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Licenciada em Ciências e Engenharia do Ambiente

Integrity monitoring of reverse osmosis membranes: Potential for naturally present viruses to verify virus removal comparing to MS2 bacteriophages

Dissertação para obtenção do Grau de Mestre em
Engenharia do Ambiente, perfil de Engenharia Sanitária

Orientador: Dr. Gertjan Medema, Chair in Water & Health, Delft University of Technology

Co-orientadora: Prof. Doutora Leonor Miranda Monteiro do Amaral, Professora Auxiliar, FCT/UNL

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Resumo

A filtração de água através de membranas de osmose inversa é considerada uma solução interessante devido a ser, teoricamente, uma excelente barreira contra microrganismos patogénicos presentes na água. Os vírus são os microrganismos patogénicos com menor dimensão presentes na água de superfície e, por isso, os mais difíceis de demonstrar a sua remoção. Para que um sistema de filtração por membranas de osmose inversa seja considerado seguro e não comprometa a saúde pública, é necessária a presença de um método de monitorização da integridade da membrana para que seja possível detetar a ocorrência de danos.

O uso de bacteriófagos MS2 é atualmente o método mais utilizado para validar a remoção de vírus em sistemas de osmose inversa. Apesar de contar com diversas vantagens como a sua semelhança em tamanho e morfologia com vírus entéricos humanos, possui desvantagens como o facto de ser necessária a introdução de bacteriófagos em concentrações elevadas na água de alimentação, não sendo aplicável em sistemas de grande escala.

Ensaios experimentais foram realizados utilizando uma instalação piloto de membranas de osmose inversa na presença de membranas intactas e danificadas para avaliar o potencial de utilização de vírus que ocorrem naturalmente em água de superfície, para verificar a remoção de vírus. Para determinar o potencial do teste acima mencionado, foram usados bacteriófagos MS2 como método de comparação.

Os resultados da presente dissertação indicam que ambos os métodos demonstraram mais do que 7 valores de remoção na presença de membranas intactas. Nas experiências realizadas com membranas danificadas com orifícios, ambos os métodos demonstraram diferentes valores de remoção consoante a severidade do dano da membrana, indicando por isso, a sensibilidade por parte dos dois métodos para detetar perdas de integridade. Um maior dano foi observado quando quatro orifícios de 1 mm de diâmetro foram provocados na membrana, seguido de um orifício de 4 mm e um orifício de 1 mm de diâmetro.

A consistência entre os resultados obtidos entre o método em avaliação em relação ao método correntemente mais utilizado, confirma o potencial de usar vírus naturalmente presentes na água para verificar a remoção de vírus, com particularidade para a aptidão do vírus natural utilizado na presente dissertação para ser um adequado indicador de remoção de vírus.

Palavras-chave: membranas de osmose inversa; integridade de membranas; MS2 bacteriófagos; vírus naturalmente presentes; remoção de vírus; indicador de remoção de vírus.

Abstract

Reverse osmosis membrane filtration is becoming an interesting solution since it is in principle an effective barrier against pathogenic microorganisms in water. Although having the ability to treat many water sources and provide safe drinking water, viruses are the smallest pathogenic microorganisms and therefore the most challenging to verify their removal in membrane filtration. For a reverse osmosis filtration system to be secure to public health, a trustful membrane integrity monitoring method is crucial to detect damages.

The use of MS2 phages is currently the most used challenge test to validate virus removal in RO membranes. Although it has numerous advantages such as their similarity in size and morphology to enteric human viruses, it still has the drawbacks of being spiked in the feed water and not being feasible in full-scale plants.

This dissertation focused on assessing the potential of a new challenge test that uses naturally occurring viruses in surface water to validate virus removal in RO membranes. This is a promising method since it discards the need of introducing components into the feed water thus being applicable to drinking water plants.

Using a reverse osmosis pilot scale, experiments were performed using intact and damaged spiral wound membranes to assess the potential of using natural present viruses in surface water for validating virus removal in comparison with the performance of the already known good method – MS2 bacteriophages.

The findings in this research demonstrate that both MS2 and naturally present viruses challenge tests achieved above 7 log removal values in the presence of intact membranes. Compromised membranes with induced pinholes achieved different log removal values according to the severity of the inflicted damages indicating sensitivity to detect impairments by both challenge tests. Greater loss of membrane integrity was observed when four pinholes with 1-mm diameter were inflicted on the membrane, followed by one 4- and one 1-mm diameter.

Based on the consistency of the achieved log removal values between the two challenge tests, these results therefore demonstrate the potential of using natural present viruses to verify virus removal, particularly the suitability of the natural virus used in this research to be an adequate virus removal indicator.

Keywords: Reverse osmosis membranes; membrane integrity; MS2 bacteriophages; naturally present viruses; challenge testing; virus removal indicator

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Abreviatures

CA	Cellulosic acetate membranes
COD	Chemical oxygen demand
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polymeric substances
KWR	KWR Watercycle Research Institute
LRV	Log removal value
NF	Nanofiltration
NGS	Next generation sequencing
NOM	Natural organic matter
NPV	Naturally present viruses
OECD	Organisation for Economic Co-operation and Development
PA	Polyamide membranes
qPCR	Quantitative polymerase chain-reaction
RNA	Ribonucleic acid
RO	Reverse osmosis
RWT	Rodhamine WT
TOC	Total organic carbon
UF	Ultrafiltration
UV	Ultraviolet radiation

1. Introduction

Water scarcity is continuously increasing mainly due to population growth, urbanisation and climate change. These factors coupled with fresh water pollution exerted by human activities will globally aggravate water shortage. OECD estimates an increase on water demand by 55% and severe water stress for nearly half of the world population in 2050 leading to an urgent need to develop new strategies for water management. For instance, countries from North Africa and the Middle East, Pakistan, India, and the northern part of China are projected to suffer from severe water scarcity by the year 2025. These circumstances will result in crises such as food shortage, regional water conflicts, limited economic development, environmental degradation and therefore it must be addressed as a severe problem. It is of utmost importance to identify and develop new alternatives that enable a more sustainable water use (Asano *et al*., 2007; Huang *et al*., 2015; Sourirajan & Matsuura, 1985).

Since fresh water supplies are becoming limited and not affordable to everyone, water reuse strategies tend to increase as the world becomes more populated and more urbanized especially near coastlines. Water reuse is defined as the use of treated wastewater for beneficial uses such as agricultural irrigation, landscape irrigation, industrial use, groundwater recharge and potable uses. This strategy is attractive because it offers an alternative water supply that is available in urban areas and resists to dry years (Asano *et al.*, 2007; USEPA, 2012).

Nevertheless, water recycling carries serious public health concerns because it enhances potential risks of infection and spreading out diseases. Therefore, drinking water quality must be carefully monitored (Australian Guidelines, 2008) as these systems contain chemical contaminants and microbial contaminants that are worrisome due to the presence of bacteria, viruses and protozoan parasites. Furthermore, assessing a risk management is imperious to assure public health not only for drinking water purposes but also for other uses that entail any human contact (National Research Council, 1998).

A solution for this challenge such as reverse osmosis filtration has become a widely preferred process for the removal of dissolved constituents in water reuse systems *(*Asano *et al.*, 2007; Bódalo-Santoyo *et al.*, 2004).

Osmosis is a fundamental process in biological systems that occurs when two solutions of different concentrations are separated by a semipermeable membrane. Water flows from the solution with the lower concentration of solute into the solution with higher concentration, through the membrane. This movement of water processes until the osmotic equilibrium is reached. A difference of height is observed between both compartments when the chemical potential is equalized, expressing the osmotic pressure. When pressure is applied to the more concentrated solution, water is forced to flow from the concentrated to the diluted side, retaining solutes in the membrane – reverse osmosis (Eisenberg & Middlebrooks, 1986; LANXESS, 2013).

This phenomenon is represented in Figure 1.1.

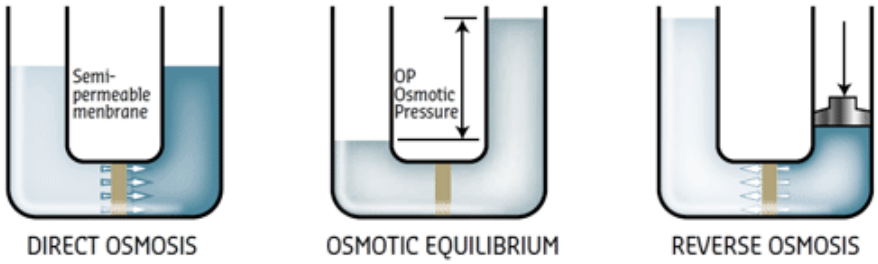

Figure 1.1 - Reverse osmosis (Hopwood, 2012)

Theoretically, reverse osmosis membranes reject all dissolved and suspended material meaning that only water is capable of passing through the membrane. Furthermore, reverse osmosis (RO) systems have been reported as the most widely used technique for high quality recycled water production. However, process failures can occur and for these reasons, it is mandatory to thoroughly monitor and test the membrane's physical state in order to detect any loss of integrity and this way avoid to compromise public health (Huang *et al*., 2015; Kitis *et al.*, 2003; Mi *et al*., 2004; Shannon *et al.*, 2008; Wagner, 2001).

This research emphasis the microorganisms related to waterborne diseases especially enteric viruses due to their consequences in human health, their limited consequential removal in membrane separation processes and the challenge to validate the removal of these viruses on reverse osmosis systems.

Membrane integrity tests were indicated by USEPA guidelines to reduce pathogens outbreak. Both direct and indirect integrity testing are required as well as a continuous monitoring. Direct methods are those applied to the membrane or the membrane module, such as pressure-based (offline technique), that assess the state of the membrane by monitoring pressure or air/water displacement under high pressure or vacuum. Indirect methods monitor some inherent aspect of permeate water quality, such as turbidity, conductivity and total organic carbon (Frenkel & Cohen, 2014; Kumar *et al*., 2007; USEPA, 2012).

Emerging techniques like challenge tests are proving to be more adequate for pathogen reduction than the aforementioned tests, and have become more popular to assess RO integrity, for instance, dye testing, pulse integrity testing, nanoparticles, and biological surrogates such as MS2 bacteriophage (Portillo, 2015). MS2 phage has a small size, it's easy to culture in large quantities and is not harmful to humans, which resulted in being one of the most used biological surrogates and indicated as the best performance on validating virus removal by achieving substantial log removal values. The removal or inactivation efficiency for a specific target such as an organism, particulate or surrogate is known as log removal value (LRV) and it is reported as presented in equation 1.

C_{in} is the concentration of the pathogen in the feed and C_{out} is the concentration of pathogen in the permeate.

$$LRV = \log_{10} \left[\frac{C_{in}}{C_{out}} \right] \quad (1)$$

Recently, virus detection techniques such as next generation sequencing (NGS) and quantitative polymerase chain-reaction (qPCR) are becoming advanced and possible to be used to assess the integrity of membranes (Ogorzaly & Gantzer, 2006). The measurement of naturally occurring viruses in, for instance, the surface water, is an interesting challenge technique because it allows the monitoring to be performed without adding compounds to the feed water (Antony *et al.*, 2012; Ferrer *et al*., 2013; Pype *et al*., 2016).

2. Literature review

2.1 Reverse osmosis membranes

A membrane is a perm-selective system that allows the passage of certain constituents. Reverse osmosis can reject even monovalent ions such as sodium and chlorine. This kind of membranes are widely used for seawater and brackish water desalination to produce both water for industrial application, for wastewater and water reuse treatments.

The configuration applied for membrane separation consists on the feed water stream flowing tangentially to the membrane surface. A fraction of the water in this feed stream passes through the membrane, whereas the majority of the feed flow travels along the surface, which results on a permeate and on a concentrate (Figure 2.1). The permeate has substantially low concentration of small particles whilst the concentrate is characterized by a high concentration of small particles. This configuration increases the potential of rejected solutes to accumulate on the membrane surface, that could lead into fouling or scaling, subjects to be discussed (Judd & Judd, 2011; LANXESS, 2013).

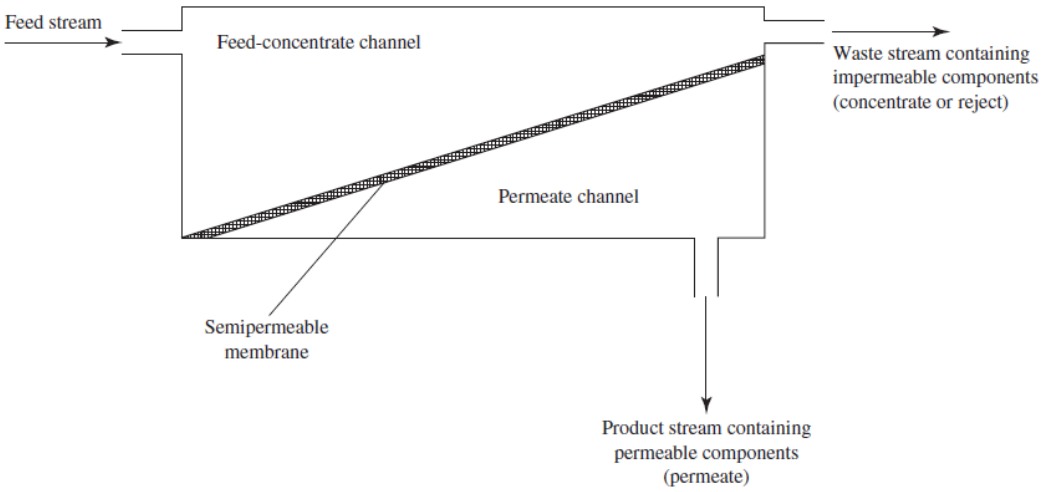

Figure 2.1 – Reverse osmosis membrane (Judd & Judd, 2011)

Membrane materials can be generally divided in two types: integral membranes and composite membranes. The most used ones are cellulosic derivatives (CA) and polyamide derivatives (PA). Although integral cellulosic acetate membranes are the common commercial material and the first to be produced, they don't tolerate elevated temperatures, they tend to hydrolyse when the pH is less than 3 or greater than 8 and they are susceptible to biological degradation. In order to achieve better performances, developments and optimizations were made and polyamide membranes were introduced. PA membranes are more resistant to biological degradation, they don't tend to hydrolyse in water and they can produce higher flux and higher rejection than CA membranes. However, Polyamide derivative membranes are more susceptible to fouling and do not tolerate free chlorine (Davis, 2011; Wagner, 2001). Regarding the performance of these membrane

materials on virus removal, it has been suggested that virus rejection achieve higher values for PA membranes due to being negatively charged (Antony *et al*., 2012) however it is not proved for reverse osmosis membranes.

The membranes' structure can be tubular membranes, flat sheet systems, ceramic systems and the most used are spiral wound element membranes. The spiral-wound membrane element has a high membrane surface area to volume ratio, it is easy to replace, it can be manufactured from a wide variety of materials, is sold by several manufacturers (Bódalo-Santoyo *et al*., 2004) and provides the highest degree of packing density using flat sheets wound around a centre pipe. The membranes are glued along three sides to form membrane leaves attached to a permeate channel (centre pipe) placed along the unsealed edge of the membrane leaf. The internal side of the leaf contains a permeate spacer designed to support the membrane sheet without collapsing under pressure. This permeate spacer is porous and conducts permeate to the centre pipe. A feed channel spacer is placed between the leaves to define the feed channel height and provide mass transfer benefits. (LANXESS, 2013) (Figure 2.2). To achieve higher recoveries, elements are placed in series.

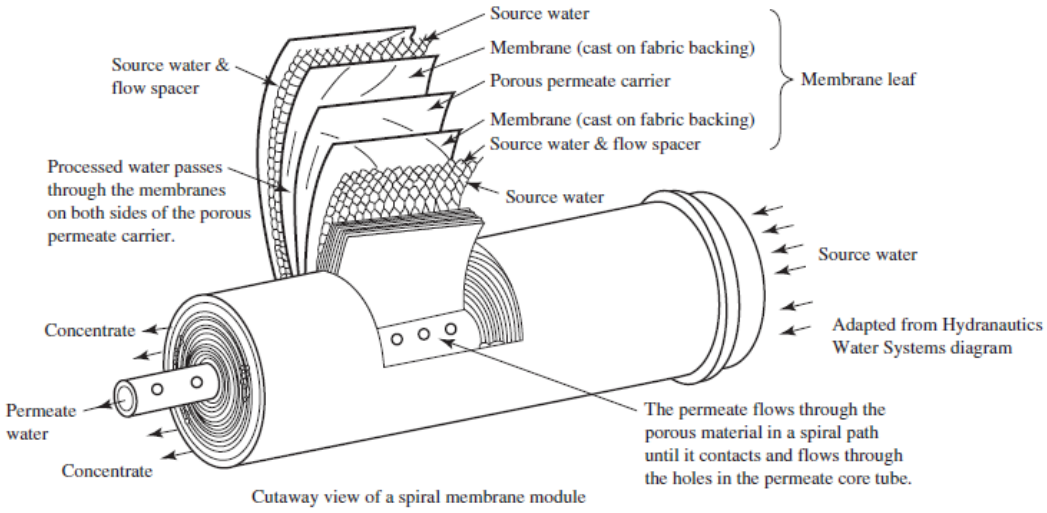

Figure 2.2 – Configuration of spiral-wound membranes (Davis, 2011)

As the process goes, the rejected particles, salts and microorganisms accumulate at the membrane surface resulting in several limitations. Membrane fouling can occur by one of the following mechanisms: deposition of silt or other suspended solids, scaling, biological fouling and organic fouling (Davis, 2011).

Deposition of particles such as silicates, sand, silt, clay can occur if these are not sufficiently removed by a previous treatment and will take place in the feed spacer and at the membrane surface leading to an increase in pressure drop - difference between the feed and concentrate

pressure during water flow through one or more RO membranes - across the membrane and a decrease in the flux.

Scaling occurs when the solubility of the salts is exceeded during filtration process and they crystalize and precipitate forming a thin layer on the membrane surface that leads to a decrease in salt passage and flux through the membrane. Extreme scaling will also increase the pressure drop across membrane elements (Escajadillo, 2016; Jong, 2014). The accumulation of solutes on the surface of the membrane results in a higher concentration than the one observed in the bulk which represents the concentration polarization. When the difference between the rate of adsorption and diffusion of those solutes to the membrane and to the feed is considerable, the concentration polarization aggravates resulting in increase of the osmotic pressure and therefore of the pressure needed (Tang *et al*., 2010).

Bio-fouling can be described by the deposition and growth of microbial cells and EPS (extracellular polymeric substances) and is one of the predominant problems in RO membrane process and causes a decline of the water permeability and an increase of the differential pressure between feed and concentrate that leads to the use of higher operating pressure of RO systems and more frequently cleaning.

Organic fouling consists in natural organic matter (NOM) which are a complex mixture of polysaccharides, humic and flulvic acids, among others that adsorb on the membrane surface by physicochemical bonds. This type of fouling also increase pressure drop and decrease flux (Jong, 2014; Pype, 2013; Zeng, 2012).

