of the plant virus being present in the backwash. On the contrary since PMMoV is a very volatile virus it may be due to cross contamination, especially since the Ct values are considerably high [31]. This was seen in the first experiment where the negative controls were contaminated. The reliability of the results was increased as the repeat experiment also gave amplification of the PMMoV virus and not in the negative control.

Indications of *Pseudomonas* phages have been seen from plaque forming units (PFU) as well as during experiments measuring the OD of bacterial cultures with added backwash water. The same experiments have been performed for *E. coli* phages which did not show any indications of the bacteriophage being present. Since the same results had been seen using both methods testing for *E. coli* and *Pseudomonas* phages, the results reliability was enhanced. In contrast, indications of *Pseudomonas* phages were only seen one time for each method and not constantly for every experiment performed. This may be due to variability of the water or low concentrations of the bacteriophage.

Bacterial growth has been seen in the backwash as suggested by the experiments described in section 9.3. As seen from the flow cytometry data, the backwash water contains a high number of cells relating to a high bacterial load, it was also evident during the plating and bacterial growth assays using OD measurements. This may be why the decrease seen for the successful bacterial growth assay first had a decrease of 0.61 log reduction after 19 h followed by a decrease of 0,36 log reduction after 24 hours.

The concentrated raw water was tested using PCR. The initial DNA concentration was as expected higher for raw water compared to eluate from the source and permeate concentration in the ultrafiltration pilot plant. This is since the water has not been treated by any microbiological barrier meanwhile the water coming into the ultrafiltration pilot plant has been treated with chemical flocculation. The elution of the raw water concentration was performed with beef extract which may have affected the results and increased the starting DNA concentration. The eluate could also not be passed by the 0,8 µm syringe filter, due to this it is likely that more bacteria was left in the sample. The measured value with qubit after DNA and RNA extraction was on average 23,8 ng/µL which can be compared to the elute from the source eluate which had a starting concentration of 0,0710 ng/µL and the starting concentration from the permeate was not measurable since it was out of range/too low. This indicates that most microbiological contaminants were removed during chemical flocculation and also a minor reduction over the membrane. PCR experiment of raw water for PMMoV, *Pseudomonas* phage, uncultured

Mediterranean phage and degenerative phage primers SP6, N4, T7 did not show any amplification. This may be due to the viruses not being present in the eluate or that they were present at levels below the detection limit for PCR.

The NanoCeram concentration of the permeate and source water was eluted using the detergent NaPP. The eluate was tested using qPCR, PCR and plating experiments as presented in section 3.2.1 and 3.2.2. The initial DNA concentration after ultracentrifugation and DNA/RNA extraction was at 0,0710 ng/µL for the source water and out of range/too low for the permeate water. The qPCR experiment testing for PMMoV, SP6, N4, DH, PP, T7 and NV did not show any amplification of the gene targets, neither did PCR experiment for DH and T7. As mentioned earlier, this may be due to the viruses not being present in the eluate or at levels below the detection limit. However, since amplification was seen for both PMMoV and *Pseudomonas* phage during PCR experiments some experimental error may have occurred. For PMMoV a reduction could also be seen over the membrane as the incoming water to the ultrafiltration membrane had an average value of 9,2 ng/µL meanwhile the outgoing value was on average 4,75 ng/µL. The experiment was repeated to increase the reliability of the result. For the repeated experiment, ultracentrifugation and DNA extraction had to be performed again. The repeated experiment showed a decrease of PMMoV and no amplification of *Pseudomonas* phages. *Pseudomonas* phages had only been seen in very low concentrations from other experiments which may be why the extracted material from the first ultracentrifugation gave amplification meanwhile the second did not.

Plating was also performed in order to investigate if any *E. coli* phages or *Pseudomonas* phages was present in the sample, as seen in section 3.2.2 some indications was seen for *Pseudomonas* phages in the source sample but most likely at very low concentrations.

The chemical reduction was seen constantly throughout absorbance and TOC measurements increasing its statistical relevance. Sampling was performed over a time of approximately one month which indicates that the membrane effectively removes contaminants with different ingoing water quality. These results indicate a general reduction over the membrane of organic material and substances. The results can be found in section 3.3. The average value of the raw water is significantly higher for both TOC and absorbance as expected since contaminants are removed during the first part of the cleaning process, for example chemical flocculation.

The bench scale model was tested using MS2 phages spiked water which have an approximate size of 27 nm and are shaped as a capsid. Therefore, according to theory all viruses should be stopped by the membrane, although in the qPCR experiment it was evident that MS2 was present also in the outlet water. This may be due to the filter not stopping all viruses larger than 20 nm. The filter may not be completely homogenous in pore size and could be affected by for example temperature or pressure. The Ct for the backwash water is also slightly higher than the inlet water, this may be due to the filter letting

some viral particles pass by the filter. This is likely since MS2 was also detected in the permeate. The bench-scale membrane had the same pore size as the one which is used in the pilot plant but was produced by another manufacturer which may have affected the results, also membrane age and storage may have affected the results.

