Fluorescence-based methods of virus estimation in wastewater:

Current challenges and future perspectives

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Abstract

Increasing freshwater scarcity drives humankind to look for alternative sources of potable water as well as water for agricultural and industrial applications. It is estimated that by 2025 half of the world population will be living in water-scarce regions. This pressure has forced governments to explore alternative water sources and more sustainable approaches to manage water services. Municipal wastewater reuse is part of the solution to address these population, industrial, and environmental challenges. However, municipal wastewater with human excreta as its' major constituent is the source of human pathogens by the mere nature of it. Of all microbial hazards present in municipal wastewater, human enteric viruses cause infection at the lowest doses while their concentration in untreated sewage can exceed 10^6 viruses per litre. Enteric viruses are also highly resistant to water treatment processes and persist in the environment. To ensure public health and safety, the wastewater industry regulations are moving toward virus testing in sewage-impacted waters. This shift requires a set of robust and well-validated methods. Despite the popularity of flow cytometric virus enumeration in marine research and some attempts to use it for engineered waters, there has been no thorough understanding of the basic mechanisms behind this method. This research focused on developing and validating a sensitive and accurate real-time method to monitor the total indigenous virus population during the treatment of municipal wastewater with intended reuse. Experiments using a set of pure-culture bacteriophages with various genome sizes demonstrated that human enteric viruses were generally below the limit of detection by current flow cytometry (flow virometry). SYBR® Green and other fluorescent nucleic acid binding dyes were shown to

form auto-fluorescent particles that interfere with the targeted virus flow cytometric signal. In addition, the presence of surfactants in the sample enhanced this non-specific signal of fluorescent dye particles that obscure the true virus signal. Hence, replacing the organic wastewater background of wastewater samples with tris-EDTA buffer reduced the non-specific flow cytometric signal and improved virus resolution. Flow virometry, however, was demonstrated to be quantitative when suitable controls were used, including a set of serial dilutions of purified bacteriophage cultures and stained viral free diluent that allowed unambiguous proof of virus identity of the flow cytometric signals of interest. Alternatively, the excitation/emission fluorescence scanning-based assay for virus quantification was shown to be a more sensitive alternative to flow virometry for real-time virus monitoring scenarios, as it measures total fluorescence that is emitted by stained viral nucleic acids. Heating the virus sample to expose viral nucleic acid to a fluorescent dye and the addition of low concentrations of humic acid sodium salt into samples improved this fluorescence scanning assay sensitivity even further. Overall, this study describes and explains the fundamental colloid and fluorescence mechanisms in sample matrices impacting virus enumeration. These along with the development of more sensitive instruments for analysis could turn flow virometry into a useful tool for water quality monitoring.

Preface

Some of the research conducted for this thesis forms part of a research project "Evaluating the Public Health Risks Associated with Wastewater Reuse in Alberta Through Quantitative Microbial Risk Assessment", led by Professors Norman Neumann and Nicholas Ashbolt at the University of Alberta in collaboration with the city of Calgary and Alberta Health Services.

The literature review in chapter 2 is my original work.

A version of Chapter 3 of this thesis has been published as:

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I was responsible for the experimental design, data collection and analysis, and the manuscript composition. Alexey M. Atrazhev assisted with the data collection. Nicholas J. Ashbolt was the supervisory author and was involved with concept formation and manuscript edits.

A version of Chapter 4 has been submitted for publication (AEM01935-20) with the title "Outer limits of flow cytometry to quantify viruses in water". I was responsible for the experimental design, data collection and analysis as well as the manuscript composition. Rafik Dey assisted with the data analysis and contributed to manuscript edits. Peter Pollard assisted with environmental samples collection and contributed to manuscript edits.

Nicholas J. Ashbolt was the supervisory author and was involved with concept formation and manuscript edits.

Chapter 5 is ready to be submitted for publication with the title “Optimizing fluorescence-based assay for virus monitoring of wastewater”. I was responsible for the concept formation, experimental design, data collection and analysis, and the manuscript composition. Peter Pollard critically reviewed the experimental design and contributed to manuscript edits. Nicholas J. Ashbolt was the supervisory author who contributed to concept formation and manuscript edits.

Appendix 1 “Staining of nucleic acids with fluorescently labeled recombinant DNA and RNA-binding proteins” is my original concept. Alexey Atrazhev provided technical advice for the experimental design.