In account with all the fouling's consequences mentioned above, an appropriate previous treatment is necessary to improve the RO system performance and therefore produce better water quality while minimizing chemical cleaning frequency (Asano *et al*. 2007; Jamaly *et al*. 2014). It's important to consider the water source in order to choose the more suitable pre-treatment. Besides the chemical treatment, physical treatment is also employed. Physical treatment processes are designed to remove particulate matter that can cause problems such as fouling of membranes and transport of bacteria and viruses whilst chemical treatment processes are designed to prevent scaling phenomena and biological attack on the membranes (Eisenberg & Middlebrooks, 1986).

The common pre-treatment processes include coagulation/flocculation, media filtration, activated carbon, disinfection and membrane filtration (microfiltration/ultrafiltration). Although conventional processes such as coagulation/flocculation and media filtration can achieve up to 1-2 log removal values for pathogens, maintaining optimised conditions is difficult and the efficiency of the processes is excessively unpredictable. It has been reported that microfiltration and ultrafiltration are theoretically the best pre-treatment upstream reverse osmosis systems removing from the feed water most of the potential elements that could lead to fouling and scaling (particles, turbidity, bacteria, and others) achieving log removal values in the range of 4-7 (Bennet, 2008; Davis, 2011; Zeng, 2012).

The resulting permeate of reverse osmosis membranes has acid properties and therefore a corrosive nature. It is usually required a post-treatment to adjust pH and water stability with the addition of chemicals and, this way, prevent the corrosion of equipment and distribution system. (Asano *et al*., 2007; Davis, 2011).

Normally, a reverse osmosis system is composed by different pressure vessels placed in parallel forming a stage. Reverse osmosis systems usually have more than one stage in which the concentrate of the previous stages is the feed to the next ones (Figure 2.3). Figure 2.4 shows some of the constituents of a pressure vessel that suffer from stress during the process and can be compromised leading to process failures such as O-ring damages. These damages allow the leakage of unfiltered feed water to the permeate side thus contaminating it (Pype *et al*., 2012).

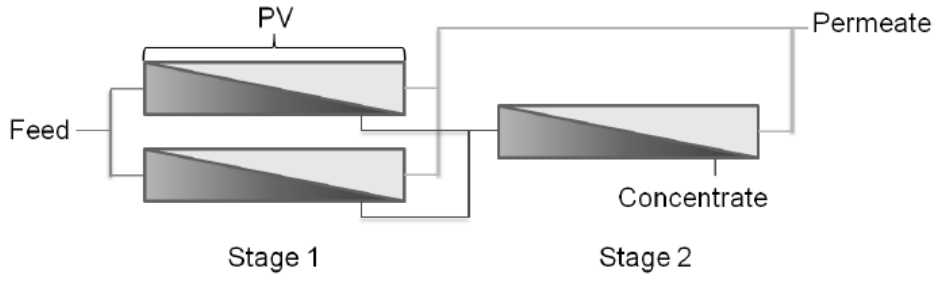

Figure 2.3 – Example of RO stages (Pype, 2013)

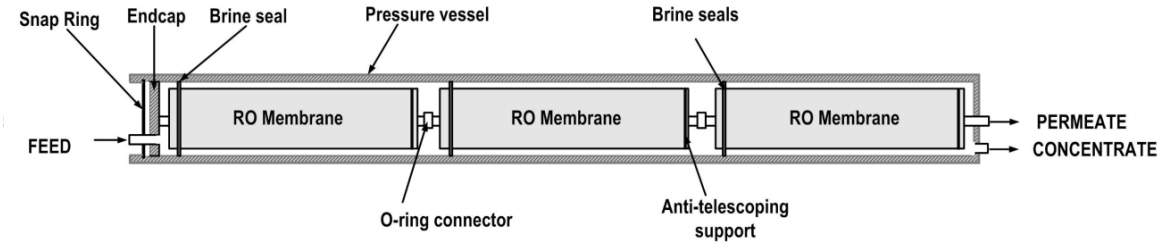

Figure 2.4 – Pressure vessel (Lenntech)

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2.2 Water and wastewater pathogens

Water reuse raises important questions regarding the levels of treatment, monitoring and testing needed to ensure public health. Wastewater contains chemical contaminants such as inorganic chemicals, natural organic matter, chemicals created by industrial, commercial, and other human activities (National Research Council, 1998). Chemical contaminants such as fuel additives, endocrine disruptors, pesticides are found in both surface and groundwater and represent a huge threat to ecosystems (Metcalf & Eddy, 2002). Furthermore, microbial contaminants are also present in wastewater and require great deal of attention. In reuse systems, bacteria, viruses and protozoan parasites are present. Those associated to waterborne diseases are primarily enteric pathogens, leaving a fecal-oral route of infection either for humans or animals and they can survive in water (National Research Council, 1998; WHO, 2011). Table 2.1 presents some of the infectious waterborne pathogens. It is also important to highlight the long persistence of viruses in water supplies and their resistance to chlorine as well as their significant levels of infectivity.

Table 2.1 – Waterborne pathogens (WHO, 2011)

Pathogen	Health significance	Persistance in water supplies	Resistance to chlorine	Relative infectivity	Important animal source
Bacteria					
Burkholderia pseudomallel	High	May multiply	Low	Low	No
Campylobacter jejuni, C.coli	High	Moderate	Low	Moderate	Yes
Escherichia coli - Pathogenic	High	Moderate	Low	Low	Yes
E.coli - Enterohaemorrhagic	High	Moderate	Low	High	Yes
Francisella tularensis	High	Long	Moderate	High	Yes
Legionella spp.	High	May multiply	Low	Moderate	No
Leptospira	High	Long	Low	High	Yes
Mycobacteria (non-tuberculous)	Low	May multiply	High	Low	No
Salmonella typhi	High	Moderate	Low	Low	No
Other Salmonellae	High	May multiply	Low	Low	Yes
Shigella spp.	High	Short	Low	High	No
Vibrio cholerae	High	Short to long	Low	Low	No
Viruses					
Adenoviruses	Moderate	Long	Moderate	High	No
Astroviruses	Moderate	Long	Moderate	High	No
Enteroviruses	High	Long	Moderate	High	No
Hepatitis A virus	High	Long	Moderate	High	No
Hepatitis E virus	High	Long	Moderate	High	Potential
Noroviruses	High	Long	Moderate	High	Potential
Rotaviruses	High	Long	Moderate	High	No
Sapoviruses	High	Long	Moderate	High	Potential
Protozoa					
Acanthamoeba spp.	High	May multiply	High	High	No
Cryptosporidium hominis/parvum	High	Long	High	High	Yes
Cyclospora cayetanensis	High	Long	High	High	No
Entamoeba histolytica	High	Moderate	High	High	No
Giardia intestinalis	High	Moderate	High	High	Yes
Naegleria fowleri	High	May multiply	High	Moderate	No

Viruses are ultramicroscopic agents with a size that ranges from 10 to 300 nm in cross-section and they are the most challenging pathogens to remove in reverse osmosis systems. These pathogens contain their genetic material that could be DNA or RNA and a protein capsid that provides protection and recognizes the correct host cell to be attacked. When the genetic material of the virus is introduced into a host cell, the genetic material takes control of the reproductive mechanism of the cell and causes the replication of more viruses (Gerardi & Zimmerman, 2005).

Enteric viruses include more than 140 types and those associated with waterborne illness include noroviruses, hepatitis A, hepatitis B, rotaviruses and enteroviruses. When it comes to water reuse, the transmission of infectious diseases, mostly by enteric viruses, is the most prominent issue due to their small size and their consequences even from a low dose exposure. For instance, enteroviruses including polioviruses and coxsackievirus A and B cause diseases such as paralysis, meningitis, fever, respiratory disease, myocarditis and heart anomalies. Rotaviruses and adenoviruses cause gastroenteritis and conjunctivitis and hepatoviruses cause hepatitis (Bosch, 1998; Gerardi & Zimmerman, 2005).

Studies have demonstrated the presence of enteroviruses in wastewater, in effluents from sewage treatment plants, in contaminated rivers and lakes and in treated drinking waters from developing as well as developed countries (Rao & Melnick, 1986). Therefore, it must be highlighted the importance to securely remove these viruses and to have a functional monitoring system in order to detect any loss of membrane integrity.

2.3 Monitoring and testing the integrity of RO membranes on virus removal

Membrane integrity loss happens due to physical and chemical damage. The integrity of the membrane should be periodically and continuously monitored in order to detect and repair the membrane damage, therefore, achieving the desired membrane performance. Theoretically, an uncompromised RO membrane has the capacity to completely reject all pathogens, however, the frequent cleaning to remove the accumulated particulates can physically damage the membrane function layer and allow constituents to pass, decreasing the removal efficiency (Antony *et al.*, 2012; Wu *et al*., 2017).

To date, there is no universal recycled water policy around the world so each agency of each country should set regulatory guidelines. Minimum log removal values for enteric viruses regarding wastewater reuse were found in the literature for California and Australia. The minimum log removal value established for enteric viruses in Australia is 9.5 LRV for potable purposes whereas the guidelines in California require a 12 log removal value for groundwater recharge (Australian Guidelines, 2008; Robillot *et al*., 2016). In the Netherlands, there is no set minimum LRV however microbiological risk analysis are required to prove that the infection risk due to drinking water consumption is less than 1/10000 people per year (Medema *et al*, 2006).

When it comes to drinking water, treatment systems include prominent levels of treatment. For instance, it features membrane filtration, reverse osmosis and advanced oxidation, providing log reduction that meets the minimum requirements. Thus, if there's an appropriate management of the processes, residual risk will be acceptable (Australian Guidelines, 2008).

The common ways to disinfect water are with free chlorine, combined chlorine, ozone, chlorine dioxide, and ultraviolet irradiation (Davis, 2011). However, enteric viruses and adenoviruses are more resistant to disinfectants than enteric bacteria (Gerardi & Zimmerman, 2005). For instance, adenovirus is one of the most resistant viruses and needs high UV doses in order to achieve the required LRV (USEPA, 2012). In addition, the formation of sub products associated with these conventional methods can be hazardous to public health, thus the need to develop new alternative control methods with minimal health and environmental impacts. Furthermore, the performance of these disinfection processes can be altered easily depending on the organism and depending on the contact time (Pype *et al*., 2016).

High pressure membranes use three distinct types of removal mechanisms: size exclusion, charge repulsion and adsorption/diffusion. The main removal mechanism for viruses is size exclusion, and charge repulsion improves their removal (Antony *et al*., 2012; Robillot *et al*., 2016) however regarding reverse osmosis membranes only few studies have been reported. Overall, for a membrane integrity method to be considered as potential especially on virus validation removal it should meet the following desired criteria: achieve significant log removal values (at least 4) (Jacangelo & Gray, 2015) , the resolution of the virus surrogate/indicator to be as similar to enteric viruses' characteristics as possible, the method must not be expensive nor time-consuming. Furthermore, it must meet the criteria for being applicable in drinking water industries.

2.3.1 Integrity monitoring tests and methods

Direct integrity testing refers to physical integrity of the membrane and indirect integrity testing focus on monitoring the quality of the permeate. The ultimate seeking goal is to correctly operate a reverse osmosis system provided with a real-time monitoring to prove the effectiveness of the membranes (Kumar *et al*., 2007; Pype *et al*., 2016).

Online (Real-time methods)

The most common indirect monitoring methods are online conductivity, online TOC (total organic carbon) and turbidity. To assess the membrane integrity, conductivity is widely used to monitor the total dissolved solids and indicate loss of integrity on the membrane that might be caused by a damaged O-ring, glue line leak, or others. Some authors point out that conductivity tests should be performed on each pressure vessel periodically (Jacangelo & Gray, 2015; Lozier & Mariñas, 2003). Total organic carbon is also an online monitoring method that is effective at detecting gross membrane failure furthermore it has been reported that TOC analysers provide more sensitivity than conductivity. Wilbert & Linton (2000) and Pype *et al.* (2012) conducted different researches in which online TOC monitoring proved a better performance than online conductivity.

Turbidity is a water quality parameter that can be also a real-time monitoring indicator, however low log removal values were reported in the range of 0,3-1,38. Additionally, sensitivity is low and it is not applicable to virus size (Jacangelo & Gray, 2015; Lozier *et al*., 2003) therefore, it does not apply to be a good method for validating virus removal.

Similar log removal values were reported in different studies regarding conductivity. The values reported by Jacangelo & Gray (2015) showed a maximum LRV of 1,8, the values reported by Pype *et al* (2016) showed an estimative of 1,5 LRV and Kruithof *et al.* (2001) reported 2 LRV for conductivity monitoring. On a research conducted by Adham *et al.* (1998), online TOC monitoring was reported to achieve 2,5-3 LRV contrasting with online conductivity monitoring which only achieved 2 LRV.

Although TOC compounds are smaller than viruses and, for this reason, could be more conservative than virus measurement (Robillot *et al.*, 2016), to date, there is no correlation between TOC removal and virus removal. TOC and conductivity indicators are considered to be inaccurate to assess virus removal since they depend on the feed water quality and on membrane operational conditions (Adham *et al*., 1998; Kumar *et al*., 2007).

Offline

Pressure hold and vacuum decay are direct testing methods. The vacuum decay test is the most common in RO process and is applicable to detect leaks that might be associated with damage in the membrane, glue line failures or leaks in O-ring seals (Lozier *et al*., 2003). These methods are mainly employed by membrane manufacturers before membrane installation. Both pressure hold and vacuum decay are sensitive tests but they depend on frequency, they are performed offline and elements must be removed (Portillo, 2015). Sulphate monitoring is an indirect test that has proved to be more sensitive than conductivity, however, some authors claim that online analytical techniques are not available at this moment, reason why some authors consider sulphate monitoring as an offline method (Pype *et al*., 2016). Nevertheless, this ion exhibits the advantage of being naturally present in the feed water (Robillot *et al*., 2016) and was reported by Pressdee *et al.* (2006) to o achieve by online monitoring a value of 3 log removals in a water facility in The Netherlands, contrarily to the previous authors. On a research conducted by Kruithof *et al.* (2001), sulphate monitoring was reported as a promising technique with the capacity of achieving up to 3 LRV, however, sulphate monitoring corresponds to an indirect integrity method meaning it depends on the feed quality and lacks sensitivity for virus removal validation (Kruithof *et al*., 2001; Portillo, 2015; Pressdee *et al*., 2006).

2.3.1.1 Challenge testing

Challenge tests can be considered as indirect methods because measurements are performed in the feed and in the permeate to assess the passage of the components through the membrane. Generally, challenge testing refers to spiking methods in which microbial or non-microbial surrogates are introduced in higher than normal concentrations in the feed water to be possible to detect in the permeate (Kumar *et al*., 2007; USEPA, 2012). Surrogate challenge tests are considered as more suitable to assess virus removal than the conventional indirect methods improving sensitivity of membrane integrity monitoring thus enhancing the probability of meeting the regulatory requirements. To be a good virus surrogate, the candidate must have similar size to the virus size and be representative of the pathogen retention characteristics as well as be easily detectable and not expensive and not harmful to humans (Guo *et al*., 2010; Portillo, 2015).

Recently, the measurement of naturally occurring viruses in water sources has been studied with more focus. This possibility brings advantages such as not being needed to spike compounds in the water (Antony *et al*., 2012; Medema *et al.*, 2006).

Table 2.2 presents the reported log removal values and major characteristics of several current challenging tests for monitoring reverse osmosis membranes.

Table 2.2 - Characteristics of current challenge tests for monitoring reverse osmosis membranes on virus removal.

Challenge Test	References	LRV	Observations
Offline			
MS2 phage	(Lozier *et al*., 2003) (Jacangelo & Gray, 2015) (Australian Guidelines 2008) (Mi *et al*., 2004)	>7 >6 >6.2 5.4-6.4	Microbial surrogate; Model of enteric viruses; Good performance
Rhodamine WT	(Lozier *et al*., 2003) (Jacangelo & Gray, 2015) (Lozier & Mariñas, 2003) (Australian Guidelines 2008)	4 >4 4 ≤4	Non-microbial surrogate; Applicable to virus size; Operating conditions dependant; Non-expensive
Online			
Fluorescent Microspheres	(Lozier *et al*., 2003) (Jacangelo & Gray, 2015) (Lozier & Mariñas, 2003)	4-4.5 >5 4	Applicable to virus size
TRASAR®	(Australian Guidelines 2008) (Portillo, 2015)	>4 2-6*	Fluorescent compound coupled with anti-scalant; *2 if injected with antiscalant; 6* if injected as neat chemical
Particle	(Huang *et al*., 2015) (Adham *et al*., 1998)	>3 (FCM) 1-2	Flow cytometry (FCM) can directly quantify virus particles and has high sensitivity
Pulsed-marker membrane integrity monitoring	(Frenkel & Cohen, 2014) (Surawanvijit *et al*., 2015)	3.8-4.40 >4	Uranine
DOM	(Pype *et al.*, 2012)	3	Naturally present
COD	(Pype *et al.*, 2016)	<3	Naturally present
Nanoparticles	(Jacangelo & Gray, 2015)	5-7*	*5 pilot scale; 7 bench scale
Virus like particles	(Pype *et al*., 2016)	>4*	*Potentially; same behaviour as authentic viruses

It is consistent in the literature that it is possible to obtain significant log removal values by using MS2 bacteriophages. Although the current technology is not sufficiently developed to perform this technique in real-time, it's still reported as the most used and as the best performance indicator achieving up to 7 log removal values according to the literature. Lozier *et al.* (2003) conducted a research in which one of the objectives was to determine the integrity of RO membrane systems regarding the passage of viruses using MS2 bacteriophages. This research achieved a minimum of 6 log removal of MS2 bacteriophage in the presence of intact membranes in flat sheet and element form, reason why it was considered as a good virus removal indicator. Also, a minimum of 6 log removal for MS2 phage was achieved on a research which compared the removal of several surrogates such as nanoparticles and Rhodamine WT. Further results demonstrated the highest removal for MS2 phage followed by nanoparticles and RWT. However, the higher error of LRV achieved was relating to MS2 bacteriophages (Jacangelo & Gray 2015). To assess the virus rejection efficiency, Australian Guidelines (2008) conducted a controlled lab experiment achieving a minimum of 6,2 LRV for MS2 bacteriophage as observable in table 2.2.

In contrast to every other report, a lower log removal value was found in the literature. Mi *et al*. (2004) reported a rejection of viruses using MS2 that ranged from 5,4 to 6,4 logs on an experiment using spiral wound membranes.

The use of MS2 bacteriophages as a biological surrogate for virus removal will be thoroughly discussed on section 2.3.2.

Lozier & Marinas (2003) stated that Rhodamine WT is a non-microbial surrogate that has proven to be applicable as a virus surrogate to assess the integrity of full-scale high-pressure membrane systems for virus removal representing an alternative to MS2 bacteriophage. Since the removal mechanisms in RO membranes consists on size-exclusion and charge repulsion, this dye has significant potential due to its high molecular weight and negative charge (Pype *et al.*, 2016). Although very promising by being non-expensive, it is an offline technique. Also, Australian Guidelines (2008) declared that any changes in operating conditions can compromise the performance of its rejection.