4.1 Viral Reduction Potential of the Ultrafiltration Membrane

A constant reduction has been seen for both the absorbance value and total organic carbon when comparing the permeate and source water. Therefore, it can be verified that the filter does take away particles and produces a cleaner outlet water then what goes into to the pilot plant. However, to determine the exact virus reduction potential of the membrane in the pilot plant further testing must be performed. Indication of PMMoV being reduced over then membrane reduction has been seen in the filter elution using PCR and qubit measurements although to verify these results more testing should be performed. Finding other viral candidates of different size and shapes would also be necessary to be able to determine the general viral reduction potential of the filter.

Qubit measurements was performed both on the eluate from the raw water filtration and on the eluate from the source and permeate water from the ultrafiltration. The starting concentration from the two raw water eluate was at 23,3 ng/µL and 24,3 ng/µL respectively. Meanwhile the eluate from the source was only 0,0710 ng/µL and the starting concentration for the permeate was too low/out of range for detection with the qubit. 400 L of raw water was filtered through the membrane meanwhile 7000L was filtered through the source and permeate water in the ultrafiltration pilot plant. The raw water was eluted with beef extract, which may affect the starting concentration, compared to the source and permeate being eluted with NaPP, but most probably much more microbiological material was present before chemical flocculation in the raw water then before and after ultrafiltration. The decreased concentration after ultrafiltration compared to before suggests that microbiological material is also removed by the filter.

4.2 Risk of Infection Spread when using Ultrafiltration Systems

Earlier it has been seen that waterborne viruses causing infection in human such as the Norovirus, Rotavirus, Hepatitis A and Adenovirus can be transmitted through water sources [13]. The ultrafiltration membrane at Norrvatten has a pore size of 20 nm and should according to theory remove all viruses larger than that size. Therefore, all mentioned viruses should be removed by the filter. Smaller or filamentous viruses may pass the filter due to their shape. One example is the bovine parvovirus which is approximately 18-26 nm. This virus is known to infect cattle but not humans [10]. Therefore, it does not cause a direct threat to the human population but may create other indirect problems if a cattle infection starts spreading.

The bench-scale experiment using spiked MS2 water indicated that viruses with a larger size than the pore size of the membrane, 20 nm, still could pass the membrane. It is therefore important to perform further testing of the membrane to get a better understanding of what can pass by and what will get stopped by the membrane. MS2 has a size of 27 nm which is not much smaller than the Norovirus which has had multiple recorded outbreaks earlier in Sweden.

The ingoing water to the pilot plant did not contain high levels of starting DNA (0,0710 ng/µL), according to the results of this study. This is probably due to Norrvattens first biological barrier, chemical flocculation, removing most of the viruses present in the incoming water to the treatment plant. Indications of some viruses could be seen in the water going into the ultrafiltration membrane, for example PMMoV and *Pseudomonas* phages. PMMoV was also reduced over the membrane according to the PCR results. Testing virus concentrations using PCR to assess the risk of infection spread is limited since the method does not differentiate between contagious and non-contagious virus particles. If the DNA or RNA investigated is present in the sample, it will still give a positive result. It is possible to determine if the viruses are removed by the filter but due to the verity of viruses that can be present in the raw water other methods may be more suitable.

5. Conclusion

The plant virus PMMoV has been detected during PCR experiments. Indications of *Pseudomonas* phages has also been seen in plating, bacterial growth assays and PCR experiments. Further investigations are needed to be able verify *Pseudomonas* phages presents in the water. The concentration and elution of the viral particles from the filter membrane with NaPP buffer was better than using beef extract. However, further optimization and development of the technology is needed to concentrate and detect virus in very low concentration in the sample especially in drinking water samples. As indication of *Pseudomonas* phages has been seen in the water in contrast to *E. coli* phages, they may be of more interest to use as a reference virus to determine how well the third biological barrier removing microbial contaminants. This also includes viruses in the drinking water treatment processes to meet Livsmedelsverket regulations.

6. Future Perspective

To minimize false negatives in the PCR experiment, positive controls may be used for all viruses tested. Having positive control enables the chance the optimize the PCR protocol for a specific virus regarding annealing temperature, denaturation and so on. It may also be good to do a viral sequencing as a first step in the project in order to investigate what viruses is present in the water from the start and narrow down the different candidates to test for. The high Ct-values for PMMoV in the backwash water may be possible to decrease by concentrating more water than suggested in the "Maxwell RSC Enviro TNA Promega KIT". If the filter allows it, instead of concentrating 40 mL, 80 mL or more may be tested.

The plant virus PMMoV has been detected during PCR experiments and may be a good indicator virus for Norrvatten to use in future projects. Indications of *Pseudomonas* phages has also been seen in plating, bacterial growth assays and PCR experiments. Further investigations are needed to be able verify *Pseudomonas* phages presents in the water, but this may also be a good indicator virus for Norrvatten to continue working with. The new restrictions from Livsmedelsverket requires water treatment process in Sweden to check for *E. coli* phages to determine the microbiological risks. As indication of *Pseudomonas* phages has been seen in the water in contrast to *E. coli* phages, they may be of more interest to use a reference virus to determine how well microbiological contaminants are removed in the drinking water treatment processes.