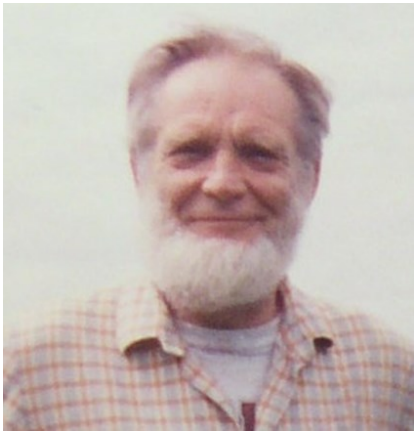
Dedication

I dedicate this body of work to two outstanding representatives of Russian and World Science, two brilliant scholars, two tireless researchers who ignited and nurtured this passion in their students…

I dedicate this work to two Masters, whose devoted disciple I am proud to be:

Dr. Olga E. Marfenina, Moscow State University Professor

and my MSc (Soil Microbiology) supervisor



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Acronyms

AMR	Antimicrobial resistance
AOP	Advanced oxidation process
ATP	Adenosine triphosphate
BCESF-AM	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxy methyl ester
BOD_5	Biochemical oxygen demand over five-day test
CFU	Colony forming unit
COD	Chemical oxygen demand
CSO	Combined sewer overflow
CT	Disinfectant concentration x Time
DALY	Disability-adjusted life years
µDALYd	Pathogen dose equivalent to 10^{-6} DALY
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DPR	Direct potable reuse
EHEC	Enterohemorrhagic *Escherichia coli*
EMA	Ethidium monoazide
FCM	Flow cytometry
FEEMS	Fluorescence excitation emission matrix scanning
FIB	Faecal indicator bacteria

FITC	Fluorescein isothiocyanate
FRET	Förster resonance energy transfer
F-RNA	Male specific bacteriophages with RNA genome
FSC	Low angle forward scatter
GFP	Green fluorescent protein
HNA	High nucleic acid content bacteria
HPC	Heterotrophic plate count
IPR	Indirect potable reuse
L	Liter
LNA	Low nucleic acid content bacteria
LRV	Log_{10} reduction value
MF	Microfiltration
NA	Nucleic acid
NADH	Nicotinamide adenine dinucleotide, reduced form
NF	Nanofiltration
OPA	Optical plankton analyzer
OTU	Operational taxonomic unit
PAA	Peracetic acid
PARAFAC	Parallel factor analysis
PCR	Polymerase chain reaction
PFU	Plaque forming unit
pH	Negative logarithm of the hydrogen ion concentration

PI	Propidium iodide
PMA	Propidium monoazide
PMMoV	Pepper mild mottle virus
QMRA	Quantitative microbiological risk assessment
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RO	Reverse osmosis
RT-PCR	Reverse Transcription polymerase chain reaction
RTU	Relative Fluorescence unit
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SFS	Synchronous fluorescence spectra
SSC	Side scatter
ST	Sequence type
$TCID_{50}$	Tissue culture infection dose at which 50% of the cells are infected
TE	Tris–EDTA buffer
TEM	Transmission electron microscopy
TOC	Total organic carbon
TSS	Total suspended solids
UF	Ultrafiltration
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
VBNC	Viable but non-culturable

VLP	Virus-like particle
WHO	World Health Organization
WSP	Water safety plan
WW	Wastewater
WWTP	Wastewater treatment plant
YLD	Years lived with disability or illness
YLL	Years of life lost

Chapter 1: Introduction

1.1 Wastewater as a necessary alternative water resource

The advent of drinking water treatment and wastewater management has been recognized as one of the major achievements of humankind in disease prevention and became the backbone of public health interventions worldwide (Gorchev & Ozolins, 2011). However, traditional source waters for communities are under increasing competition due to irrigation and energy production demands on freshwater, along with a significant change in climate and precipitation patterns in many areas of the world, all exacerbated by a noticeable growth of the urban population. Hence, scarcity of suitable freshwater is a growing problem worldwide (Rijsberman, 2006; Singh & Kumar, 2014). Mekonnen and Hoekstra (2016) reported that four billion people live under severe water scarcity at least one month a year and half a billion live in such conditions all year round. In some countries water consumption exceeds natural capacity for water source restoration and self-purification (Tomei *et al.*, 2016). It is estimated that by 2025 half of the world population will be living in water-scarce regions (https://www.who.int/news-room/fact-sheets/detail/drinking-water). This pressure has forced governments to explore alternative water sources and sustainable methods of water management. Wastewater reuse is now the preferred method, amongst others, to address these population, industrial, and environmental challenges with water (Zhang *et al.*, 2017). Overall, wastewater reuse (recycling) is the use of treated wastewater for beneficial purposes like agricultural or urban irrigation, industrial needs and to supplement drinking water sources (https://www3.epa.gov/region9/water/recycling/). Water recycling has been