The maximum LRV reported in the literature for Rhodamine WT was 4. However, a new fluorescent compound attached with an anti-scalant denominated TRASAR® has becoming interesting, reporting between 2 to 6 log removal values. TRASAR® achieved 6 LRV when dosed as a neat chemical. This new compound has the capability to be an online monitoring technique that detects ultra-low concentrations. The drawbacks of this compound is that it must be spiked into the feed water and its rejection in presence of compromised membranes are still unknown (Portillo, 2015; Pype *et al*., 2016).

As Table 2.2 shows, the literature reported 4 to 5 LRV regarding fluorescent microspheres. According to Lozier *et al.* (2003), these microspheres have virus sizes and their presence is measured in the feed water and in the permeate using a flow cytometer or a spectrofluorometer. This method has the advantage of potentially being monitored in real-time. However, this method lacks sensitivity (Jacangelo & Gray, 2015) and it's still too expensive and complex to analyse.

Huang *et al.* (2015) conducted a study that reported at least 3 LRV achievable by FCM (Flow cytometry). FCM is an accurate and fast method for analysing biological particles in suspension, but for virus detection is still a challenge due to their small sizes. Particle enumeration techniques have the advantage of particles being naturally present in the water and the possibility to be monitored online. On a research that used a more conventional online particle counting, the log removals achieved were no more than 1-2 (Adham *et al*., 1998).

Recently, Pulsed-marker membrane integrity monitoring (PM-MIM) is becoming an interesting technique. Based on the pulsed spiking of a high concentration of fluorescent detection in the permeate, this online method was reported to achieve 3,8-4,40 LRV using uranine (Frenkel & Cohen, 2014) and Surawanvijit *et al.* (2015) demonstrated greater than 4 LRV also via uranine, both results are higher than the usual LRV using RWT as presented above (up to 4 LRV).

Naturally present indicators in the feed water, such as DOM (dissolved organic matter) and COD (chemical oxygen demand) are considered as online techniques. DOM is detected by fluorescence and both can achieve up to 3 LRV However, it's not clear the suitability of DOM as a virus surrogate and operational conditions can also have an impact on DOM and COD rejection (Pype *et al*., 2016, 2012).

The monitoring of membrane integrity using nanoparticles became more popular over the last year due to their surface's possibility to be modified to have equivalent properties to viruses. It is also possible for them to be added fluorescent dyes and improve even more the method's potential to verify virus removal. Nanoparticles have a unique optical/light scattering properties that allowed a new technique to be developed. Jacangelo & Gray (2015) reported a technique based on light scattering that achieved more than 7 log removal values in bench scale and around 5 LRV at a pilot scale. In addition, a comparison between the removal of MS2, nanoparticles and RWT by different RO elements showed the lowest error for nanoparticle detection (Jacangelo & Gray, 2015; Takimoto *et al*., 2010). On the other hand, these indicators can aggregate and, therefore, foul the membrane. Moreover, these techniques currently have high costs and the consequences for human health are not completely clear (Pype *et al*., 2016).

The use of virus-like particles in membrane integrity challenge tests is promising because they're composed of viral structural proteins without the genetic material whose organization and characteristics resemble to authentic viruses (Roldão *et al.*, 2017). Regarding their performance on virus removal the literature has reported to achieve up to 4, nevertheless, the detection method ELISA (enzyme-linked immunosorbent assay) still has a high limit of detection (LOD) meaning that other particle counting methods were used (Pype *et al*., 2016).

2.3.2 MS2 bacteriophage as a virus surrogate

Viruses that infect bacteria are classified as bacteriophages. Bacteriophages can be isolated from faeces and wastewater and are also very common in soil. There are bacteriophages that are morphologically like enteroviruses, fact that plays an important role in using them as virus surrogate to monitor the performance of reverse osmosis membranes on virus removal. There are a few morphological groups of bacteriophages: filamentous phages, isosahedral phages without tails, phages with tails, and even several phages with a lipid containing envelope or contain lipids in the particle shell. This makes bacteriophages the largest viral group in nature (Kurtboke, 2012; Ogorzaly & Gantzer, 2006; Pype *et al*., 2016).

The replication cycle of bacteriophages is represented on Figure 2.5.

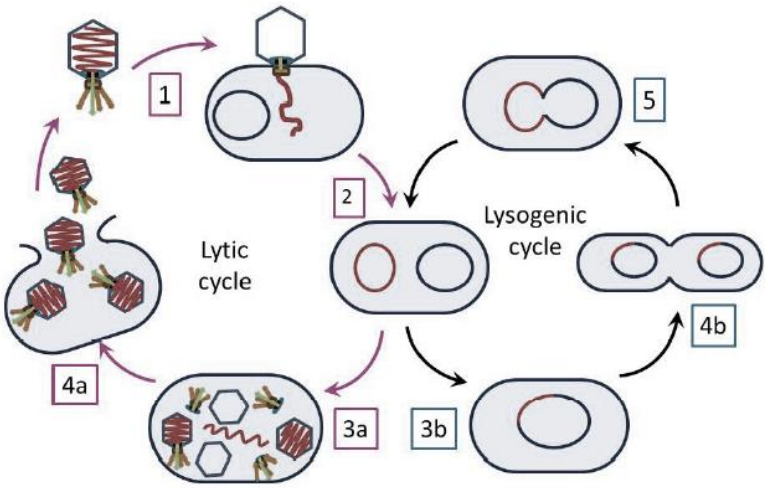

Figure 2.5 – Bacteriophages life cycle (Kurtboke, 2012)

The lytic cycle represented by 1, 2, 3a and 4a consists in the infection of the bacteria by the bacteriophage followed by the replication of numerous bacteriophages and finally the death of their infected host cells which results in the lyse of the cell and the release of new phages to the extracellular space. In the lysogenic cycle, the bacteriophages integrate the bacteria and insert its genetic material into the bacterial genome. Although this cycle can be stable for an unknown amount of time, the genetic material of the bacteriophage will be replicated thus forming fully assembled bacteriophages (2, 3b, 4b, 5) (Clokie *et al.*, 2011).

MS2 bacteriophage belongs to a group that have a simple capsid containing single-stranded RNA as the genome – F-specific RNA bacteriophages. This group infects bacteria that possesses the F-pili or sex-pili produced by male bacterial cells which possess the F plasmid. The F plasmid is transferable to a wide range of Gram-negative bacteria such as *E.coli* and *Salmonella typhimurium* (ISO 10705-1, 1995).

F-RNA bacteriophages appear to be well suited as a model organism to monitoring purposes due to their similarity regarding the size, shape, morphology and physiochesmistry to many pathogenic human waterborne viruses (Kurtboke, 2012). For instance, F-RNA bacteriophages have sizes of 25 nm and isoeletric point of 3,9 (i.e., the pH at which the electrophoretic mobility of the particle is zero) and, similarly, human enterovirus have sizes of 22-30 nm and an isoelectric point of 4-6,4. This means that they are not expected to be adsorbed by negatively charged membranes. Haveelar *et al.* (1993) conducted a research that confirmed the effectiveness of RNA phages as model organisms for human viruses in a wide range of environments and treatment processes. In all the different cases, F-specific RNA bacteriophages were the best predictors of virus concentrations and their behaviour in individual water treatment processes was strongly similar to human enteric viruses. Furthermore, F-RNA bacteriophages MS2 are morphologically similar to enteroviruses and are frequently used to study viral resistance to environmental stressors, disinfection and other treatment processes (Havelaar *et al*., 1993).

Apart from the main advantages already mentioned related to their morphological similarity to enteric viruses and survival characteristics in aquatic environments, this surrogate can be cultured in high quantity at high concentrations, it's non-pathogenic to humans nor it's significantly influenced by operating conditions and possesses slightly hydrophobicity properties thus decreasing the possibility of adsorption by the membrane. However, there are some aspects that remain as drawbacks. Its quantification in the laboratory is time consuming and it is impractical to implement this test on a full-scale operational plant due to inflated costs and effort to culture and to plate the necessary amount. Considerable expertise in microbiology is also required to avoid phenomena such as contamination and particle aggregation (Antony *et al*., 2012; Antony & Leslie, 2014; Robillot *et al*., 2016).

Regarding high pressure membranes, bacteriophages are mainly rejected by size exclusion, adsorption and electrostatic repulsion between the membrane surface and the bacteriophages. Notwithstanding, when the dimensions of the bacteriophages are smaller than the membrane pores the rejection will depend on both bacteriophages and membrane surface properties such as surface charge or hydrophobicity and bacteriophage shape/aggregation phenomena (Ferrer *et al*., 2013).

2.3.3 Naturally present viruses as removal indicators

Although using MS2 bacteriophages to validate virus removal in reverse osmosis systems is currently reported as the best challenge test, it still has major drawbacks. The ideal method to monitor the integrity of reverse osmosis membranes would be to use naturally occurring viruses in water to successfully verify virus removal discarding the necessity to introduce components in the feed water. Additionally, the capability of a method that already meet the previously mentioned features to provide real-time integrity monitoring information would culminate in the ideal scenario (Antony *et al*., 2012; Huang *et al*., 2015).

The advancing technology allowed for some virus detection techniques to be improved especially regarding their limit of detection. It has been reported by researchers that the quantitative polymerase chain-reaction (qPCR) technique is a good alternative to the conventional plaque-assay method (Langlet *et al*., 2009; Ogorzaly & Gantzer, 2006).

Recently, it has been proved that it is possible to detect and quantify naturally occurring viruses by using the qPCR technique. The qPCR virus quantification method combined with the NGS method - next generation sequencing - is promising and can be a huge advance in membrane integrity monitoring if a certain virus could be authenticated as virus removal indicator. The next generation sequencing (NGS) method allows to determine the exact sequence of the nucleotides of a DNA/RNA molecule. In a report where the goal was to identify possible virus indicators for verifying virus removal in purification processes, NGS was used to search for viruses in surface water that would meet requirements such as being present in high concentration, being detectable in analysed samples and being always present in the surface water without the influence of location or seasonal changes (Hornstra, 2016).

Shirasaki *et al*. (2017) evaluated the efficacy of membrane filtration processes to remove human enteric viruses using a natural occurring virus in surface water - a plant virus that might be a potential virus removal indicator. Despite this research was conducted with low pressure membranes, in accordance with these authors, pepper mild mottle virus (PMMoV) infects pepper species and can be found in environmental waters including drinking water sources at higher concentrations than human enteric viruses. The experiments revealed removal ratios strongly correlated to adenoviruses, CV, hepatitis A and MNV thus inducing that PMMoV is a potential indicator for human enteric viruses in ultrafiltration membranes (Shirasaki *et al.*, 2017).

This new challenge test represents enormous potential for assessing the integrity of membranes due to the simplicity of collecting samples without the need nor the expertise to add components to the feed water. Moreover, in case of drinking water production this method's advantages clearly overcome the others. Essentially, if a certain virus is identified by NGS and could have similar behaviour as enteric viruses it would be possible to use qPCR to amplify and quantify and therefore calculate its concentration in the feed and in the permeate and the associated log removal values (Hornstra, 2016).

In terms of costs, Robin et al. (2016) has reported that next generation sequencing has potential to be cost-effective. The qPCR method is already mentioned in the literature as having numerous advantages over the plaque-assay technique, also regarding the cost savings (Edelman & Barletta, 2003; Perkins & Webb, 2005).

2.3.4 Integrity monitoring on virus removal of compromised reverse osmosis membranes

Apart from assessing virus removal on intact reverse osmosis membranes, it's important to understand what happens when integrity loss is present in spiral would membranes. The most common integrity problems are caused by damaged O-rings, glue line leaks, piping leaks, chemical damage and damaged caused by fouling. These membrane impairments can occur during manufacturing, transport, installation and operation. For instance, when O-rings are compromised, pathogens can possibly pass into the feed stream due to deprivation of proper sealing (Jacangelo & Gray, 2015; Johnson & MacCormick, 2003).

A study was held on a pilot scale to assess the impact of distinct types of membrane damage regarding the removal of MS2 bacteriophage, conductivity, Rhodamine WT and TRASAR. It was deducted from the results that a damaged O-ring represents the type of impairment that more significantly compromises the indicators' rejection (Jacangelo & Gray, 2015). The results of this experiment are depicted in Figure 2.6.

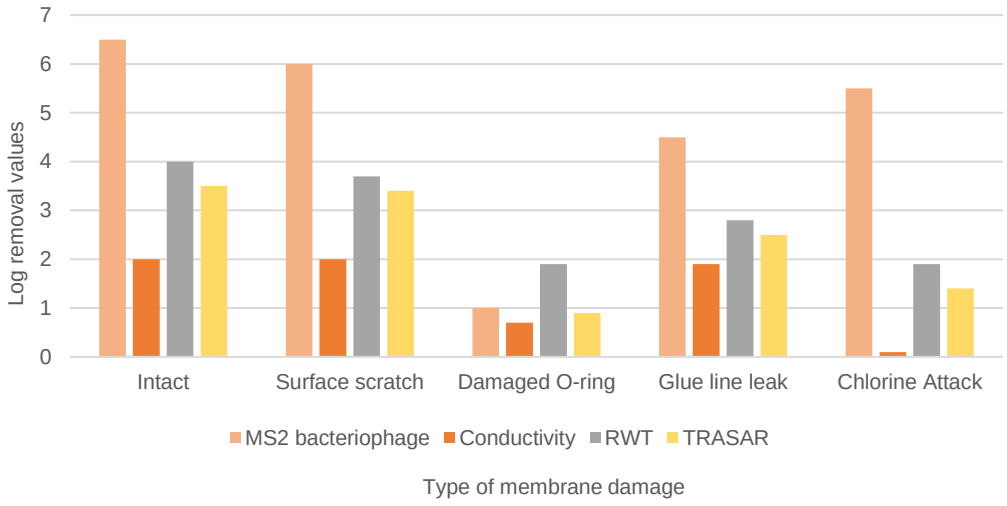

Figure 2.6 –Membrane damage type influence on achieved log removal values (Jacangelo & Gray, 2015)

In contrast, Mi *et al.* (2004) observed a narrow decrease on MS2 phage removal when in presence of a cracked O-ring located in the connection of the permeate tube to the element vessel. The rejection of MS2 still achieved above 6 log removals. The authors suggest these considerable LRV were achieved because the cracked O-ring sealed inside its groove by the compression exerted by the permeate tube resulting in a non-significant passage of the surrogate to the feed stream (Mi *et al.*, 2004). This theory can be corroborated by another study using MS2, microspheres and RWT which also concluded that a cracked O-ring is not sufficient to cause loss of integrity. However, it was experimented that when small fractions of the O-ring were removed, the log removal values decreased proportionally with the extension of the removed fraction (Adham *et al.*, 1998; Lozier & Mariñas, 2003) .

The literature reported another type of damage - small pinholes. Some authors affirm that small pinholes do not substantially influence the removal values as they are filled up by scaling and bio-fouling phenomena (Wilbert & Linton 2000; Antony *et al*. 2012). However, Kitis *et al.* proved a deterioration of the RO system performance when a 400µm pinhole was impaired on the membrane's surface after removing the tape wrap and the membranes leaves and associated feed spacers were unrolled.

Pype *et al.* (2015) determined the influence of aged membranes on virus removal and integrity testing. MS2 bacteriophages were used as a virus surrogate whilst Rhodamine WT, sulphate, DOM and salt were used as indicators. The results in the presence of aged membranes demonstrated a reduction of the membranes performance as lower log removal values were achieved. Nevertheless, this decrease was not significant since the MS2 bacteriophage was still able to remove above 4 LRV and the non-microbial indicators were also able to achieve up to 3 LRV. These amounts still provide considerable validation for virus removal and are in accordance, for instance, with the minimum value set by the Australian guidelines regarding single barriers – 4 log removal values (Pype *et al*., 2015).

The findings associated with compromised membranes and RO system components enhance the importance of having a trustful and functional membrane integrity monitoring method (Kitis *et al*., 2003).

3. Objectives

The main goal of this dissertation is to understand the potential of using naturally present viruses in surface water to monitor the integrity of reverse osmosis membranes on virus removal by comparing their performance to the performance of using MS2 bacteriophages. Furthermore, this research aims to assess the impact of different types of membrane damage on virus removal by reverse osmosis membranes.

The intended outcome of this thesis is to demonstrate the suitability of using naturally present viruses in surface water to securely monitor the integrity of reverse osmosis membranes through a simple and spiking-free method.

4. Experimental plan

To achieve the goals in this research, the following plan was carried out.

Using a reverse osmosis pilot unit provided by Oasen in Kamerik, samples from the feed water and from the permeate were collected to assess the potential of using naturally present viruses to validate virus removal. A challenge test using MS2 bacteriophages was performed and samples were collected also from the feed water and the permeate after spiking the MS2 stock solution into the feed water tank. The MS2 challenge test will provide a mean of comparison for the performance of the naturally present viruses in surface water being a technique that is already considered as one of the best approaches to validate virus removal on reverse osmosis membranes. Control samples were also collected from the feed and from the permeate. Further details will be attained in the following chapter.
The samples corresponding to the naturally present virus were transported to KWR Watercycle Institute Research to be analysed by the qPCR method whilst the ones corresponding to MS2 were analysed in TU Delft (Figure 4.1).
Experiments were performed in the presence of compromised membranes that followed the same procedure.

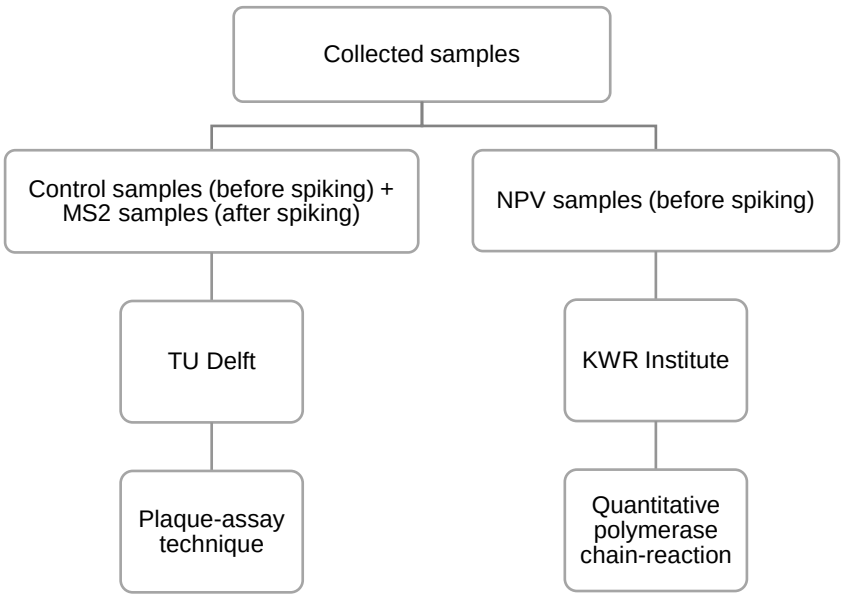

Figure 4.1 – Destination of the collected samples from the RO unit (feed and permeate)

5. Materials and methods

5.1 Reverse osmosis unit

Oasen is a Dutch water company that supplies reliable and fresh drinking water to 750 000 people and 7500 companies in the eastern part of South Holland. The company contains seven drinking water purification plants, nine pump stations and delivers around 48 billion litres of drinking water per year and its main priority is public health. The treatment plant “De Hooge Boom” located in Kamerik, Oasen has more than ten pilot installations using full-flow reverse osmosis membranes followed by remineralization. This is the facility where the reverse osmosis unit used for the experiments is located. The set-up used for this research was a Single Element Unit (SEU8”) (Figure 5.1 and 5.2).