Due to the low virus concentration present in this stage of the cleaning process, assessing the virus reduction potential using NanoCeram filtration and qPCR is most likely not possible in a sustainable way. The recovery rate is low [28] and the species recovered will vary in type and concentration depending on season and water coming into the treatment plant. The chances of finding the right viral species and be able to do this with an optimized PCR technique is very low. To see the viral reduction potential in the pilot plant a spiking experiment would be suggested, this does on the other hand require a lot of resources and may therefore not be suitable.

Other methods for detecting the viral reduction such as flow virometry may be a better option since it can detect a broader range of viruses compared to PCR or plating/bacterial growth assays. Flow virometry may also be combined with other techniques such as functional assays to be able to be access the virulency of the viral contaminants in the water. PCR can detect both infectious and non-infectious viruses if the nucleic acid still is present in the sample and may therefore not be a suitable technique for directly determining the risk of infection spreading.

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9. Appendices

9.1 Preparation for Sequencing

Three NanoCeram filters were processed to perform metagenomic sequencing. The metagenomic sequencing was performed by Eurofins using next generation sequencing allowing for detection of bacteria, one cell organisms, fungi and double stranded DNA. The pre-processing was performed at Norrvatten during the project. One filter was placed on the raw water coming into the treatment plant 03-04-23 until 06-04-23 filtering a total amount 429 L. 13 680 L of permeate water and 8 825 L of source water (see figure 5 for reference) was filtered during the same time period. The filtered volume was calculated based on average flow (20 s/L for the permeate water and 31 s/L for the source). The filtered volume for the raw water was based on online measurements. The filters were then eluted using the same protocol as described in section 2.3 for the raw water filtration. The results of the sequencing were sent to Norrvatten after the finish of this project.

9.2 Results from Experiment 1 and 2 (Growth Assays described in section 2.5.1)

The results of Experiment 1 and 2 as described in section 2.5.1 is found in table A1 and A2. During Experiment 1 one absorbance measurement was performed and the starting OD for the *E.Coli* culture was at 2,96 and for the *Pseudomonas* culture the starting OD was 2,1. During experiment 2, replicate measurements was performed for each sample. The starting OD for the *E.Coli* culture was at 2,4 and for the *Pseudomonas* culture the starting OD was 2,8.

Table A1: Results from Experiment 1 as described in section 2.5.1.

Sample	Absorbance 3h	Dilution 3h	OD 3h	Absorbance 3 days	Dilution 3 days	OD 3 days
EC W100	1,26	20	25,2	0,18	200	36
EC C100	1,14	20	22,8	0,213	200	42,6
EC W200	1,18	20	23,6	0,214	200	42,8
EC C200	1,02	20	20,3	0,35	200	69,2
PS W100	0,74	20	14,7	0,23	200	46,8
PS C100	0,59	20	11,8	0,19	200	38,6
PS W200	0,55	20	11	0,24	200	48
PS C200	0,57	20	11,3	0,09	200	17,2

Table A2: Results from Experiment 2 as described in section 2.5.1.

Sample	Average Absorbance 2h	Standard Deviation 2h	Dilution 2h	OD 2h	Average Absorbance 1 day	Standard Deviation 1 day	Dilution 1 day	OD 1 day
PS W300	0,99	0,015	20	19,9	0,42	0,057	200	84,1
PS C300	0,90	0,11	20	18,1	0,39	0,069	200	77,5
PS W200	0,98	0,028	20	19,6	0,49	0,076	200	97,3
PS C200	0,75	0,021	20	15,1	0,46	0,005	200	91,3
EC W300	1,68	0,019	20	33,6	0,42	0,014	200	83,8
EC C300	1,48	0,136	20	29,6	0,43	0,07	200	86,6
EC W200	1,47	0,047	20	29,3	0,42	0,015	200	83,9
EC C200	1,37	0,04	20	27,3	0,5	0,09	200	101,7

9.3 Bacterial Growth Experiment on Backwash water

To assess the bacterial growth, 100 µL of concentrated backwash (10 min centrifugation at 10 000 RPM and 4°C), 1 mL backwash water, 100 µL and 1 mL of sterile water (used as controls), was added to nutrient broth. The mixture was incubated for 72 hours in 30 and 37 degrees. OD measurements was taken after 19.5, 24 and 72 hours to measure the bacterial growth in the samples. Backwash water was also plated on agar plates, MacConkey agar for gram-negative bacteria and FE-plates for gram-positive bacteria and left for a time period of 14 day. Number of bacterial colonies were counted after 4,7 and 14 days.

Figure A1 illustrated the logarithmic growth when adding 100 µL concentrated backwash water, 1 mL backwash water, 100 µL and 1 mL of sterile water to nutrient broth in a 30- and 37-degrees incubator. As can be seen in figure A1 most growth is seen in the concentrated backwash followed by backwash water and no growth was seen when adding sterile water. This indicated bacteria being present in the backwash.

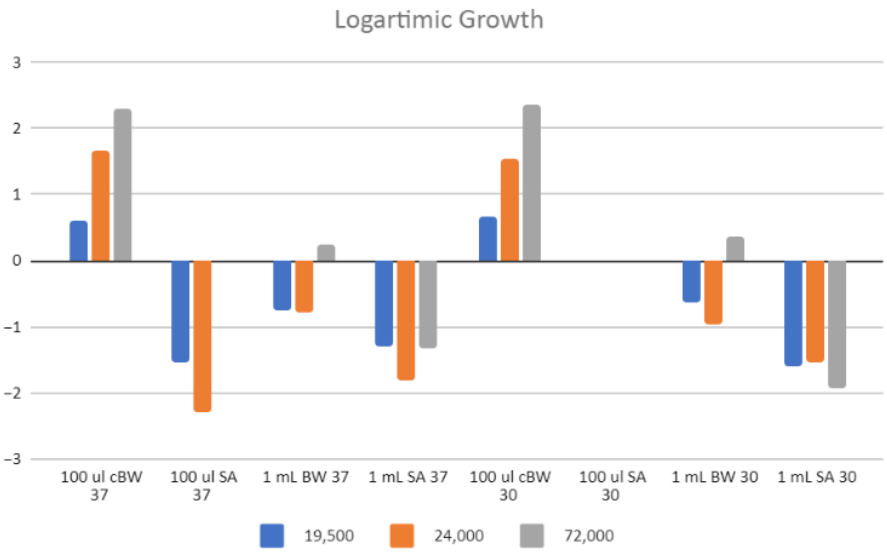


Figure A1: Logarithmic growth in samples taken 19.5, 24 and 72 hours after addition of backwash (BW), concentrated backwash (cBW) and sterile water (SA).

Bacteria were growing on the agar plates for all temperatures tested (room temperature, 30 ºC and 37 ºC). Bacterial growth was on the other hand not always seen on all the MacConkey (McC) or FE plates testing for gram-negative and gram-positive bacteria respectively. The average amount of colonies from 4,7 and 14 days of incubation can be seen in the table below. As seen in table A3 most bacteria grow at room temperature, followed by 30 ºC and 37 ºC.

Table A3: Average amount of colonies counted after 4,7 and 14 days.

Average amount of colonies	FE Backwash	McC Backwash	Agar Backwash	FE Concentrated Backwash	McC Concentrated Backwash	Agar Concentrated Backwash
37 ºC	1	0	6,67	0	0	57,33
30 ºC	0	1	26,67	0	9,33	77,33
Room Temperature	3,33	2	46,67	3,67	17,67	116,67

The figure below shows some of the agar plates where colonies has been grown. As seen in the figure colonies of different color and size are visible on the plate indicating that different types of bacteria present in the backwash water.

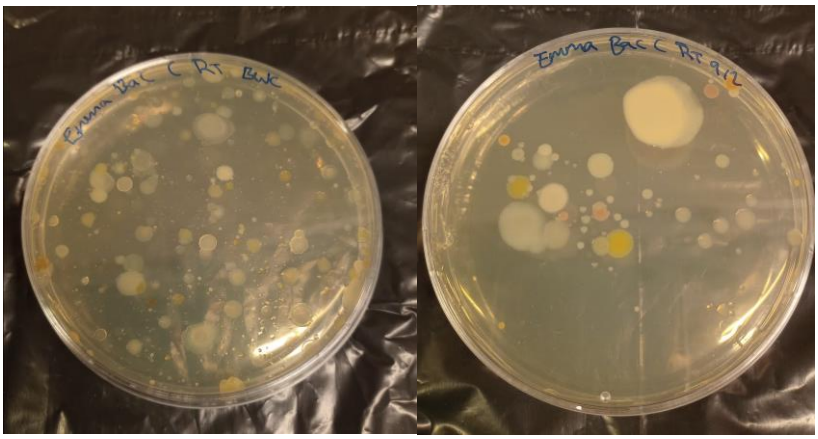


Figure A2: Bacterial colonies of different color and size grown on agar plates incubated in room temperature.

To determine if any bacteria was present in the backwash (BW), concentrated backwash (cBW, meaning the pellet from centrifugation for 10 min at 10 000 RPM and 4°C of the backwash water) and raw water samples (RW1, RW2) PCR was performed using a universal bacterial primer. The amplicon was also added to an agarose gel together with 1 µL of 6x DNA loading dye. 5 µL of GeneRuler DNA ladder was added to a separate well. The gel picture taken with GZ imager can be seen in figure A3. The figure indicates amplicon being present with an approximate size of 1,4 kB in the raw water, circled in red. Since other experiments indicated bacterial growth, it is likely that in the three sample that did not give any amplification had an experimental error, for example unsuccessful DNA extraction.