Figure 5.1 – Reverse osmosis unit

Figure 5.2 – Pressure vessels of the RO unit

The unit features a tank with a mixer that contains the feed water. Immediately before this tank one cooler is attached to keep the water at a certain temperature. Once the unit starts, the water flow through the system to the first pump before entering the cartridge filter (5µm pore) where larger particles will be retained. Afterwards, the water heads to the second pump which has larger size than the first one and is immediately placed before two pressure vessels. The unit features two parallel pressure vessels (8") that accommodate single standard 8" reverse osmosis elements and both are used independently.

Throughout the unit, several valves are placed and they can be operated automatically or manually. Control devices such as flow rate meters, pressure meters and conductivity meters are placed throughout the installation, including a screen that provides real-time information about the unit's operation and status.

5.1.1 Operation mode

The circulation mode reassembles a normal reverse osmosis filtration process where the feed water, that comes from the tank, is pumped through the membrane producing permeate and concentrate. This unit was operated as a closed system, which means that both permeate and concentrate returned to the tank. Attached to the tank, the cooler kept the water's temperature in the tank at a constant value (around 12ºC) and simultaneously caused turbulence in the tank thus contributing to proper mixing.

The system recovery is 15% that equals to the module recovery and the flow rate is 6,8 m^3/h.

The diagram that represents the circulation mode and configuration of the installation is represented in Appendix I.

5.1.2 Experimental and sampling procedure

The tank was filled with drinking water and the membrane was flushed for 1-2 days at a flow rate of 6,8 m^3/h and recovery of 15% every time before any experiment took place. After this period, the tank was emptied and refilled with the test water - surface water from the Grecht channel located in Woerden.

The sampling procedure was based on this thesis's objective, therefore four samples regarding NPV were collected 30 and 60 minutes after stabilization of the setup at the same time as bacteriophages control samples, from the feed and permeate. Afterwards, bacteriophages with a concentration of 2 x 10^7 PFU/ml were spiked into the feed water tank and the mixing took place for 15 minutes. Four samples for the MS2 assay were taken 30 and 60 minutes from the feed and from the permeate after stabilization of the unit. Based on the set-up flow rate and the capacity of the tank, it is estimated that in 30 minutes the tank was "refreshed" three times thus being an appropriate time to start the experiment. It is important to mention that due to technical problems with the feed water sampling point, the sampling point used was the one after the cartridge filter. Therefore, a sample from the feed tank was collected to assess the influence of the cartridge filter on the experiments.
Blank samples were collected prior to each experiment to confirm the proper disinfection of the system.
Control samples were collected to assess the influence of naturally present bacteriophages in the surface water on the results.
Finally, the system was disinfected with sodium hypochlorite for a minimum of four hours to guarantee that no spiked bacteriophages were left in the system. After repeating this process with a damaged membrane, the membrane was disposed and swapped by a new one for the next experiment.

Table 5.1 presents the overview of the collected samples.

Table 5.1 – Overview of the collected samples

Samples	Time (min)
Blank	0
Naturally present viruses + Controls	35
	45
	65
	75
Tank	0
MS2 bacteriophages	35
	45
	65
	75

The unit's sampling points connected to taps where the samples were collected are shown in Figure 5.3.

Figure 5.3 – Sampling taps

The sampling procedure was carried out carefully to avoid contamination. Sterile gloves and sterile sample flasks were used and the taps were sterilized by using a burner before taking the samples. All the sample flasks were labelled with the correspondent identification and time and immediately placed in an ice cooling box. The deviation of time between the samples were ±1,5 min. The samples corresponding to MS2 bacteriophages were transported to the Delft University of Technology's laboratory whilst the ones corresponding to naturally present viruses were delivered KWR Institute's laboratory to be analysed by their specialists. Cross contamination was avoided placing the influent and effluent samples in separated ice cooling boxes.

The detailed protocol that was created and followed for the performance of these experiments is presented in Appendix II.

5.1.3 Membrane damages

Experiments were carried out with damaged membranes to assess the impact of different types of impairments on the performance of virus removal of both methods – naturally present viruses and MS2 bacteriophages.

The experimental and sampling procedure protocol used for these experiments was the same as for the intact membranes.

The resume of the inflicted impairments is depicted in Table 5.2.

Table 5.2 – Resume of the performed membrane impairments

Experiment	Type of damage	Observations
1	10 pinholes Pore: 0,6 mm	Inflicted between the membrane sheets using a common needle (Attempt)
2	1 pinhole Pore: 4 mm	Inflicted across the membrane sheets through the housing of the membrane using an electric drill
3	1 pinhole Pore: 1 mm	Inflicted across the membrane sheets through the housing of the membrane using an electric drill
4	4 pinholes Pore: 1 mm	Inflicted across the membrane sheets through the housing of the membrane using an electric drill

Figure 5.4 shows the 4-mm pinhole executed on “damaged membrane 2” and Figure 5.5 shows the 1 mm hole present on “damaged membrane 3” and “damaged membrane 4”.

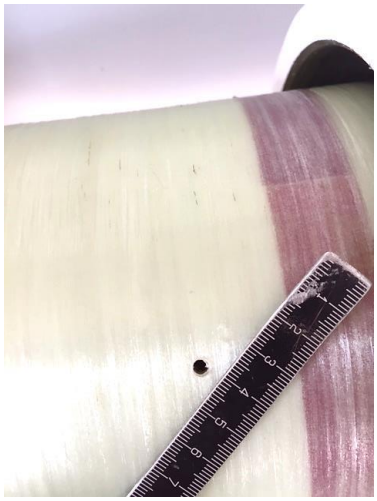

Figure 5.4 – 4 mm pinhole

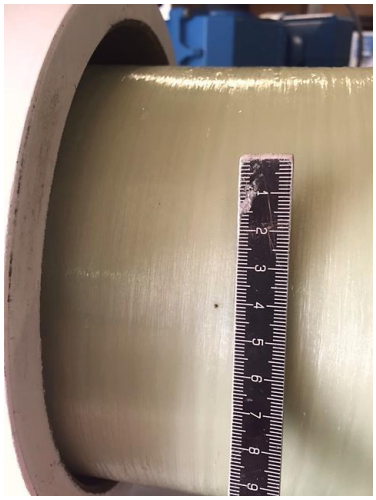

Figure 5.5 – 1 mm pinhole

These pinholes were performed with the intention of puncturing one or two membrane sheets. For this, the drilling was interrupted shortly after penetrating the membrane housing. The selection of the pinholes’ location was done without criteria thus being randomly selected. It is noteworthy to mention that these damages cannot be considered as precise due to the uncertainty of knowing how deep the membrane was drilled.

5.1.4 Membrane characteristics

The membranes used on this research’s experiments were supplied by Hydranautics Nitto Group Company. These 8” composite polyamide membranes had a spiral wound configuration with an active area of 40.9 m^2. Two ESPA2 MAX membranes were used for the first four experiments: Intact 1, Damaged 1, Intact 2, Damaged 2.

For the fifth experiment, the used membrane was the same as Damaged 1. For the last experiment, another ESPA2MAX membrane was used.

Because they are preserved in sodium biosulfite, flushing the membranes with drinking water prior to the experiments is mandatory as mentioned before.

Further information regarding membranes specifications can be consulted in Appendix III.

5.2 Enumeration of MS2 bacteriophages - plaque-assay technique

From the total of 26 samples collected from each experiment, 18 were transported to TU Delft's microbiology laboratory and analysed by the plaque assay method. Appropriate dilutions were done considering each expected concentration on each sample as the original MS2 stock had a concentration of 2 x 10^{11} PFU/ml and the spiking was performed with a concentration of 2 x 10^{7} PFU/ml with the goal to demonstrate the removal of 7 logs. Surface water contains naturally present bacteriophages therefore 8 out of 26 samples were taken before the spiking of the bacteriophages to verify their influence on the experiments. It is also important to mention once again that because the samples relating to the feed water were collected from the sampling point after the cartridge filter, a sample was taken from the feed water tank to assess the removal associated with the filter after spiking the MS2 and assay through this method. Furthermore, blank samples were collected and analysed by the plaque-assay method.

As aforementioned on the experimental plan, the samples from the feed and permeate that were analysed in TU Delft by the plaque-assay method are presented in Table 5.3.

Table 5.3 – Overview of the samples taken to be analysed by the plaque-assay technique

Samples	Time (min)
Blank	0
Controls	35
	45
	65
	75
Tank	0
MS2 bacteriophages	35
	45
	65
	75

The following method was based on the International Organization for Standardization - ISO 10705-1 that focuses on the detection and enumeration of bacteriophages by the plaque-assay technique. This method consists on the sample being mixed with a small volume of semi-solid nutrient medium and a culture of host strain added and plated on a solid nutrient medium. After this, incubation and reading of plates for counting plaques is possible. By counting the plaques, it is calculated the concentration present in PFU/ml as defined in ISO - 10705-1 present in Appendix IV. The subsequent log removal calculation is obtained by equation 1.

$$LRV = \log_{10}\left[\frac{C_{in}}{C_{out}}\right] \qquad (1)$$

Where,

C_{in} – Concentration of the pathogen present in the influent (PFU/ml);

C_{out} - Concentration of the pathogen present in the effluent (PFU/ml).

The mean calculation of log removal values was done through the following adequate equation for logarithmic values:

$$\text{Mean} = -\log\left(\frac{10^{-LRV1} + 10^{-LRV2} + 10^{-LRV3} + 10^{-LRV4}}{4}\right) \qquad (2)$$

The materials, diluents and reagents were used according to the ISO 10705-1:1995 and can be consulted on the Appendix IV as aforementioned.

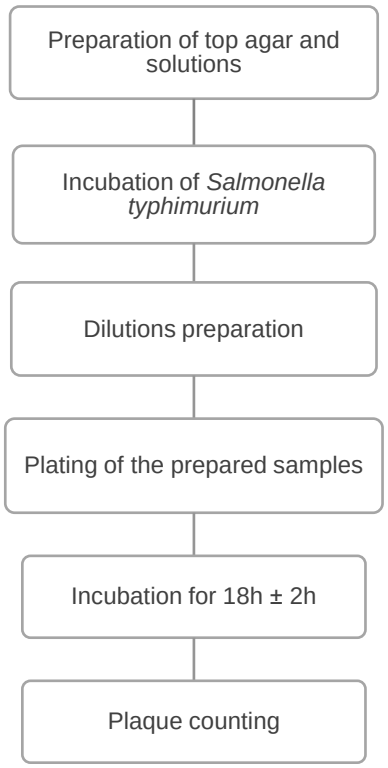

Figure 5.6 – Steps of the plaque-assay technique - resume

The plaque counting results can be consulted in Appendix V.

5.3 Next generation sequencing and Quantitative polymerase chain-reaction

The techniques used by KWR Watercycle Research Institute to detect naturally present viruses in surface water samples were the next generation sequencing (NGS) and quantitative polymerase chain-reaction (qPCR).

Next generation sequencing was used to identify a virus sequence that appear to be present in high concentrations in surface water and have interesting properties to be a virus removal indicator. The steps to obtain the virus sequence used in this research followed the BTO protocol – "*Nieuwe indicatororganismen om de verwijdering van virussen door zuiveringsprocessen te bepalen".*

Once a virus sequence was identified, the design of primer pairs candidates took place. After, quantitative PCR was used to amplify and quantify nucleotides present in a DNA sample. The previously prepared samples were placed in the qPCR machine. The PCR cycle after which the fluorescence signal of the amplified DNA is detected (threshold cycle [C_T]) was used to quantify the gene copy concentrations (van der Wielen & van der Kooij, 2013). However, due to lack of available virus concentration, the obtained virus concentrations in this research were calculated through the number of cycles (Ct values) therefore they are indicative.

Because during the extraction phase some DNA can be lost, a concentration correction had to be performed based on the DNA recovery for each sample. It is important therefore to have in account these recoveries as the higher the value the more reliable the result.

The designed PCR-primer pair by the KWR Institute was the primer pair 2314 which targets a DNA sequence with similarity with a phage: *Phormidium phage* Pf-WMP3. The primer used to amplify viral sequences is shown in Table 5.4. This virus was detected in high concentrations in the Grecht channel thus having the potential to indicate virus removal.

Table 5.4 – Primer pair 2314

Forward	Reverse	Sequence length
ACCAGGGGCGGTGTATATTG	GACGCCGTTGAAATGTCAGG	102

The virus' concentrations present in feed and effluent samples were provided by KWR Institute and the log removal values were estimated through equation 1. The mean calculation of log removal values was done through equation 2.

$$\text{Mean} = -\log\left(\frac{10^{-LRV1} + 10^{-LRV2} + 10^{-LRV3} + 10^{-LRV4}}{4}\right) \qquad (2)$$

The detailed description followed by KWR's analysts of the applied qPCR method is described in the article "*Nontuberculous Mycobacteria, Fungi, and Opportunistic Pathogens in Unchlorinated Drinking Water in the Netherlands*" which can be consulted in Appendix VI.

6. Results and discussion

The results from the experimental procedures performed with intact and damaged membranes are outlined in this chapter. These experiments were performed in Oasen's installations and the samples corresponding to MS2 and controls were transported to TU Delft's microbiology laboratory and analysed by the plaque-assay method as explained in the previous chapter. The naturally occurring viruses' samples were analysed in KWR Watercycle Research Institute by the qPCR method and the data was provided to be studied in this research.

This chapter will detail the MS2 and NPV challenge test's results for each experiment followed by another sub-chapter dedicated to the overview and comparison between the challenge tests.

6.1 Experimental results

As mentioned in the previous chapter, the feed water tap was not functional therefore the feed samples were collected from the tap corresponding to the feed after the cartridge filter. In each experiment, a sample was collected from the tank to assess the influence of the cartridge filter in the removal process. The concentrations presented in the next table are average estimated values for each experiment. The results are depicted in Table 6.1.

Table 6.1 – Cartridge filter removal estimation

Experiment	Influent (tank) (PFU/ml)	After cartridge (PFU/ml)	Log removal value
Intact 1	1,65 x 10^7	1,05 x 10^7	0,20
Intact 2	1,43 x 10^7	1,35 x 10^7	0,03
Damaged 1	1,79 x 10^7	1,11 x 10^7	0,21
Damaged 2	1,35 x 10^7	1,53 x 10^7	-0,05
Damaged 3	1,68 x 10^7	1,45 x 10^7	0,06
Damaged 4	1,77 x 10^7	1,63 x 10^7	0,04

The influence of the cartridge filter is not significant as low associated LRV are indicated in table 6.1. These results indicate that the collecting the feed samples after the filter did not importantly influence the experiments.

Blank samples from the tank were collected prior to every experiment aiming to demonstrate that the system was properly disinfected, not leaving MS2 bacteriophages traces from the previous

experiment in the feed tank. The lab analysis was performed without diluting the sample. The system's disinfection was successfully achieved as significantly low counts were detected in the influent and none in the effluent, as expected in case of proper killing of MS2. This information can be consulted in Appendix V.

As aforementioned, control samples were collected before the MS2 spiking took place in order to understand the significance of bacteriophages that occur naturally in surface water in each experiment. The results were obtained by the plaque-assay technique and the average values can be consulted in Table 6.2.

Table 6.2 – Naturally present bacteriophages in each experiment

Experiment	Influent	Effluent
Intact 1	0	0
Intact 2	12	0
Damaged 1	0	0
Damaged 2	3	0
Damaged 3	2	0
Damaged 4	90	0

As understandable from Table 6.2, no plaques were detected in the samples from "Intact 1" and "Damaged 1". Relatively small number of plaques were detected in the influent control samples in experiment "Intact 2", "Damaged 2" and "Damaged 4" before the spiking of MS2. These are expected readings since it is anticipated for a few bacteriophages to be found naturally in surface water. In the last experiment, higher number of plaques were detected. It is also acceptable to perceive that few phages could remain in the system since the blank sample had, as well, the highest value in this experiment (Table V.13 - Appendix V). Although they differ more significantly from all the previous control results, there was no influence on the outcome of the experiment since the influent values were still low and furthermore the effluent remained null.

6.1.1. Intact membrane 1

The first experiment was performed with an intact membrane aiming to prove that it can achieve the log removal values that it was validated for. This experiment occurred with a feed pressure of 9,5 bar and a permeate conductivity value of 2,3 µS/cm.

MS2 bacteriophages

The results with regard to MS2 challenge test in this chapter are presented in plaque-forming units per millilitre. The plaque counting results can be consulted in Appendix V. The results for each sample collected during the first experiment are shown in Table 6.3.

The log removal values achieved in this experiment were calculated through Equation 1, explained in the Materials and methods chapter. However, the detection limit associated to the plaque-assay method is 1 PFU/ml therefore the calculation was done by considering the effluent's concentration as 1 PFU/ml for estimation matters in the cases where the real effluent concentrations were null. Therefore, the log removal values presented in Table 6.3 should be considered as reliable however lower than the real values.

Table 6.3 – Achieved LRV by the MS2 challenge test (Intact membrane 1)

Sampling time (min)	Influent Concentration (PFU/ml)	Effluent Concentration (PFU/ml)		Log removal value
35	$1{,}04 \times 10^7$	0	>	7,02
45	$1{,}15 \times 10^7$	0	>	7,06
65	$1{,}09 \times 10^7$	0	>	7,04
75	$1{,}06 \times 10^7$	0	>	7,03

The MS2 challenge test results are in agreement with the expected. In the presence of an unimpaired membrane, the expected number of plaques in the effluent plates is zero, suggesting the system was capable to remove all components from the feed water, especially MS2 bacteriophages.

Each sample demonstrated to achieve above 7 log removal values. Although it is a lower bound value, it strongly suggests the ability of an intact reverse osmosis membrane being capable of rejecting all dissolved and suspended material as the real value is expected to be higher. The virus removal validation on this experiment is achieved because the bacteriophages'

concentration spiked in the feed tank was 2 x 10^7 PFU/ml and the concentration in the effluent is null, meaning that no virus was capable to pass through the membrane thus resulting in high removal values.

Naturally present viruses

The results regarding the naturally present viruses challenge test in this chapter are presented in DNA copies/l and refer to primer pair 2314. DNA recoveries are depicted along with the results.

As the methods used for the naturally present viruses challenge test are new, proper information regarding the detection limit is still limited. Therefore 1 DNA copy/ml was considered for the effluent's concentration in the cases where the real effluent concentrations were null.

The results obtained by the quantitative polymerase chain-reaction are shown in Table 6.4. Similarly, to the MS2 results, the same approach was done regarding the log removal values estimation. Accordingly, the results in the referred table should be considered as a lower bound from the real values.

Table 6.4 – Achieved LRV by the NPV challenge test (Intact membrane 1)

Sampling time (min)	Influent Recovery (%)	Influent concentration (DNA copies/l)	Effluent recovery (%)	Effluent concentration (DNA copies/l)		LRV
35	69,3	2,47 x 10^7	51,8	0	>	7,39
45	65,2	2,57 x 10^7	59,8	0	>	7,41
65	68,3	2,19 x 10^7	55,9	0	>	7,34
75	72,3	2,10 x 10^7	55,2	0	>	7,32

As shown in Table 6.4, this method demonstrates that the virus was not detected in the effluent therefore achieving significant removal in all samples above 7, suggesting that this method can demonstrate virus removal in the presence of an unimpaired membrane.

6.1.2. Intact membrane 2

The second experiment using a new intact membrane was performed following the same approach as the previous experiment. A second test in these circumstances was carried out to understand the consistency of the results obtained from the first experiment.

This experiment occurred with a feed pressure of 9,5 bar and a permeate conductivity value of 2,3 µS/cm.

MS2 bacteriophages

The data relating to the “Intact membrane 2” experiment was managed and calculated with the same approach as “Intact membrane 1”. Table 6.5 presents the MS2 assay results.

Table 6.5 - Achieved LRV by the MS2 challenge test (Intact membrane 2)

Sampling time (min)	Influent Concentration (PFU/ml)	Effluent Concentration (PFU/ml)		Log removal value
35	$1,27 \times 10^7$	0	>	7,10
45	$1,45 \times 10^7$	0	>	7,16
65	$1,23 \times 10^7$	0	>	7,09
75	$1,32 \times 10^7$	0	>	7,12

Table 6.5 shows that there were no MS2 bacteriophages detected in the effluent samples, hence proving the full removal of MS2 phages by the membrane. Based on the intention of double checking the first experimental results, it is noticeable by Table 6.5 the consistency between them. Above 7 log removal values were achieved in all the samples by the second intact membrane supporting the obtained results from “Intact membrane 1” experiment thus suggesting the suitability in using MS2 challenge test to validate virus removal in presence of intact membranes.

Naturally present viruses

The results obtained by the quantitative polymerase chain-reaction regarding the naturally present viruses' concentrations in the influent and effluent samples are shown in Table 6.6. Since the effluent concentration is null, the calculated log removal values should be considered as lower than the real value, as previously explained.

Table 6.6 - Achieved LRV by the NPV challenge test (Intact membrane 2)

Sampling time (min)	Influent Recovery (%)	Influent concentration (DNA copies/l)	Effluent recovery (%)	Effluent concentration (DNA copies/l)		LRV
35	70,0	$8{,}88 \times 10^6$	6,8	0	>	6,95
45	1,3	$3{,}15 \times 10^7$	65,9	0	>	7,50
65	1,7	$2{,}55 \times 10^7$	55,7	0	>	7,41
75	73,3	$8{,}90 \times 10^7$	63,5	0	>	6,95

Similarly, to the results from the first experiment, above 7 log removal values were achieved by every sample. It can be stated that the outcome of the current experiment is in accordance with the obtained results in the first intact membrane as both achieved above 7 LRV. These are noteworthy results that imply and reinforce the potential suitability for the NPV challenge test to validate virus removal in the presence of intact membranes. Although the recoveries corresponding to samples Influent 45, Influent 65 and Effluent 35 are considerably low, satisfactory concentrations were still attained meaning that no interference was held in the outcome of the results.

6.1.3. Damaged membrane 1 (Ten 0,6 mm pinholes)

The first experiment using a damaged membrane was performed with the same membrane as "Intact membrane 1" after chlorine disinfection. This experiment followed the same experimental protocol using a membrane that was already used and disinfected. Apart from the damage that chlorine disinfection might inflict on the membrane, it was decided to impair the membrane causing 10 small pinholes with a 0,6-mm pore needle. However, since the membrane sheets were considerably compact, it is not possible to affirm the needle stabbed across the sheets thus producing the planned damage.

This experiment occurred with a feed pressure of 9,5 bar and a permeate conductivity value of 2,4 µS/cm.

MS2 bacteriophages

The results from the first experiment using a compromised membrane are shown in Table 6.7.

Table 6.7 - Achieved LRV by the MS2 challenge test (Damaged membrane 1)

Sampling time (min)	Influent Concentration (PFU/ml)	Effluent Concentration (PFU/ml)		Log removal value
35	1,18 x 10^7	0	>	7,07
45	1,43 x 10^7	0	>	7,16
65	8,05 x 10^6	0	>	6,91
75	1,29 x 10^7	0	>	7,11

It should be noted that since no bacteriophages were detected in effluent samples, the log removal estimation followed the same approach as for the intact membrane experiments.

Although it should be expected that a certain number of bacteriophages would have passed through the membrane due to the impairment inflicted by the needle, these results are however similar to the ones obtained in the two previous experiments performed with intact membranes. These results demonstrate high virus removal which is corroborated with the removal of approximately 7 logs for each sampling time.

This outcome suggests the unsuccessfully attempt to perforate membrane sheets. It seems acceptable to consider this interpretation as the needle was not appropriate for the task due to its weakness and short length. Since no damage was inflicted by the needle, these results also

suggest that the chlorine disinfection itself it's not enough to damage the membrane and cause integrity loss.

Furthermore, some authors have reported that fouling of small pinholes can occur by the particles present in the test water, which sometimes results in higher log removal values (Mi *et al*., 2004; Wilbert & Linton, 2000).

Naturally present viruses

The recovered results for the first damaged membrane experiment can be consulted in Table 6.8. Since the effluent concentration is null, the log removal values represented in the table below should be consider as a lower bound, as previously explained.

Table 6.8 - Achieved LRV by the NPV challenge test (Damaged membrane 1)

Sampling time (min)	Influent Recovery (%)	Influent concentration (DNA copies/l)	Effluent recovery (%)	Effluent concentration (DNA copies/l)		LRV
35	33,7	$1{,}24 \times 10^7$	55,03	0	>	7,1
45	68,6	$1{,}43 \times 10^7$	60,61	0	>	7,2
65	67,5	$1{,}01 \times 10^7$	63,67	0	>	7,0
75	62,9	$1{,}10 \times 10^7$	64,83	0	>	7,0

The obtained results by the naturally present viruses challenge test reassemble with the ones achieved by the MS2 challenge test. As Table 6.8 shows, the virus was not detected in the effluent suggesting that despite the attempted inflicted pinholes on the membrane, the virus was successfully removed by the membrane. Similarly, to the intact membranes' results, more than 7 log removal values were achieved by this method in each sample.

The possible explanations and interpretations regarding these results are equivalent to those previously mentioned relating to the MS2 challenge test results.

6.1.4. Damaged membrane 2 (One 4 mm pinhole)

The second damaged membrane experiment was performed with the same membrane as “Intact membrane 2” after chlorine disinfection. The experiment followed the same experimental protocol using the referred membrane that was already used and disinfected.

The type of impairment performed in this experiment was influenced by the obtained results for the “Damaged membrane 1” experiment in which ten 0,6 mm pinholes were not enough to compromise the membrane’s integrity. Therefore, another strategy to damage the membrane was experimented. Accordingly, a 4-mm pinhole was executed with an electric drill through the housing of the membrane aiming the drilling of 3 membrane sheets maximum.

The operational conditions regarding this experiment were the following:

Pressure: 9 bar;

Conductivity: 3,9 µS/cm.

MS2 bacteriophages

The influent and effluent concentrations and subsequent log removal values calculated for this experiment were calculated through Equation 1 and are presented in Table 6.9.

Table 6.9 - Achieved LRV by the MS2 challenge test (Damaged membrane 2)

Sampling time (min)	Influent Concentration (PFU/ml)	Effluent Concentration (PFU/ml)	Log removal value
35	$1,46 \times 10^7$	$1,45 \times 10^4$	3,0
45	$1,75 \times 10^7$	$5,80 \times 10^4$	2,5
65	$1,51 \times 10^7$	$3,75 \times 10^4$	2,6
75	$1,86 \times 10^7$	$2,25 \times 10^4$	2,9

In this experiment, plaques were detected in the effluent plates. In this case, the passage of MS2 and the perforation of membrane sheets clearly occurred (Table 6.9). The presence of one pinhole with 4 mm diameter successfully damaged the membrane, decreasing the log removal values to an average of 2,1. These results indicate that MS2 challenge test is capable of detecting membrane damage.

Naturally present viruses

The results regarding the experiment in which one 4-mm pinhole was induced on the membrane are shown in Table 6.10.

Table 6.10 - Achieved LRV by the NPV challenge test (Damaged membrane 2)

Sampling time (min)	Influent Recovery (%)	Influent concentration (DNA copies/l)	Effluent recovery (%)	Effluent concentration (DNA copies/l)	LRV
35	95,0	$1,25 \times 10^7$	71,8	$2,64 \times 10^4$	2,7
45	94,5	$1,14 \times 10^7$	70,2	$2,23 \times 10^4$	2,7
65	88,4	$9,45 \times 10^7$	67,6	$2,57 \times 10^4$	2,6
75	74,2	$1,79 \times 10^7$	81,1	$1,82 \times 10^4$	3,0

It is clear in Table 6.10 that there is significant loss of integrity comparing to the previous experiments achieving in this experiment an average of 2,2. These results suggest that this challenge test is sensitive and capable of detecting this type of membrane impairment. The detection of the virus (primer pair 2314) in the effluent is consistent with the expectation and with the results obtained from MS2 challenge test.

6.1.5. Damaged membrane 3 (One 1 mm pinhole)

“Damaged membrane 3” experiment was performed using the same membrane as “Damaged membrane 1” since its results showed that the membrane was not compromised therefore its availability to be used for the third damaged membrane experiment.

The inflicted damage on the membrane was executed with an electric drill through its housing producing one 1 mm pinhole. The aim of this experiment is to understand the membrane’s performance in the presence of diverse types of damage.

The operational conditions regarding this experiment were the following:

Pressure: 9,2 bar;

Conductivity: 3,3 µS/cm.

MS2 bacteriophages

The MS2 concentration corresponding to each taken sample during the experiment and subsequent LRV are shown in Table 6.11.

Table 6.11 - Achieved LRV by the MS2 challenge test (Damaged membrane 3)

Sampling time (min)	Influent Concentration (PFU/ml)	Effluent Concentration (PFU/ml)	Log removal value
35	$1{,}56 \times 10^{7}$	$1{,}52 \times 10^{3}$	4,0
45	$1{,}46 \times 10^{7}$	$2{,}95 \times 10^{2}$	4,7
65	$1{,}54 \times 10^{7}$	$8{,}40 \times 10^{2}$	4,3
75	$1{,}48 \times 10^{7}$	$7{,}15 \times 10^{2}$	4,3

It is shown in Table 6.11 the passage of MS2 phages through the membrane. However, the achieved log removal values in the presence of one 1 mm pinhole were higher than the ones in the presence of a 4 mm, as expected. Although the achieved log removals decreased when compared to the ones regarding intact membranes, these results suggest that even with this type of damage, the membrane was still capable to achieve an average of 3,7 LRV. These findings can corroborate the known sensitivity of MS2 challenge test for monitoring the integrity of reverse osmosis membranes.

Naturally present viruses

The results obtained through the provided virus concentrations by KWR Institute are shown in Table 6.12.

Table 6.12 - Achieved LRV by the NPV challenge test (Damaged membrane 3)

Sampling time (min)	Influent Recovery (%)	Influent concentration (DNA copies/l)	Effluent recovery (%)	Effluent concentration (DNA copies/l)	LRV
35	72,4	$1,26 \times 10^7$	55,7	$3,26 \times 10^3$	3,6
45	26,5	$2,64 \times 10^7$	66,7	$2,50 \times 10^3$	4,0
65	73,5	$1,09 \times 10^7$	57,8	$2,39 \times 10^3$	3,7
75	71,6	$1,12 \times 10^7$	62,7	$2,68 \times 10^3$	3,6

According to the expected, the virus was detected in the effluent due to the inflicted impairment on the membrane. Around 3,2 LRV were achieved by the NPV challenge test which is higher than the obtained results regarding the 4-mm pinhole. These are interesting findings for this method as they suggest its sensitivity in detecting different types of membrane damage.

6.1.6. Damaged membrane 4 (Four 1 mm pinholes)

This experiment was performed using a used membrane provided by the treatment plant. This experiment was done with the intention of having at least three experiments with different compromised membranes. In this case, four 1-mm pinholes were inflicted using the electric driller mentioned before.

The operational conditions regarding this experiment were the following:

Pressure: 7,9 bar;

Conductivity: 6,9 µS/cm.

MS2 bacteriophages

The results obtained regarding the last experiment are shown in Table 6.13.

Table 6.13 - Achieved LRV by the MS2 challenge test (Damaged membrane 4)

Sampling time (min)	Influent Concentration (PFU/ml)	Effluent Concentration (PFU/ml)	Log removal value
35	$1,72 \times 10^7$	$1,35 \times 10^5$	2,1
45	$1,52 \times 10^7$	$1,35 \times 10^5$	2,1
65	$1,69 \times 10^7$	$1,30 \times 10^5$	2,1
75	$1,52 \times 10^7$	$1,51 \times 10^5$	2,0

The results in the table above show the passage of MS2 bacteriophages through the membrane due to the executed four holes. The average log removal values obtained when four holes with 1 mm of diameter were performed on the membrane was 1,6 (Table 6.13). These results show significant decrease on the log removal values when compared to the previous two experiments. Moreover, indicating that multiple 1 mm pinholes lead to worse membrane damage than a single 4 mm pinhole. This result therefore suggests the four induced pinholes perforate a higher number of membrane sheets than the 4-mm pinhole.

Naturally present viruses

The results for “Damaged membrane 4” are shown in Table 6.14.

Table 6.14 - Achieved LRV by the NPV challenge test (Damaged membrane 4)

Sampling time (min)	Influent Recovery (%)	Influent concentration (DNA copies/l)	Effluent recovery (%)	Effluent concentration (DNA copies/l)	LRV
35	76,2	$1,23 \times 10^7$	91,6	$2,78 \times 10^4$	2,65
45	74,2	$8,62 \times 10^6$	74,6	$2,95 \times 10^4$	2,47
65	77,9	$7,33 \times 10^6$	73,1	$3,93 \times 10^4$	2,27
75	79,0	$6,72 \times 10^6$	58,9	$3,50 \times 10^4$	2,28

As shown in Table 6.14, the virus was detected by the qPCR method in the effluent samples which proves the membrane was successfully compromised hence the virus passage through the membrane. Furthermore, significant low LRV (average of 1,9) were achieved in this experiment suggesting the inflicted pinholes severely damaged the membrane’s integrity. This challenge test achieved higher LRV for the 1 mm pinhole, secondly 4 mm and lastly four 1 mm pinholes.

6.2 Overview

After reviewing each individual experimental result, a more thorough analysis is presented in this section.

Concerning the influence of collecting the feed samples after the cartridge filter, it is possible to state, based on the results from the previous subchapter, that it did not significantly influence the outcome of the final results. The results from the blank samples that were collected from the feed tank prior to every experiment in order to guarantee that a proper disinfection of the system was carried out are in accordance to the desired scenario as null or significantly low phage counts in the influent and none in the effluent were detected by the plaque-assay method this meaning that the results were not compromised. Furthermore, these low counts are expected because bacteriophages occur naturally in surface water. For this reason, control samples were collected at the same time as the naturally present viruses samples however analysed through the plaque-assay technique to assess the role of naturally present bacteriophages on the experiments. Overall, it is clear the coherence of these results since only a few number of plaques were detected in the influent samples and none in the effluent samples thus being possible to assume that the natural presence of bacteriophages in the surface water did not interfere with this research's findings.

The following graphics (Figure 6.1) intend to demonstrate the log removal values achieved by the MS2 bacteriophage challenge test and by the naturally present viruses challenge test on each experiment throughout operating time. It should be noted that for the cases where the effluent concentration was null (Intact membrane 1, Intact membrane 2 and Damaged membrane 1) lower values than the real ones are shown due to impractical calculations as explained in the previous sub chapter.

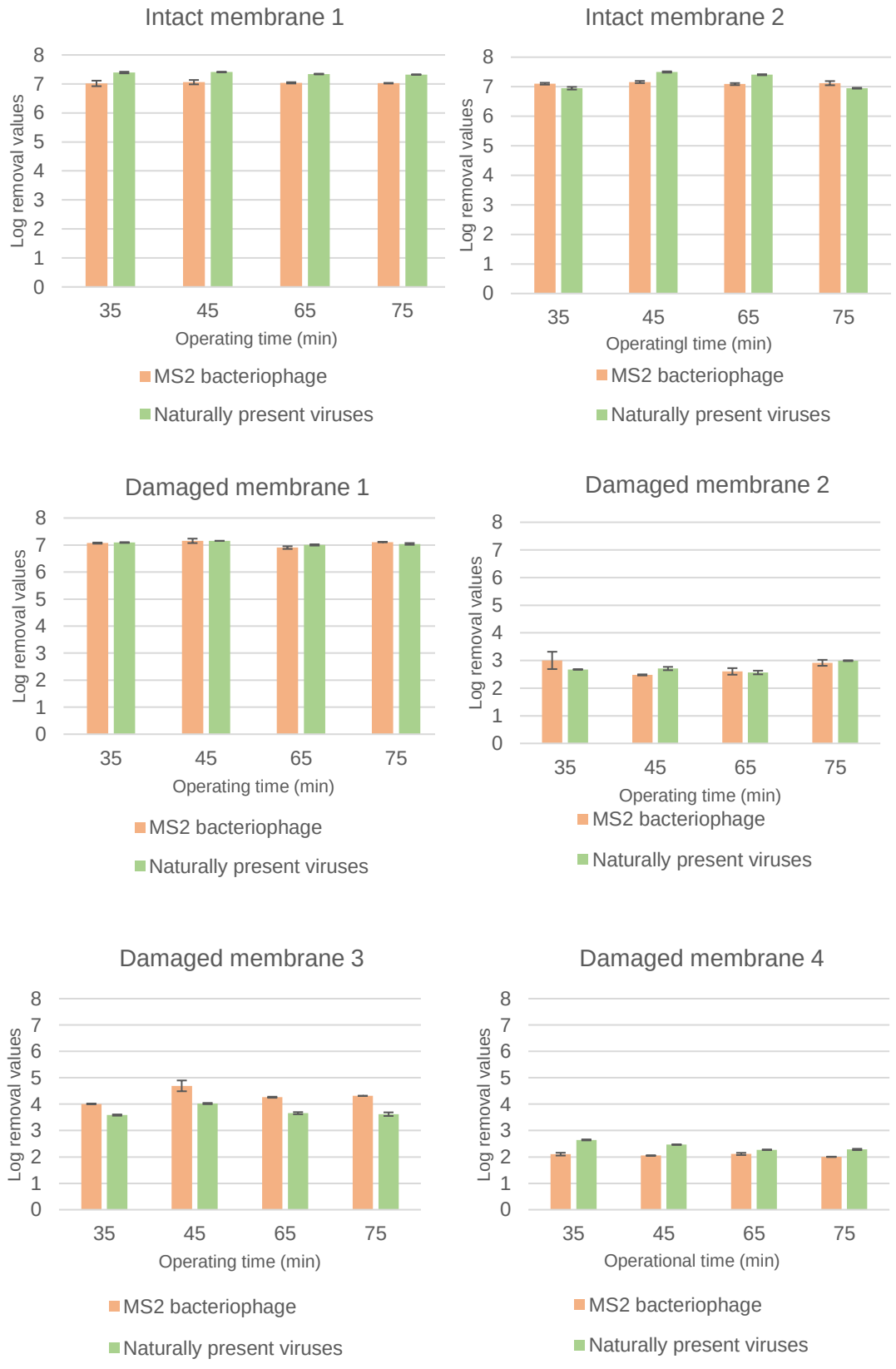

Figure 6.1 – Log removal values achieved by MS2 and NPV challenge tests throughout operating time in each experiment

According to the presented Figure, the log removal values were overall consistent for both methods during each experiment. For the cases in which intact membranes were used, the removal achieved more than 7 logs by the plaque-assay technique as well as by the qPCR method. However, the first damaged membrane experiment was not successfully damaged as explained before, resulting in the achievement superior to 7 logs which might imply that chemical damage did not play a role in these experiments as the dosed concentrations were not sufficiently harmful to the membrane nor the exposure time. Furthermore, in case the membrane perforation did occur, there is no viable way to prove whether the pinholes were clogged by the particles present in surface water. Therefore, and based on these results, the third experiment could represent a third intact membrane experiment.