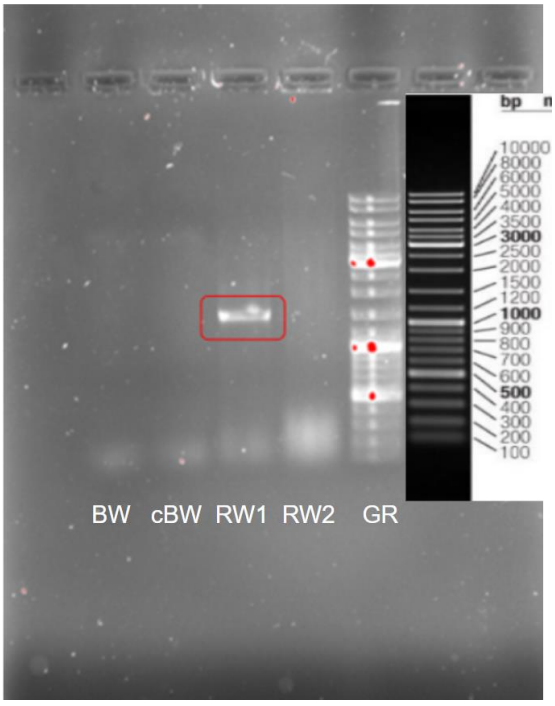


Figure A3: Gel image indicating bacteria being present in the extracted raw water solution.

The number of live and dead cells present in the sample was measured using flowcytometry and BD Biosciences Flow Cytometer BD Accuri™ C6 Plus. Backwash water was colored using SYBR Green and PI dye. 5 µL of SYBR Green was added to 495 µL backwash water and 4 µL of PI was added to 496 µL backwash water. The backwash water was both tested untreated, with a 10x dilution using milliQ water and with filtrating the water through a 5,0 µM filter. All samples were done in duplicates. The backwash water was tested using flowcytometry 14/2-2023 and 5/4-2023. 14/2-2023 the backwash water was not tested with a 5,0 µM filter pretreatment.

The number of cells present in the backwash water was measured using flow cytometry 2023-02-14 and 2023-04-06. The result of the backwash is compared to flow cytometry results from the carbon filter (inlet water to the ultrafiltration pilot plant). The average of 16 samples from the carbon filter between the time period 221012 and 221214 is presented in table A4 together with the analysis of the backwash water.

Table A4: Summarized flow cytometry data from backwash sampling 2023-02-14 and 2023-04-06 compared to data from carbon filtration.

Sample	**Total amount of cells [10^3 cells/mL]**	**Std Cells [10^3 cells/mL]**	**PI [10^3 cells/mL]**	**Std PI [10^3 cells/mL]**	**Damaged Cells [10^3 cells/mL]**
Carbon filter	1452	6,58	1281	11,9	172
Backwash 2023-02-14	4915	473,3	3832,6	90,6	1008
Backwash 2023-04-06	14612	77,58	13135	14	1476

As seen in table A4, the number of cells is much larger 6/4 compared to 14/2, this may be due to weather conditions effecting the water quality. Dot plots of the flow cytometry data from the backwash sample analyzed 2023-04-06 can be seen in figure A4 and figure A5.

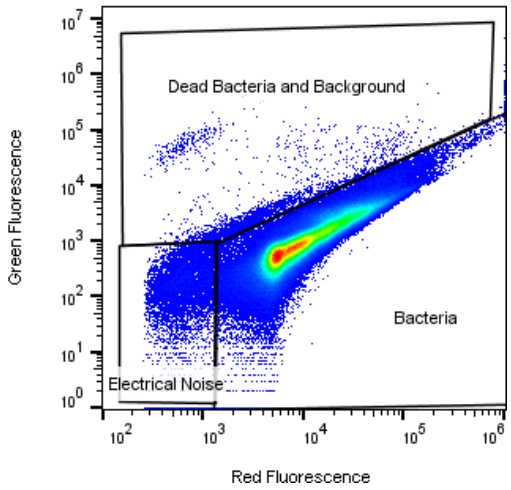


Figure A4: Flowcytometry dot plot of red versus green fluorescence. The backwash water sample (2023-04-06) has been added undiluted and was stained using SYBR Green.