Three successfully damaged membrane experiments were performed. The inflicted damages were done by drilling the membrane across several sheets with an electric drill in which the first membrane was damaged with a 4-mm pinhole, the second with a 1-mm pinhole whilst the third one was impaired with four 1-mm pinholes. As Figure 6.1 shows, in the last three experiments the log removals achieved by both methods were overall consistent with each other and, as expected, lower than the ones performed using intact membranes.

It should be noted that no visible trend was detected through the operating time regarding the removal values suggesting that the concentration of the spiked MS2 did not decay during the referred time.

To get an overview of the achieved LRV by intact and damaged membranes, Figure 6.2 is presented.

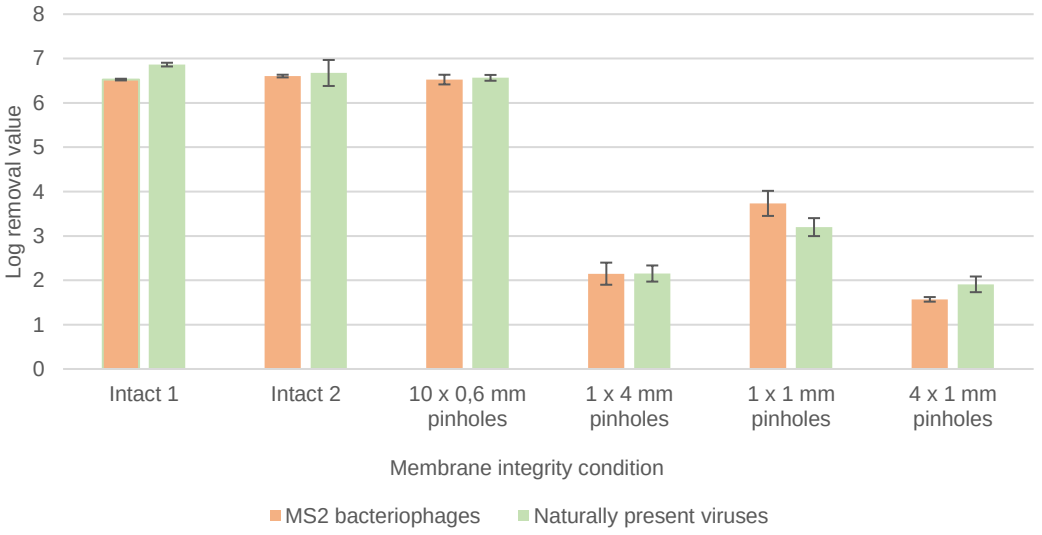

Figure 6.2 – Comparison of log removal values between challenge test

Both methods were capable to detect the presence of integrity loss and appear to be sensitive to diverse types of damage on the membrane. The removal logs achieved by the qPCR method are consistent with the ones obtained by the plaque-assay method being the latter an already known and widely used method to monitor membrane integrity. Based on this research's outcome it can be indicated that the primer pair 2314 is suitable as an indicator for virus removal since it has demonstrated comparable log removal values to the MS2 challenge test.

The standard deviation was estimated for both methods. As Figure 6.2 shows, it is not possible to affirm that the NPV challenge test has lower error than the MS2. However, the potential associated with this new challenge test is unquestionable.

A few aspects such as the log removal values achieved, resolution, frequency, applicability and regarding the two performed methods in this research, were gathered in Table 6.15.

Table 6.15 – Comparison between challenge tests

Characteristics	**MS2 bacteriophage**	**Naturally present viruses**
Log removal value	- Intact membranes: Above 7 - Damaged membranes: 1 x 4 mm pinhole: ~ 2,1 1 x 1 mm pinhole: ~ 3,7 1 x 4 mm pinhole: ~ 1,6	- Intact membranes: Above 7 - Damaged membranes: 1 x 4 mm pinhole: ~ 2,2 1 x 1 mm pinhole: ~ 3,2 1 x 4 mm pinhole: ~ 1,9
Resolution	Similar to virus size: 25 nm	Natural occurring viruses
Frequency	3 days	1-2 days
Applicability	- Simple method however great lab effort is required - Some operator expertise needed - Offline - Spiking into feed water needed - Not applicable to drinking water production - System shutdown is required	- Simple method - No operator expertise needed - Offline - No spiking needed - Applicable to drinking water production - System shutdown not required
Cost	Expensive	Moderate

The advantages of using naturally present viruses to monitor the integrity of reverse osmosis membranes are evident when compared to MS2's. The log removal values that both can achieve are substantially high and comparable representing the suitability of the primer pair used in this thesis to validate virus removal in reverse osmosis membranes. In addition, it is estimated that performing the plaque-assay method is more time consuming than the qPCR method thus requiring laboratory effort. As discussed in chapter 2, performing the MS2 challenge test in full-scale plants remains impractical due to the effort needed to culture and plate all the samples which is one of many reasons for this new challenge test to be so appealing in the scientific community. Moreover, this method uses natural viruses already present in surface water fact that rule out the need to introduce components that reassembles to viruses into the feed water. Although both challenge tests are offline, MS2 needs the system to be shut down at the time of each monitoring test to ensure disinfection, contrarily to the natural viruses. Therefore, it is concluded that the NPV challenge test requires less expertise from the operators and less laboratory effort than the MS2. The estimative of the costs present in the table above were obtained through personal communication and based on the experience of the involved companies in this dissertation. The plaque-assay technique carries high associated costs due to the enormous required laboratory effort whilst the qPCR costs are based on an estimation that the method is fully developed and commercialized. Therefore and considering the qPCR's labour costs, the cost per sample will decrease due to the possibility of processing more than one sample per single experiment. Although the reagents cost is higher for the qPCR method, it is becoming clear that this method's further advantages overcome this fact.

The current challenge tests that are being discussed in the scientific community still have major drawbacks when compared to the using of naturally occurring viruses to verify virus removal. If an analysis is to be done comparing these tests to the using of NPV, it is evident that none of them validates as many advantages. Although challenge tests such as Rhodamine WT, Fluorescent Microspheres, TRASAR, PMMIM and Nanoparticles have already been reported as being capable of achieving at least 4 LRV using intact membranes, they cannot be performed without introducing components into the feed water hence their inapplicability to be used in drinking water treatment. Furthermore, several of these challenge tests require expertise from the operators to spike the components and to disinfect the system afterwards.

Lastly, it is important to have in account the operating conditions in which the experiments occurred. For this matter, a few parameters such as pressure, feed conductivity, permeate conductivity, flow rate and recovery were noted in the end of each experiment. Additionally, Table 6.16 contains the information regarding the removal values achieved for conductivity, MS2 and NPV is presented.

Table 6.16 – Overview of the experimental parameters and conditions

Experiment	Pressure (bar)	Feed conductivity (µS/cm)	Permeate Conductivity (µS/cm)	LRV Conductivity	LRV MS2	LRV NPV	Flow rate (m^3/h)	Recovery (%)
Intact 1 -	9,5	617,7	2,3	2,43	>6,5	>6,9	6,8	15
Intact 2 -	9,5	620,4	2,3	2,43	>6,6	>6,7		
Damaged 1 10 x 0,6 mm pinholes	9,5	660,2	2,4	2,44	>6,5	>6,6		
Damaged 2 1 x 4 mm hole	9	732,8	3,9	2,27	2,1	2,2		
Damaged 3 1 x 1 mm hole	9,2	635,7	3,3	2,28	3,7	3,2		
Damaged 4 4 x 1 mm holes	7,9	638,8	6,9	1,97	1,6	1,9		

Although it is not the goal of this research to determine the log removals associated to conductivity parameter, an estimation was done based on the values from each experiment. As Table 6.16 indicates, conductivity was not capable of achieving more than 2,4 LRV which corroborates its inability to assess virus removal. It is however noticeable that when the permeate conductivity increases from around 3 to around 7 µS/cm, the removals decrease considerably thus suggesting severe membrane damage which can be easily detected by the real-time values provided by the RO unit and detect notable changes in the permeate conductivity.

The relation between the operating conditions and the log removals achieved by MS2 and NPV challenge tests is visible in Figure 6.3.

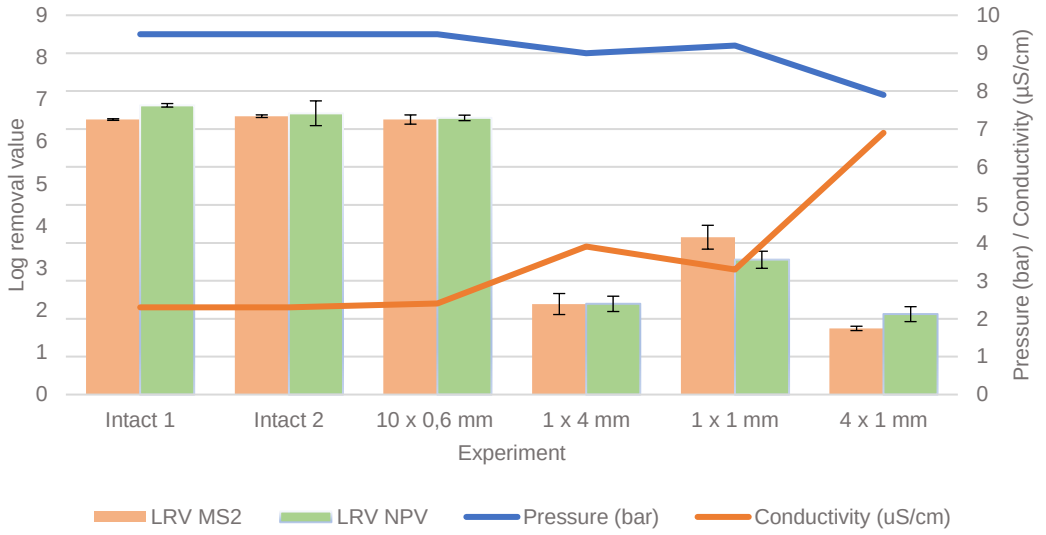

Figure 6.3 – Experimental conditions in relation with achieved LRV

Figure 6.3 indicates that for intact membranes, when the log removal values are high (above 7), the pressure values are also high (around 9 bar) and the permeate conductivity is low (around 2,5 µS/cm), according to the expectation. It is understandable that when the impairment on the membrane is more serious the amount of salts passing through the pinhole is higher therefore the increase in the conductivity values for the damaged membrane experiments, especially regarding the one with four 1-mm pinholes.

7. Conclusions

This dissertation's' findings indicates that the demonstrated removal values by the naturally present viruses challenge test were overall substantially consistent with the ones achieved by the MS2 bacteriophages which was performed as a comparison test.

Both MS2 and NPV challenge tests achieved above 7 log removal values in the presence of intact membranes. On the third experiment where the first impairment was executed it was expected the passage of viruses through the membrane, however it did not happen suggesting that the damage was not truthfully compromised. Nevertheless, it cannot be excluded the hypothesis of the inflicted pinholes to had been fouled by the particles present in the surface water.

Three successfully compromised membranes achieved different log removal values according to the severity of the inflicted damage furthermore indicating sensitivity by the NPV challenge test as well as by the MS2 challenge test. It is concluded by these experiments' results that higher loss of membrane integrity was observed by both challenge tests when four pinholes with 1-mm diameter were inflicted on the membrane, followed by one 4 and one 1-millimetre diameter pinholes, achieving below 2 LRV, up to 3,7 LRV and approximately 2 LRV, respectively.

The objective of this thesis was accomplished as the potential for using naturally present viruses in surface water was demonstrated and compared with the reported best integrity monitoring method for virus removal (MS2). The primer pair 2314 used in this dissertation exhibited to be an adequate virus removal indicator due to the achievement of high log removal values and the ability of detecting membrane failure. Moreover, features such as being present in high concentrations in surface water and apparent behaviour that reassembles human enteric viruses meet the requirements to be a virus removal indicator.

The searching goal consists in finding a virus or virus surrogate able to demonstrate higher that 4 log removal values and that can be applied in drinking water production facilities. In addition, it is desired that the analysis would not be time-consuming neither expensive. The perfect integrity monitoring scenario is not however currently possible, because although the new challenge test studied in this thesis validates all the requirements, the exception relies in featuring online/real-time monitoring.

In conclusion, using naturally occurring viruses in surface water has significant potential to safely monitor the integrity of reverse osmosis systems validating virus removal and consequentially ensuring public health.

8. Future research and recommendations

The current dissertation revealed interesting results worthy of further research and investigation. The following topics are based on the demonstrated potential of using naturally present viruses to verify virus removal in reverse osmosis membranes.

- The performed experiments in this research should be tested in full-scale installations to validate the suitability of the NPV challenge test in greater scale.
- Including distinct types of damages apart from puncturing pinholes is recommended with emphasis on O-ring damages. Further experiments involving membrane impairments must be precisely performed.
- The presence of the primer pair 2314 in distinct locations must be confirmed to assess whether this virus is only applicable for water industries using surface water from the Grecht channel. This virus's presence and concentration should also be assessed regarding seasonal influences.
- It is important for the techniques and technologies used in the virus naturally present challenge test to continue to develop regarding their limit of detection and viruses' databases in order to be possible to attempt this challenge test in facilities where the source water is groundwater.
- Further investigation to accurately determine and confirm this natural virus's physicochemical characteristics to determine if it is comparable, for instance in size and surface properties, to enteric human viruses would improve confidence to authenticate its suitability for verifying virus removal.
- Provided that this new challenge test is successfully performed in full-scale installations this will have major impact in the scientific community. Possibilities such as each country detecting their own natural occurring virus indicator to demonstrate virus removal is a future perspective that is to be taken in account.

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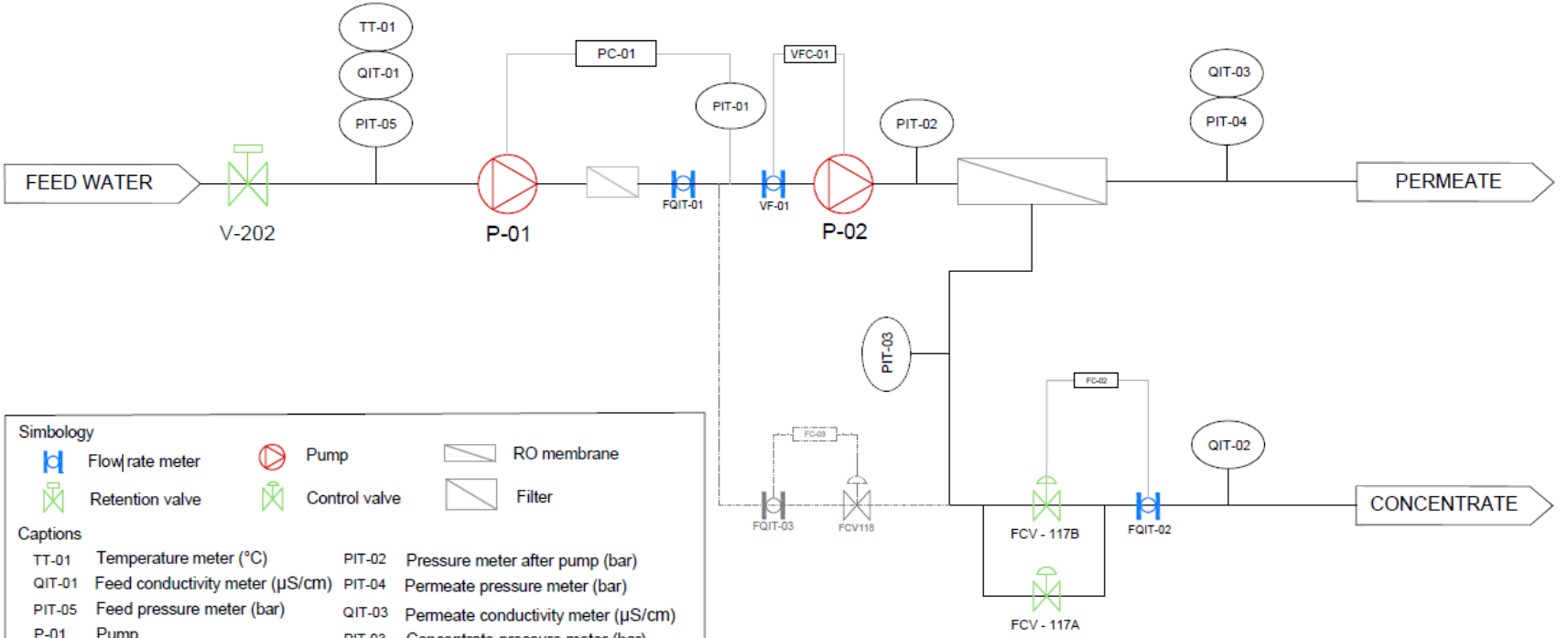
APPENDIX I. Diagram of the RO unit