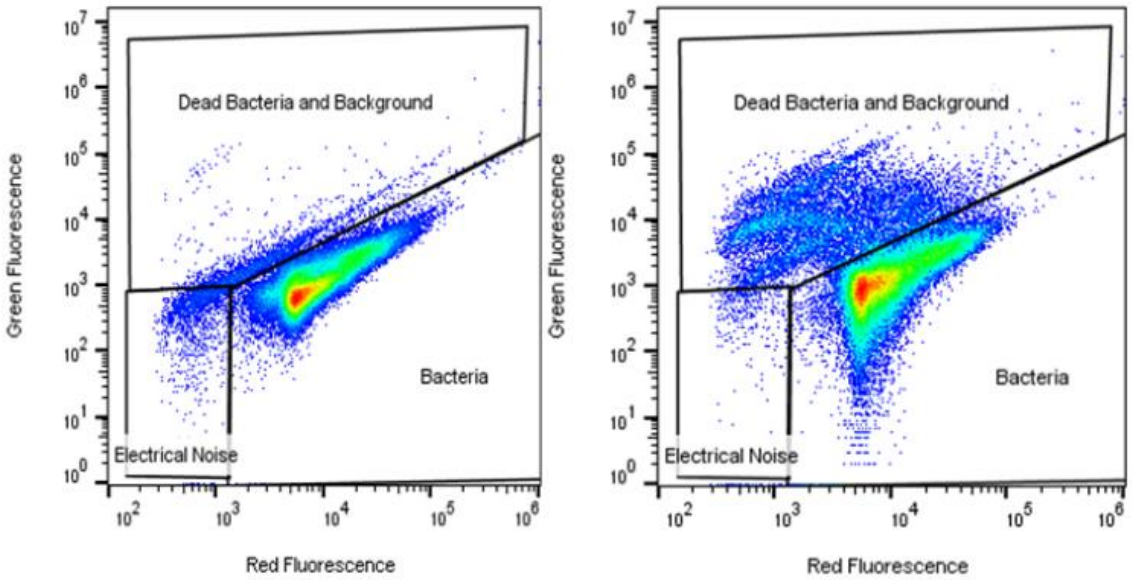


Figure A5: Flowcytometry dot plot of red versus green fluorescence. The figure to the left used SYBR Green staining meanwhile the figure to the right used propidium iodide. The backwash water (2023-04-06) has been diluted 10 times.

The experiments above indicate a high bacterial load in the backwash water, meaning that the ultrafiltration membrane efficiently removes bacterial contaminants from the water being treated by the system.

9.4 PCR on Raw Water Elution

The raw water was tested for *Pseudomonas* phages and PMMoV since earlier testing had indicated it being present in the backwash water from the ultrafiltration membrane. The testing was performed by

using PCR and Qubit measurements. No amplification was seen in the experiment, the result can be seen in table A5. Starting concentration of the two raw water samples were at 23,3 ng/µL and 24,3 ng/µL the samples were diluted to a concentration of 17,47 and 18,22 ng/µL.

Table A5: Qubit measurement for raw water sample tested for PMMoV and *Pseudomonas* phage in raw water.

Sample name	Replicate 1 [ng/µL]	Replicate 2 [ng/µL]
PMMoV raw water sample 1	15,9	15,7
PMMoV raw water sample 2	15,1	14,9
Pseudomonas phage raw water sample 1	14,7	14,9
Pseudomonas phage raw water sample 2	14,5	14,7

The raw water was also tested for uncultured Mediterranean phage and degenerative phage primers SP6, N4 and T7. The starting concentration for the raw water was measured to 30,5 and 31,5 ng/µL. The result of the PCR experiment can be seen in table A6.

Table A6: Raw water tested with PCR using UMP, SP6, N4 and T7 primers.

Sample name	Replicate 1 [ng/µL]	Replicate 2 [ng/µL]
UMP 1	26,2	26,7
UMP 2	29,3	29,25
SP6	29,7	29,9
N4	24,5	24,5
T7	27,6	28,3

9.5 PCR and qPCR experiment on Permeate and Source Concentration

The eluate from the first concentration of the permeate and source water in the ultrafiltration pilot plant (see figure 5 for reference) was tested for Doroceras hygrometricum and the degenerative phage primer T7 using PCR and qubit measurements. The results did not show any significant differences between the samples and was therefore treated as unreliable. The increased concentration may be due to contaminants or measurement errors. The initial concentration for the concentrated backwash was at 0,966 ng/µL, the backwash had a starting concentration of 0,542 ng/µL and source of 0,0710 ng/µL meanwhile the DNA concentration of permeate could not be determined since it was too low/out of range.

Table A7: PCR results from testing for Doroceras hygrometricum.

Sample name	Replicate 1 [ng/µL]	Replicate 2 [ng/µL]
cBW	3,48	3,92
BW	4,23	4,21
Source	3,92	3,85
Permeate	3,83	3,78

Table A8: PCR results from testing for T7.

Sample name	Replicate 1 [ng/µL]	Replicate 2 [ng/µL]
cBW	3,7	3,61
BW	3,18	3,18
Source	3,94	3,91
Permeate	4,57	4,54

The source and permeate was also tested using qPCR. The NanoCeram Eluate was first tested for PMMoV, DH, SP6, N4, T7, PP and NV. The experiment did not show any amplification in either the samples or the positive controls for PMMoV. Therefore, an experiment only testing the positive control was conducted. The experiment only showed amplification in half of the wells where a new set of primers had been used, therefore the old PMMoV primers were discarded.

9.6 Bacterial Growth Analysis of NanoCeram filter Elution using NaPP.

To see if the detergent NaPP may have affected the bacteria when it was used as the elution buffer for the NanoCeram filters, a bacterial growth experiment was performed comparing *Pseudomonas* phage growth with and without addition of NaPP. The bacterial growth with and without the addition of detergent NaPP did not show any difference as can be seen in the figure below. Therefore, it may be assumed that adding 1% NaPP detergent does not affect the microbiological environment greatly. The pellet, filtrate and supernatant are all sample which NaPP has been added to meanwhile elution buffer is without.

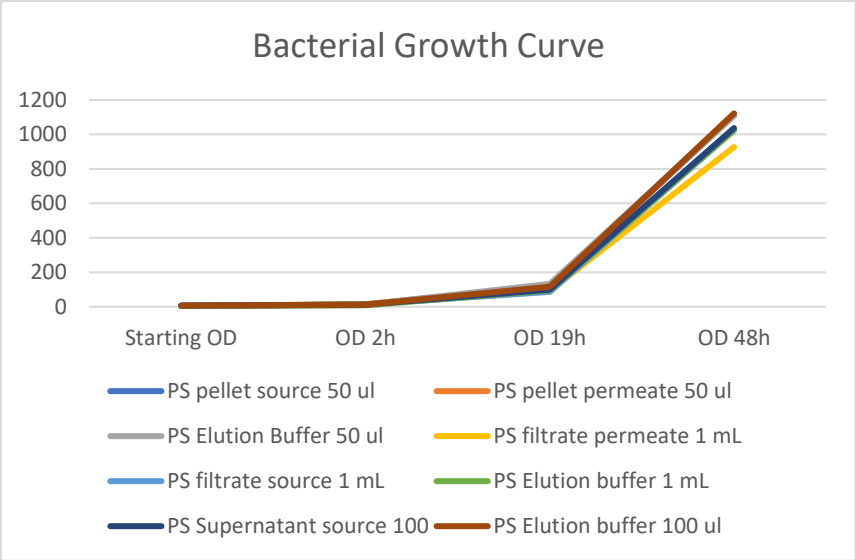


Figure A6: Bacterial Growth Curve produced by measuring the OD of *Pseudomonas* cultures 2, 19 and 48 hours after the sample has been added.

9.7 MS2 qPCR Standard Curve Experiment

A stock solution of 2,9x 10^10 PFU MS2 phages/mL was diluted 10, 100, 1000, 10 000 times and then extracted using the same method as in the section 2.4.1. The extracted material was tested using qPCR to be able to create a standard curve. The extracted stock solution was first tested using SYBR Green detection chemistry giving no amplification. Therefore, a probe and ROX dye was tested giving amplifications in some wells. Details about the probe and primer sequences can be seen in section 2.4.2. Different starting concentrations was tested and in the final experiment all concentrations gave amplification. The Ct values was more similar to each other than suggested by the initial concentration, the results can be seen in table A9. A suggestion for future experiments would be to test for lower concentration and having a larger variation between the wells. Other extraction methods may also be tested such as sonication.

Table A9: Starting sample concentration and Ct-values of the MS2-standard curve experiment is presented.

Starting Sample Concentration (PFU)	Ct-value
2,9*10^6	17,43
5,8*10^6	16,33
8,7*10^6	15,99
11,6*10^6	16,69
14,5*10^6	16,39
17,4*10^6	20,35

9.8 Data Sheet for Ultrafiltration Membrane used for Bench-Scale Experiment

PENTAIR

X-FLOW RX300 0.83UFC
ULTRAFILTRATION MEMBRANE

MEMBRANE ELEMENT DATASHEET

1" RX300 0.83UFC 0.83mm
ARTICLE CODE : 1051BL885A

GENERAL INFORMATION

RX300 0.83UFC is an ultrafiltration pilot module, used for production of process and potable water. Typical applications are the filtration of surface water, potable water and WWTP effluent. Mode of operation is feed-and-bleed with a minor crossflow or dead-end mode with regular backwash (permeate only) and chemically enhanced backwash.

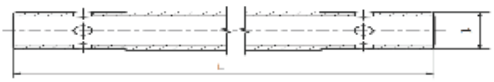
MEMBRANE CHARACTERISTICS

Materials of Construction

Housing	PSF
Potting	EP resin
Membrane	PES/PVP

ELEMENT SPECIFICATIONS

Hydraulic membrane diameter (mm/mil)	Membrane area (m^2/ft^2)	Element length L_{OA} (mm/inch)	Element outer diameter (mm/inch)
0.83 [32.7]	0.08 [0.86]	300 [11.8]	23.9 [0.94]



OPERATING SPECIFICATIONS

Max. system pressure	Max. trans-membrane pressure	Max. backflush pressure	Max. temp.
[kPa/psi]	[kPa/psi]	[kPa/psi]	[°C/°F]
at 20 °C 800 [116]	at 0-30 °C 300 [43]	at 0-30 °C 300 [43]	60 [140]
at 40 °C 600 [86]	at 30-60 °C 200 [29]	at 30-60 °C 150 [21.5]	
at 60 °C 400 [58]			

- Final maximum operating limits are determined by the lowest values of the membrane and element pressure and temperature specifications

PROCESS CHARACTERISTICS (WATER 20 °C)

Membrane diameter	Flow rate (*)	Pressure-drop across module at 1 m/s	Pressure-drop across module at 2 m/s
[mm/mil]	[m^3/h/gpm]	[kPa/psi]	[kPa/psi]
0.83 [32.7]	0.27 x v [1.19 x v]	11 [1.6]	24 [3.5]

(*) superficial velocity (v) in m/s [ft/s]