APPENDIX II. Experimental protocol - MS2 and NPV challenge tests

1st Part - Intact

1. One to two days before running an experiment, empty the tank and refill it with drinking water and let it flush for 2 days;
2. In the day of the experiment put everything in the van:
   - Ice cooling boxes with ice
   - Take 200 ml of MS2 stock from the lab
   - Take 2 glass bottles of 1L
3. Have all the stuff ready for the experiment
4. Take things out of the van and place the MS2 stock in the fridge
5. Put on safety shoes
6. Empty the tank and refill it with test water (surface water)
7. Make sure the cooler is on and the temperature is stabilized
8. Turn on the RO installation and let it stabilize for ~ 20 minutes making sure everything is clear on the monitor
9. Label all the sample containers
10. Get the gloves, the sample containers and the burner
11. Make sure you have all the sample containers ready as well as the ice cooling boxes
12. After waiting 30 minutes burn the taps to sterilize them with gloves on
13. Open the taps and let run a bit before taking samples because of the dead volume
14. Wait 5 minutes and take one sample for the controls and one sample for the NPV, both from the feed and from the permeate
15. Wait another 5 minutes and repeat
16. Put all the samples in the fridge/ice cooling box and move them away as soon as you collect the samples
17. Wait another 30 min and repeat the previous sampling procedure
18. Prepare 100 ml of MS2 stock into a 1L bottle and fill it with surface water
19. Make sure the setup is shutdown. Spike the MS2 into the feed water tank and turn on the mixer with the first valve closed (to assure proper mixing before turning on the installation)
20. Wait 30 minutes and before taking samples take all the precautions mentioned above
21. Take the influent 0 sample
22. Wait 5 minutes and take one sample for the MS2, both from the feed and from the permeate.
23. Wait 5 minutes more and repeat

24. Wait another 30 min and repeat the previous sampling procedure

25. Take all the samples and ice cooling boxes away

26. Shutdown the installation and dose 100 ml of sodium hypochlorite into the tank and let it mix around 15 min before starting the installation

27. Let it flush for a minimum of 4 hours

28. Deliver the samples to KWR Institute

29. Transport the remaining samples to TU Delft

2nd Part – Damages

30. Go to the treatment plant and empty the tank and fill with surface water

31. Open the caps of the pressure vessel in order to reach the membrane

31.1 Prepare the 0,6-mm pore needle and puncture the membrane across its sheets (10 times) (On the first damaged membrane attempt)

31.2 Prepare the electric drill and drill one 4-millimetre hole into the housing trying to perforate 1-2 sheets in a random location (On the second membrane damage experiment)

31.3 Prepare the electric drill and drill one 1-millimetre hole into the housing trying to perforate 1-2 sheets in a random location (On the third membrane damage experiment)

31.4 Prepare the electric drill and drill four 1-millimetre holes into the housing trying to perforate 1-2 sheets (On the fourth damaged membrane experiment)

32. Repeat the procedure as explained in the 1st part.

APPENDIX III. Membranes' characteristics

Nitto

HYDRANAUTICS
Nitto Group Company

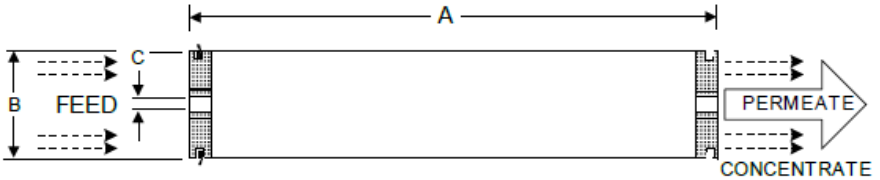
	Membrane Element	ESPA2 MAX
Performance	Permeate Flow:	12,000 gpd (45.4 m^3/d)
	Salt Rejection:	99.6% (99.5% minimum)
Type	Configuration:	Spiral Wound
	Membrane Polymer:	Composite Polyamide
	Membrane Active Area:	440 ft^2 (40.9m^2)
Application Data*	Maximum Applied Pressure:	600 psig (4.14 MPa)
	Maximum Chlorine Concentration:	< 0.1 PPM
	Maximum Operating Temperature:	113 °F (45 °C)
	pH Range, Continuous (Cleaning):	2-10.6 (1-12)*
	Maximum Feedwater Turbidity:	1.0 NTU
	Maximum Feedwater SDI (15 mins):	5.0
	Maximum Feed Flow:	75 GPM (17.0 m^3/h)
	Minimum Ratio of Concentrate to Permeate Flow for any Element:	5:1
	Maximum Pressure Drop for Each Element:	15 psi

* The limitations shown here are for general use. For specific projects, operating at more conservative values may ensure the best performance and longest life of the membrane. See Hydranautics Technical Bulletins for more detail on operation limits, cleaning pH, and cleaning temperatures.

Test Conditions

The stated performance is initial (data taken after 30 minutes of operation), based on the following conditions:

1500 PPM NaCl solution
150 psi (1.05 MPa) Applied Pressure
77 °F (25 °C) Operating Temperature
15% Permeate Recovery
6.5 - 7.0 pH Range



A, inches (mm)	B, inches (mm)	C, inches (mm)	Weight, lbs. (kg)
40.0 (1016)	7.89 (200)	1.125 (28.6)	36 (16.4)

Notice: Permeate flow for individual elements may vary + or - 15 percent. Membrane active area may vary +/-4%. Element weight may vary. All membrane elements are supplied with a brine seal, interconnector, and o-rings. Elements are enclosed in a sealed polyethylene bag containing less than 1.0% sodium meta-bisulfite solution, and then packaged in a cardboard box.

Hydranautics believes the information and data contained herein to be accurate and useful. The information and data are offered in good faith, but without guarantee, as conditions and methods of use of our products are beyond our control. Hydranautics assumes no liability for results obtained or damages incurred through the application of the presented information and data. It is the user's responsibility to determine the appropriateness of Hydranautics' products for the user's specific end uses. 5/13/16

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APPENDIX IV. ISO 10705-1 Part 1: Enumeration of F-specific RNA bacteriophages

Water quality — Detection and enumeration of bacteriophages —

Part 1:
Enumeration of F-specific RNA bacteriophages

1 Scope

This part of ISO 10705 specifies a method for the detection and enumeration of F-specific ribonucleic acid (RNA) bacteriophages by incubating the sample with an appropriate host strain. The method can be applied to all kinds of water, sediments and sludges, where necessary after dilution. In the case of low numbers, a preconcentration step may be necessary for which a separate part of ISO 10705 will be developed. The method can also be applied to shellfish extracts. Depending on the relative abundance of F-specific RNA bacteriophages to background organisms, additional confirmatory tests may be necessary and are also specified in this part of ISO 10705.

The presence of F-specific RNA bacteriophages in a water sample generally indicates pollution by wastewater contaminated by human or animal faeces. Their survival in the environment, removal by widely used water treatment processes and concentration or retention by shellfish resembles that of foodborne and waterborne human enteric viruses, for example the enteroviruses, hepatitis A virus and rotaviruses.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 10705. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 10705 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods.*

ISO 5667-1:1980, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes.*

ISO 5667-2:1991, *Water quality — Sampling — Part 2: Guidance on sampling techniques.*

ISO 5667-3:1994, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of samples.*

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

ISO 8199:1988, *Water quality — General guide to the enumeration of micro-organisms by culture.*

3 Definition

For the purposes of this part of ISO 10705, the following definition applies.

3.1 F-specific RNA bacteriophages: Bacterial viruses which are capable of infecting a specified host strain with F-pili or sex-pili to produce visible plaques (clearance zones) on a confluent lawn grown under appropriate culture conditions, whereas the infectious process is inhibited in the presence of a concentration

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of 40 (occasionally 400) µg/ml of RNase in the plating medium.

4 Principle

The sample is mixed with a small volume of semi-solid nutrient medium. A culture of host strain is added and plated on a solid nutrient medium. After this, incubation and reading of plates for visible plaques takes place. Where necessary, simultaneous examination of parallel plates with added RNase for confirmation by differential counts is carried out. The results are expressed as the number concentration of plaque-forming particles (C_{pfp}) per unit of volume.

5 Safety precautions

The host strain used is a *Salmonella typhimurium* mutant of low pathogenicity and should be handled in accordance with the appropriate national or international safety procedures for this bacterial species. F-specific RNA bacteriophages are non-pathogenic for man and animals, but are very resistant to drying. Appropriate precautions should therefore be taken to prevent cross-contamination of test materials, particularly when examining or handling cultures of high titre or when inoculating cultures of the host strains. Such procedures must be carried out in a biohazard cabinet or a separate area of the laboratory.

6 Diluent, culture media and reagents

6.1 Basic materials

Use ingredients of uniform quality and chemicals of analytical grade for the preparation of culture media and reagents, and follow the instructions given in annex A. For information on storage see ISO 8199, except where indicated in this part of ISO 10705. Alternatively, use dehydrated complete media and follow strictly the manufacturer's instructions.

For the preparation of media, use glass-distilled water or deionized water free from substances which might inhibit bacterial growth under the conditions of the test, and in accordance with ISO 3696.

6.2 Diluent

For making sample dilutions, use peptone saline solution as indicated in A.8.

6.3 Reagents

6.3.1 RNase from bovine pancreas, specific activity approximately 50 units/mg (Kunitz).

6.3.2 Antibiotic discs, for susceptibility testing with nalidixic acid (130 µg; 9 mm) and kanamycin (100 µg; 9 mm).

6.3.3 Glycerol, 870 g/litre.

6.4 Microbiological reference cultures

Salmonella typhimurium strain WG49, phage type 3 Nal^r (F′ 42 *lac*::Tn5), NCTC 12484.

Bacteriophage MS2, NCTC 12487 or ATCC 15597-B1.

Escherichia coli K-12 Hfr from appropriate culture collection, e.g. NCTC 12486 or ATCC 23631.

NOTE 1 The NCTC strains are available from the National Collection of Type Cultures, 61 Colindale Avenue, London NW9 6HT, England. The ATCC strains are available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A.

7 Apparatus and glassware

Usual microbiological laboratory equipment, including

7.1 Hot-air oven for dry-heat sterilization and an autoclave. Apart from apparatus supplied sterile, glassware and other equipment shall be sterilized according to the instructions given in ISO 8199.

7.2 Incubator or water bath, thermostatically controlled at 37 °C ± 1 °C.

7.3 Incubator or water bath, thermostatically controlled at 37 °C ± 1 °C and equipped with a rotary platform at 100 min^{-1} ± 10 min^{-1}.

7.4 Water bath, thermostatically controlled at 45 °C ± 1 °C.

7.5 Water bath or equivalent device, for melting agar media.

7.6 pH-meter.

7.7 Counting apparatus, with indirect, oblique light.

7.8 Deep freezer, thermostatically controlled at − 20 °C ± 5 °C.

7.9 Deep freezer, thermostatically controlled at − 70 °C ± 10 °C.

7.10 Spectrometer, capable of holding 1 cm cuvettes or side-arm of nephelometric flasks (7.17) and equipped with a filter in the range 500 nm to 650 nm with a maximum bandwidth of ± 10 nm.

Usual sterile, microbiological laboratory glassware or disposable plastics ware according to ISO 8199 and including the following.

7.11 Petri dishes, of diameter 9 cm or 15 cm.

7.12 Graduated pipettes, of capacities 1 ml, 5 ml and 10 ml.

7.13 Glass bottles, of suitable volumes.

7.14 Culture tubes, with caps.

7.15 Measuring cylinders, of suitable capacity.

7.16 Conical flasks, of capacity 250 ml to 300 ml, with cotton wool plugs or suitable alternatives.

7.17 Cuvettes, of optical path length 1 cm or **nephelometric conical flasks**, of capacity 250 ml to 300 ml, with cylindrical side-arms which can be fitted to the spectrometer (7.10) and with cotton wool plugs or suitable alternatives. (See figure 1.)

7.18 Membrane filter units, for sterilization, pore size 0,2 µm.

7.19 Plastics vials, lidded, of capacity 1,5 ml to 2 ml.

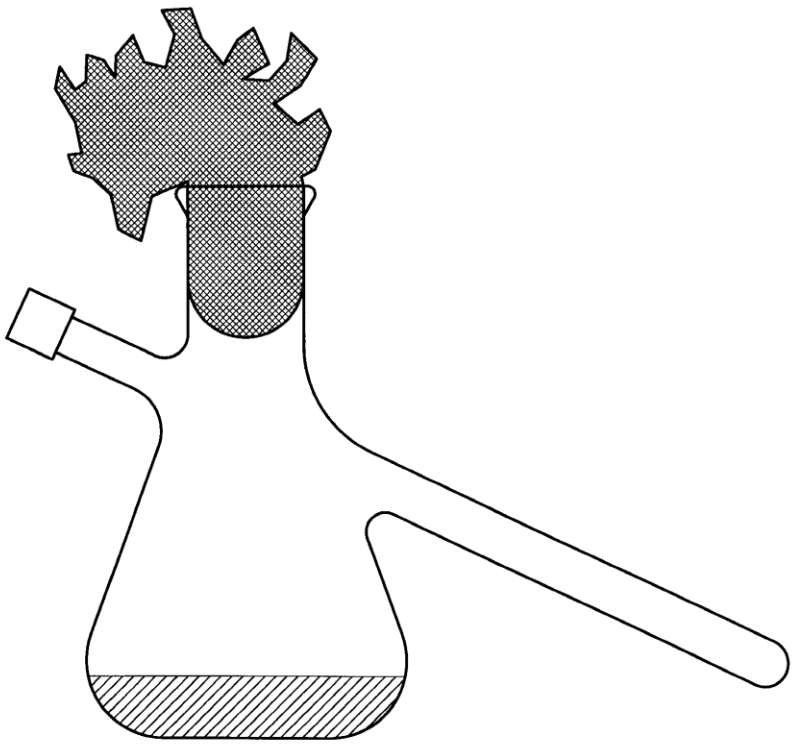

Figure 1 — Nephelometric conical flasks for culturing the host strain

8 Sampling

Take samples and deliver them to the laboratory in accordance with ISO 8199, ISO 5667-1, ISO 5667-2, and ISO 5667-3.

9 Preparation of test materials

9.1 Culturing and maintenance of host strains WG49 and *E. coli* K12 Hfr

The culturing and maintenance of host strains involves several stages which are summarized in figure 2. The figure also indicates the stages where quality control of the host culture is performed.

9.1.1 Preparation of stock cultures

Rehydrate the contents of a lyophilized ampoule of the reference culture of the host strains in a small volume of TYGB (A.1) using a Pasteur pipette. Transfer the suspension to 50 ml of TYGB in a 300 ml conical flask (7.16). Incubate for 18 h ± 2 h at 37 °C ± 1 °C while shaking at 100 min^{-1} ± 10 min^{-1}. Add 10 ml of glycerol (A.6) and mix well. Distribute into plastics vials (7.19) in 1,2 ml aliquots and store at − 70 °C ± 10 °C.

NOTE 2 This first culture of the host strain should be stored as a reference standard in the laboratory.

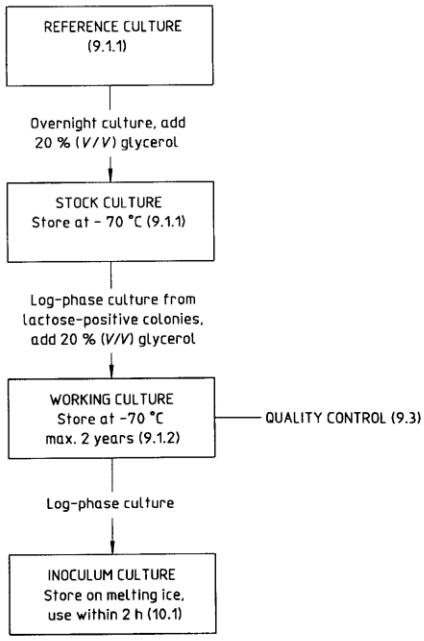

Figure 2 — Scheme for culturing, maintenance and quality control of host strain WG49

9.1.2 Preparation of working cultures

Thaw one vial of stock culture (9.1.1) at room temperature and inoculate on a plate of McConkey agar (A.7), or another lactose-containing medium, in such a way that single colonies will be obtained. Incubate at 37 °C ± 1 °C for 18 h ± 2 h. Add 50 ml of TYGB to (A.1) a 300 ml conical flask (7.16) and warm to room temperature. Select three to five lactose-positive colonies from the McConkey agar and inoculate material from each of these colonies in the flask with TYGB. Incubate for 5 h ± 1 h at 37 °C ± 1 °C while shaking at 100 min^{-1} ± 10 min^{-1}. Add 10 ml of glycerol (A.6) and mix well. Distribute into plastics vials (7.19) in 1,2 ml aliquots and store at − 70 °C ± 10 °C for a maximum of 2 years. Control the quality of the working culture according to 9.3.

NOTES

3 If a great number of tests is anticipated, several conical flasks can be inoculated in parallel.

4 If quality control fails, prepare new inocula from the stock culture. After repeated failures, or if the stock culture is depleted, obtain a new lyophilized ampoule of the reference culture. Do not subculture repeatedly in the laboratory.

9.2 Calibration of turbidity measurements

Take one vial of working culture of host strain WG49 from the freezer and thaw at room temperature. Add 50 ml of TYGB (A.1) to a nephelometric conical flask (7.17), warm to room temperature, adjust the spectrometer reading to 0 on the filled side-arm. Alternatively, use a plain conical flask (7.16) and adjust the spectrometer reading to 0 on broth transferred to a cuvette (7.17). Inoculate 0,5 ml of working culture. Incubate at 37 °C ± 1 °C while shaking at 100 min^{-1} ± 10 min^{-1} for up to 3 h. Every 30 min, measure the turbidity and withdraw a 1 ml sample for viable counts, assuring that the flask is taken from the incubator for as short a time as possible.

Dilute samples to 10^{-6} and spread 0,1 ml volumes of the 10^{-4}, 10^{-5} and 10^{-6} dilutions on TYGA plates in duplicate (A.2); incubate at 37 °C ± 1 °C for 24 h ± 2 h. Count the total number of colonies on each plate yielding between 30 and 300 colonies and calculate the number of cfp/ml (consult ISO 8199 if necessary).

NOTE 5 This procedure should be carried out several times to establish the relationship between turbidity measurements and colony counts. If sufficient data have been obtained, further work can be based on turbidity measurements only.

9.3 Quality control of host strain WG49

Use a culture as prepared in 9.2.

At times $t = 0$ h and $t = 3$ h, also inoculate two plates of McConkey agar (A.7), or another lactose-containing medium with the same dilution series, and incubate at 37 °C ± 1 °C for 24 h ± 2 h. From plates yielding between 30 and 300 colonies, count the number of lactose-positive and lactose-negative colonies and calculate the percentage of lactose-negative colonies.

At times $t = 0$ h and $t = 3$ h, spread 0,1 ml of the 10^{-2} dilution on a plate of McConkey agar or alternative, place one disk with nalidixic acid (Nal) and one disk with kanamycin (Km) on the plates and incubate for 24 h ± 2 h at 37 °C ± 1 °C.