- Backwash water should be free of particulates and should be of permeate quality or better
- Backwash pumps should preferably be made of non-corroding materials, e.g., plastic or stainless steel. If compressed air is used to pressurize the backwash water, do not allow a two-phase air/water mixture to enter the element
- To avoid mechanical damage, do not subject the membrane module or element to sudden temperature changes, particularly decreasings. Do not exceed 60 °C process temperature. Bring the module or element back to ambient operating temperature slowly (typical value 1 °C/min). Failure to adhere to this guideline can result in irreparable damage

9.9 Summary of PCR and qPCR programmes

qPCR/PCR	Primers used	Samples	Programme
qPCR	COVID-19, PMMoV	Backwash water 31/1	50 °C 10 min, 95 °C 30 s, followed by 45 cycles of 95 °C 10 s, 60 °C for 30 s
qPCR	COVID-19, PMMoV	Backwash water 31/1	50 °C 10 min, 95 °C 30 s, followed by 45 cycles of 95 °C 10 s, 60 °C for 30 s
qPCR	PMMoV, DH, SP6, N4, T7, PP,NV	NanoCeram eluate with 1% NaPP (week7): Source pellet from ultracentrifugation (UC)*2 Permeate pellet from UC *2 Source sample with direct capture concentration	Stage 1 (hold stage): 50C, 10 min. 95C, 30 sek (1,6C/s increase). Stage 2 (PCR stage): 95C, 10 sek, 60 C, 30 sek (1,6C/s decrease) X45. Stage 3 (melt curve stage): 95C 10 sek, 65C 0,05 sek (1,6C/s decrease). 95C 0,05 sek (0,5C/s increase – dissociation stage)
PCR	PMMoV, PP	NanoCeram eluate with 1% NaPP (week7): Source pellet from ultracentrifugation (UC) Permeate pellet from UC. Backwash (31/1) and Concentrated Backwash (31/1)	Stage 1: 50C, 10min. 95C, 30 sek. X1. Stage 2: 95C, 10sek. 60C, 30 sek. X45. Stage 3: 95C 10sek 65C, 5 sek. X1
PCR	DH,T7	NanoCeram eluate with 1% NaPP (week7): Source pellet from ultracentrifugation (UC) Permeate pellet from UC. Backwash (31/1) and Concentrated Backwash (31/1)	Stage 1: 50C, 10min. 95C, 30 sek. X1. Stage 2: 95C, 10sek. 60C, 30 sek. X45. Stage 3: 95C 10sek 65C, 5 sek. X1
PCR	PMMoV, PP	NanoCeram eluate from Raw water filtration	Stage 1: 50C, 10min. 95C, 30 sek. X1. Stage 2: 95C, 10sek. 60C, 30 sek. X45. Stage 3: 95C 10sek 65C, 5 sek. X1
PCR	PMMoV, PP	NanoCeram eluate with 1% NaPP (week7): Source pellet from ultracentrifugation (UC) Permeate pellet from UC. Backwash (31/1) and Concentrated Backwash (31/1)	Stage 1: 50C, 10min. 95C, 30 sek. X1. Stage 2: 95C, 10sek. 60C, 30 sek. X45. Stage 3: 95C 10sek 65C, 5 sek. X1

PCR	16 sRNA	NanoCeram eluate with 1% NaPP (week7): Source pellet from ultracentrifugation (UC) Permeate pellet from UC. Backwash (31/1) and Concentrated Backwash (31/1)	98 °C 30 s, followed by 30 cycles of 98 °C 10 s, 61 °C for 30 s, 72 °C for 1 min 30s, followed by one cycle of 72 °C for 10 s then cooling to 4 °C
PCR	UMP, SP6, N4, T7	Raw Water	3 min, 95 °C; followed by 39 cycles, 10 s, 95 °C; 45 s, 60°C
qPCR	MS2	Bench-scale inlet and outlet water and backwash water	55°C for 30 min, 95°C for 2 min, followed by 45 cycles of 95 °C for 15 sec and 58°C for 30°C
qPCR	PMMoV	NanoCeram eluate with 1% NaPP (week7): Source pellet from ultracentrifugation (UC) Permeate pellet from UC.	50 °C 10 min, 95 °C 30 s, followed by 45 cycles of 95 °C 10 s, 60 °C for 30 s
qPCR	MS2	Positive controls for standard curve	55°C for 30 min, 95°C for 2 min, followed by 45 cycles of 95 °C for 15 sec and 58°C for 30°C
qPCR	MS2	Repeated experiment of positive controls for standard curve	55°C for 30 min, 95°C for 2 min, followed by 45 cycles of 95 °C for 15 sec and 58°C for 30°C
qPCR	MS2	Repeated experiment of positive controls for standard curve (changed starting DNA)	55°C for 30 min, 95°C for 2 min, followed by 45 cycles of 95 °C for 15 sec and 58°C for 30°C
qPCR	MS2	Repeated experiment of positive controls for standard curve (optimization)	55°C for 30 min, 95°C for 2 min, followed by 45 cycles of 95 °C for 15 sec and 58°C for 30°C
qPCR	MS2	Repeated experiment of positive controls for standard curve (optimization)	55°C for 30 min, 95°C for 2 min, followed by 45 cycles of 95 °C for 15 sec and 58°C for 30°C