Measure inhibition zones around the antibiotic disks.

The host strain is acceptable if the following criteria are met:

- plate count on TYGA (9.2) at 0 h: 0,5 to 3×10^7 cfp/ml;
- plate count on TYGA (9.2) at 3 h: 7 to 40×10^7 cfp/ml;
- lactose-negative colonies (plasmid segregation) < 8 %;
- inhibition zone around Nal disk: absent;
- Km disk: < 20 mm diameter.

NOTE 6 Antibiotic disks with a different diameter or concentration can be used; another criterion for the maximum inhibition zone around the Km disk should be set.

Check the host strain for sensitivity for F-specific RNA bacteriophages as follows.

Prepare a stock culture of bacteriophage MS2 as described in annex C and store at 4 °C ± 2 °C. Prepare a decimal dilution series and plate out according to 10.1; but use the *E. coli* K-12 Hfr host strain. Store the dilution series at 4 °C ± 2 °C overnight. Count the number of plaques, from the dilution series, and prepare 100 ml to 1 000 ml of a suspension of MS2 in peptone-saline solution (A.8) which is expected to contain approximately 100 pfp/ml. Add glycerol (5 g/l).

Distribute over plastics vials (7.19) in 1,2 ml aliquots and store at − 20 °C ± 5 °C or − 70 °C ± 5 °C.

Thaw four vials at room temperature, combine them in one tube and plate out 1 ml volumes in duplicate

on the *E. coli* K-12 Hfr strain and on WG49 according to 10.1. Count the number of plaques on each plate and calculate the recovery on WG49 relative to the *E. coli* strain. Accept WG49 if the recovery is > 80 %.

10 Procedure

10.1 Standard procedure

Take one vial of working culture from the freezer and thaw it at room temperature. Add 50 ml of TYGB (A.1) to a nephelometric conical flask (7.17), or plain conical flask (7.16). Adjust the spectrometer reading to 0 as described in 9.2 and prewarm to room temperature. Inoculate 0,5 ml of working culture. Incubate at 37 °C ± 1 °C while shaking at 100 min^{-1} ± 10 min^{-1}. Measure turbidity every 30 min. At a turbidity corresponding to a cell density of approximately 10^8 cfu/ml (based on data obtained in 9.2), take the inoculum culture from the incubator and quickly cool on melting ice. Use within 2 h.

NOTE 7 It is essential that the culture is quickly cooled to prevent loss of F-pili by the cells, which will negatively influence recovery.

Melt bottles of ssTYGA (A.3), cool to 44 °C to 50 °C, aseptically add calcium-glucose solution (A.1) (0,5 ml/50 ml) and distribute 2,5 ml aliquots into culture tubes with caps, placed in a water bath at 45 °C ± 1 °C. To each tube, add 1 ml of sample (or dilution or concentrate). Examine each volume or dilution step at least in duplicate.

Add 1 ml of inoculum culture, mix carefully and pour the contents over the surface of a 9 cm TYGA plate (A.2). Distribute evenly, allow to solidify on a perfectly horizontal, cool surface and incubate the plates upside-down at 37 °C ± 1 °C for 18 h ± 2 h.

NOTES

8 Do not stack more than 6 (preferably 4) plates.

9 The addition of ice-cold sample and host culture to the top-agar may lead to a sharp drop in temperature and solidification of the medium. Assure a sufficient time interval between these two steps to allow reheating. However, make sure that inoculated tubes remain in the water bath for not more than 10 min.

Count the number of plaques appearing on each plate within 4 h, using indirect oblique light.

10.2 Method for samples with high bacterial background flora

Add nalidixic acid to ssTYGA (A.3) until a final concentration of 100 µg/ml is obtained.

NOTE 10 Nalidixic acid is stable when heated. It can either be added from a filter-sterilized solution (A.4) (0,2 ml/50 ml) after melting of ssTYGA or can be added to TYGA before autoclaving.

10.3 Confirmatory test

In parallel with the series of plates described under 10.1, prepare a similar series with RNase-solution (A.5) added to the tubes of ssTYGA until a final concentration of 40 µg/ml is obtained (i.e. 100 µl of RNase solution to 2,5 ml of ssTYGA in a tube).

NOTES

11 Confirmatory tests should at least be carried out

a) when examining new sampling points;

b) regularly at fixed sampling points when N_{RNase}/N (see clause 11) is usually less than 10 %;

c) always at fixed sampling points when N_{RNase}/N is usually > 10 %;

d) if large, circular, clear plaques with smooth edges (probably somatic *Salmonella* phages) are regularly seen.

12 In rare cases, RNA-phages may not be inhibited by RNase at 40 µg/ml and it may be necessary to increase the concentration of RNase to 400 µg/ml.

10.4 Samples with low phage counts

Proceed according to 10.1 but with the following modifications:

— 10 ml of ssTYGA, 1 ml of host culture and 5 ml of sample in duplicate per dilution step;

— pour over 50 ml of TYGA in a 14 cm Petri dish.

NOTE 13 This procedure will be able to detect up to 1 pfu/50 ml or 100 ml, if 10 or 20 plates are inoculated in parallel. Due to the high consumption of culture media, it may be advisable to use concentration methods which will also be necessary for even lower counts.

10.5 Quality assurance

With each series of samples, examine a procedural blank using sterile diluent as the sample and a stan-

dard preparation of MS2 (see 9.3). Plot the results on a control chart.

Optionally, also use a naturally polluted standard sample, taken from sewage or surface water, diluted to approximately 100 pfp/ml in peptone saline solution and glycerol (5 g/l) and stored at − 20 °C ± 5 °C or − 70 °C ± 5 °C. Discard the standard samples if the concentration of RNA-phages decreases.

NOTE 14 In the absence of easily available standardized reference materials, any programme for the exchange of standard samples between laboratories should be encouraged.

If sensitivity to phages is lost (this is unusual but it may happen very suddenly and completely), prepare a new set of inocula according to 9.1.2.

11 Expression of results

Select plates with 30 to 300 plaques. From the number of plaques counted, and taking into account the results of previous confirmatory tests, calculate the number concentration of (plaque-forming particles of) F-specific RNA bacteriophages in 1 ml of the sample as follows:

$$C_{pfp} = \frac{N - N_{RNase}}{n} \times F$$

where

C_{pfp} is the confirmed number concentration of F-specific RNA bacteriophages per millilitre;

N is the total number of plaques counted on WG49 plates according to 10.1, 10.2 or 10.4;

N_{RNase} is the total number of plaques counted on WG49 plates with RNase according to 10.3;

n is the number of replicates;

F is the dilution (or concentration) factor (1/5 in the case of 10.4).

12 Test report

The test report shall contain the following information:

a) a reference to this part of ISO 10705;

b) all details necessary for complete identification of the sample;

c) if a confirmatory test was used and the ratio of N_{RNase} to N, as a percentage;

d) the results expressed in accordance with clause 11;

e) any other information relevant to the method.

APPENDIX V. Plaque assay results

Table V.1 – Plaque readings of MS2 samples from Intact membrane 1 experiment

MS2 samples – Intact membrane 1									
Dilution	-4	-4	-5	-5	**Dilution**	0	0	-1	-1
Sampling time (min)					**Sampling time (min)**				
Influent 35	TMTC	TMTC	120	88	**Effluent 35**	0	0	0	0
Influent 45	TMTC	TMTC	129	101	**Effluent 45**	0	0	0	0
Influent 65	TMTC	TMTC	113	105	**Effluent 65**	0	0	0	0
Influent 75	TMTC	TMTC	104	108	**Effluent 75**	0	0	0	0

Table V.2 - Plaque readings of MS2 samples from Intact membrane 2 experiment

Dilution	**-4**	**-4**	**-5**	**-5**	**Dilution**	**0**	**0**	**-1**	**-1**
Sampling time (min)					**Sampling time (min)**				
Influent 35	TMTC	TMTC	133	120	**Effluent 35**	0	0	0	0
Influent 45	TMTC	TMTC	136	153	**Effluent 45**	0	0	0	0
Influent 65	TMTC	TMTC	116	130	**Effluent 65**	0	0	0	0
Influent 75	TMTC	TMTC	147	117	**Effluent 75**	0	0	0	0

Table V.3 - Plaque readings of MS2 samples from Damaged membrane 1 experiment

Dilution	**-4**	**-4**	**-5**	**-5**	**Dilution**	**0**	**0**	**-1**	**-1**
Sampling time (min)					**Sampling time (min)**				
Influent 35	TMTC	TMTC	114	122	**Effluent 35**	0	0	0	0
Influent 45	TMTC	TMTC	162	124	**Effluent 45**	0	0	0	0
Influent 65	TMTC	TMTC	87	74	**Effluent 65**	0	0	0	0
Influent 75	TMTC	TMTC	128	130	**Effluent 75**	0	0	0	0

Table V.4 - Plaque readings of MS2 samples from Damaged membrane 2 experiment

Dilution	-4	-4	-5	-5	Dilution	-2	-2	-3	-3
Sampling time (min)					Sampling time (min)				
Influent 35	TMTC	TMTC	142	150	**Effluent 35**	143	150	21	8
Influent 45	TMTC	TMTC	174	175	**Effluent 45**	TMTC	TMTC	56	60
Influent 65	TMTC	TMTC	136	166	**Effluent 65**	TMTC	311	41	34
Influent 75	TMTC	TMTC	189	182	**Effluent 75**	202	198	19	26

Table V.5 - Plaque readings of MS2 samples from Damaged membrane 3 experiment

Dilution	-4	-4	-5	-5	Dilution	-1	-1	-2	-2
Sampling time (min)					Sampling time (min)				
Influent 35	TMTC	TMTC	150	161	**Effluent 35**	149	155	17	17
Influent 45	TMTC	TMTC	162	130	**Effluent 45**	41	50	4	4
Influent 65	TMTC	TMTC	170	138	**Effluent 65**	23	36	0	1
Influent 75	TMTC	TMTC	162	134	**Effluent 75**	90	78	7	5

Table V.6 - Plaque readings of MS2 samples from Damaged membrane 4 experiment

Dilution	-4	-4	-5	-5	Dilution	-2	-2	-3	-3
Sampling time (min)					Sampling time (min)				
Influent 35	TMTC	TMTC	161	183	**Effluent 35**	TMTC	TMTC	138	132
Influent 45	TMTC	TMTC	150	154	**Effluent 45**	TMTC	TMTC	132	138
Influent 65	TMTC	TMTC	174	163	**Effluent 65**	TMTC	TMTC	125	134
Influent 75	TMTC	TMTC	148	155	**Effluent 75**	TMTC	TMTC	149	152

Table V.7 - Plaque readings of blank samples in each experiment

Blank samples				
Dilution	0	0	-1	-1
Experiment				
Intact 1	0	0	0	0
Intact 2	2	2	0	0
Damaged membrane 1	0	0	0	0
Damaged membrane 2	1	3	0	0
Damaged membrane 3	0	0	0	0
Damaged membrane 4	27	40	0	0

Table V.8 – Plaque reading of Intact membrane 1 control results

Dilution	**0**	**0**	**-1**	**-1**	**Dilution**	**0**	**0**	**-1**	**-1**
Sampling time (min)					**Sampling time (min)**				
Influent 35	0	0	0	0	**Effluent 35**	0	0	0	0
Influent 45	0	0	0	0	**Effluent 45**	0	0	0	0
Influent 65	0	0	0	0	**Effluent 65**	0	0	0	0
Influent 75	0	0	0	0	**Effluent 75**	0	0	0	0

Table V.9 - Plaque reading of Intact membrane 2 control results

Dilution	**0**	**0**	**-1**	**-1**	**Dilution**	**0**	**0**	**-1**	**-1**
Sampling time (min)					**Sampling time (min)**				
Influent 35	9	9	0	0	**Effluent 35**	0	0	0	0
Influent 45	9	11	0	0	**Effluent 45**	0	0	0	0
Influent 65	12	9	0	0	**Effluent 65**	0	0	0	0
Influent 75	14	23	0	0	**Effluent 75**	0	0	0	0

Table V.10 - Plaque reading of Damaged membrane 1 control results

Dilution	0	0	-1	-1	Dilution	0	0	-1	-1
Sampling time (min)					Sampling time (min)				
Influent 35	0	0	0	0	Effluent 35	0	0	0	0
Influent 45	0	0	0	0	Effluent 45	0	0	0	0
Influent 65	0	0	0	0	Effluent 65	0	0	0	0
Influent 75	0	0	0	0	Effluent 75	0	0	0	0

Table V.11 - Plaque reading of Damaged membrane 2 control results

Dilution	0	0	-1	-1	Dilution	0	0	-1	-1
Sampling time (min)					Sampling time (min)				
Influent 35	4	3	0	0	Effluent 35	0	0	0	0
Influent 45	3	2	0	0	Effluent 45	0	0	0	0
Influent 65	1	2	0	0	Effluent 65	0	0	0	0
Influent 75	6	4	0	0	Effluent 75	0	0	0	0

Table V.12 - Plaque reading of Damaged membrane 3 control results

Dilution	0	0	-1	-1	Dilution	0	0	-1	-1
Sampling time (min)					Sampling time (min)				
Influent 35	1	1	0	0	Effluent 35	0	0	0	0
Influent 45	2	3	0	0	Effluent 45	0	0	0	0
Influent 65	2	4	0	0	Effluent 65	0	0	0	0
Influent 75	2	1	0	0	Effluent 75	0	0	0	0

Table V.13 - Plaque reading of Damaged membrane 4 control results

Dilution	0	0	-1	-1	Dilution	0	0	-1	-1
Sampling time (min)					Sampling time (min)				
Influent 35	107	103	0	0	**Effluent 35**	0	0	0	0
Influent 45	69	90	0	0	**Effluent 45**	0	0	0	0
Influent 65	101	120	0	0	**Effluent 65**	0	0	0	0
Influent 75	87	90	0	0	**Effluent 75**	0	0	0	0

APPENDIX VI. Quantitative polymerase chain-reaction protocol

Nontuberculous Mycobacteria, Fungi, and Opportunistic Pathogens in Unchlorinated Drinking Water in the Netherlands – Materials and Methods

MATERIALS AND METHODS

Sampling. The distributed unchlorinated drinking water from five treat- ment plants that used surface water (plants SW1, SW2, SW3, SW4, and SW5) and three treatment plants that used groundwater (plants GW1, GW2, and GW3) were analyzed. The drinking water produced at these plants differed in total organic carbon (TOC) and easily assimilable or- ganic carbon (AOC) concentrations, and the heterotrophic plate counts (HPC) in the distributed drinking water differed as well (Table 1). These eight distribution systems were sampled in the winter (January through March) and the summer (August through September) of 2010. Drinking water samples (2 liters each) were taken from the kitchen cold-water tap of different houses (plant SW1, 13 houses; SW2, 13 houses; SW3, 11 houses; SW4, 11 houses; SW5, 13 houses; GW1, 12 houses; GW2, 10 houses; GW3, 10 houses) connected to the distribution system of each treatment plant, and the water temperature was measured immediately after each sample was taken. One sample was also taken of the treated water at each treat- ment plant in the summer, except for plant SW3. Samples at the tap were taken according to the Dutch drinking water decree so that they would represent drinking water from the distribution system. In short, each tap was flushed until the water temperature remained stable for 30 s, and the drinking water was subsequently sampled. Exceptions to this procedure were made for samples collected from the distribution systems of plants SW2 and SW3 in the summer, which were taken directly from the tap without prior flushing. These samples represented drinking water from the premise plumbing systems. The water samples were transported and stored at 4°C and processed within 24 h after collection.

DNA isolation. A volume of 1,000 ml was filtered through a 25-mm polycarbonate filter (0.22-µm pore size, type GTTP; Millipore, the Neth- erlands). The filter and a DNA fragment of an internal control were added to phosphate-MT buffer in a lysing matrix E tube of the FastDNA spin kit for soil (Qbiogene, Inc., Carlsbad, CA) and stored at —20°C. The internal control was used to determine the recovery efficiency of DNA isolation and PCR analysis (20). DNA was isolated according to the supplier's pro- tocol. The filter, DNA fragment, and buffer were processed for 30 s at speed 5.5 in a FastPrep instrument. Subsequently, the lysing matrix E tubes were centrifuged for 30 s at 14,000 × *g*. The supernatant was trans- ferred to a clean tube, and 250 µl protein precipitation solution (PPS) reagent was added and subsequently mixed by hand for 10 min. The tubes were centrifuged for 5 min at 14,000 × *g*. Subsequently, the supernatant was transferred to a clean 15-ml tube, and 1 ml binding matrix suspension was added. Tubes were subsequently inverted by hand for 2 min and placed in a rack for 3 min. Five hundred microliters of supernatant was carefully removed and discarded. Approximately 600 µl of the mixture was added to a spin filter and centrifuged for 1 min at 14,000 ×

g. Subse- quently, the catch tube was emptied, and the remaining supernatant was added to the spin filter and centrifuged again for 1 min at 14,000 × *g*. Next, 500 µl salt-ethanol wash solution (SEWS-M) was added to the spin filter and subsequently centrifuged for 1 min at 14,000 × *g*. Subsequently, the spin filter was replaced in the catch tube and centrifuged for 2 min at 14,000 × *g*. Afterward, the spin filters were placed in fresh kit-supplied catch tubes, and the filters were air dried for 5 min. After drying, 200 µl DNA elution solution (DES) was added, and the matrix on membrane was gently stirred with a pipette tip. Finally, the spin filters and the catch tubes were centrifuged for 1 min at 14,000 × *g*. The eluted DNA was subse- quently kept at —80°C.

Quantitative PCR analyses. The numbers of gene copies of the opportunistic pathogens in the drinking water samples were determined with previously developed quantitative PCR (qPCR) analyses for drinking water samples (18). These qPCR methods target the macrophage infectiv- ity potentiator (*mip*) gene of *L. pneumophila*, the 16S rRNA gene of *My- cobacterium* spp., the 16S rRNA gene of *M. avium* complex, the 18S rRNA gene of fungi, the 28S rRNA gene of *Aspergillus fumigatus*, the *regA* gene of *P. aeruginosa*, the *chiA* gene of *S. maltophilia*, and the 18S rRNA gene of *Acanthamoeba* spp. (18). Reaction mixtures for PCR analyses contained 25 µl of 2× IQTM SYBR green supermix (Bio-Rad Laboratories BV, Veenendaal, the Netherlands), 0.2 µM each primer and, if applicable, probe, 0.4 mg ml^{-1} bovine serum albumin, and 10 µl DNA template in a total volume of 50 µl. Amplification, detection, and data analysis were performed in an iCycler iQ real-time detection system (Bio-Rad Labora- tories BV). Primer-probe sequences and the amplification programs are shown in Tables S1 and S2 in the supplemental material. The PCR cycle after which the fluorescence signal of the amplified DNA is detected (threshold cycle [C_T]) was used to quantify the gene copy concentrations. Quantifications were based on comparison of the sample C_T value with the C_T values of a calibration curve based on known copy numbers of the respective gene from the different microorganisms.