implemented in Namibia, Israel, Australia, USA, and numerous other countries so as to meet the needs of their growing population, and the World Health Organization (WHO) recently issued guidelines for potable water reuse (WHO, 2017b). Reuse also reduces agricultural production costs by means of (wastewater) nutrient recovery and reuse for crop production, and increasingly so by creating a pool of reusable water fit for purpose (Apostolidis *et al.*, 2011; Jiménez & Asano, 2008; Lahnsteiner & Lempert, 2007). Yet, public concerns about microbial safety of reclaimed water still linger (Cooper, 1991; Marecos do Monte, 2007; Po *et al.*, 2003; Po *et al.*, 2005) and have limited its wider use (Purnell *et al*. 2020; Turner et al., 2016).

1.2 Key hazards in wastewater relevant to reuse

There are three major groups of hazards that might be present in drinking water and treated wastewater intended for reuse as defined by the WHO (WHO, 2017a, 2017b): radiological, chemical and microbial.

Radiological hazards result from radionuclides present in water. Although most radionuclides are naturally occurring, such as dissolved radon and daughter products (Baeza *et al.*, 2012 and 2017; Salas *et al.*, 2014), some radioactive contaminants result from medical or industrial usage (Khan *et al.*, 2019). However, most medical radionuclides have short half-lives to reduce their persistence following shedding from patients. It is also reported that standard wastewater treatment processes employed for potable reuse are sufficient for radionuclides removal (WHO, 2017b).

Chemical hazards of concern in water reuse include a wide range of naturally occurring and synthetic organic or inorganic chemicals. These include heavy metals, inorganic toxic compounds like ammonia, nitrate, or fluoride, and organic pharmaceuticals, endocrine disruptors, pesticides, cyanobacterial toxins, disinfection by-products, *etc*. (Benotti *et al.*, 2009; Calderon, 2000; Van Leeuwen, 2000). However, Khan *et al.* (2019) admit that for many chemical contaminants toxicology data are scarce and their public health impacts are unknown.

In recycled wastewaters, microbial contaminants pose the highest risk to public health due to the nature of the water source itself. Municipal wastewater systems are designed to collect human wastes and inevitably contain waterborne, enteric pathogens that are classically known for their faecal-oral route of transmission. Major groups of microorganisms and pathogens present in wastewater are listed in Table 2.2 of Chapter 2, but generally summarised here. The three major classes of pathogens present in wastewater are enteric and opportunistic bacteria, parasites (helminths and protozoa), and viruses.

1.2.1 Enteric bacterial pathogens in wastewater

Bacteria were the first pathogen group to be addressed by water treatment processes. Waterborne typhoid fever and cholera outbreaks were rampant before and during the time Robert Koch identified the “contagion” that caused these diseases and proved their infectious nature. The “germ theory” predetermined the whole idea of water treatment and sanitation (National Research Council (US) Safe Drinking Water Committee., 1977).

The environmental conditions during the biological (secondary) wastewater treatment process are designed to favour the growth of microorganisms responsible for removal of excess nutrients and organic pollutants, and considered detrimental for most human bacterial pathogens (Tchobanoglous *et al*., 2003). Most enteric bacteria during secondary treatment are either ingested by indigenous protozoa in activated sludge or fail to compete with indigenous bacteria and decay. Nonetheless, there is recent evidence of some treatment-resistant enteric bacteria, such as a subset of *E. coli* and *Arcobacter* spp. (Banting et al., 2016; Zhi et al., 2019) , which may be particularly problematic in passing antimicrobial resistance (AMR) on (Böhm *et al*., 2020). In addition, residual faecal bacteria may survive in secondary effluent as either culturable or viable but non-culturable (VBNC) forms (Mansfeldt *et al*., 2020; Rizzo *et al*., 2013).

In acute gastrointestinal infection, a person can shed up to $10^{9\text{-}10}$ colony-forming units (CFU)/g of faeces. However, most enteric bacteria are not highly infectious with pathogens like *Salmonella* Typhi and *Vibrio cholerae* requiring some 10^{5}-10^{6} cells to cause a 50% infection probability. Of all *Enterobacteriaceae* pathogens, enterohemorrhagic *E. coli* (EHEC) is the most infectious reported – likely with as few as 10 cells (Health Canada, 2013). Surveillance reports from 1990 to 2000 implicated EHEC in roughly 6-7% of drinking water outbreaks in the UK and USA (Craun *et al.*, 2006; Smith *et al.*, 2006). However municipal wastewater seems to be an unlikely source of EHEC due to their generally low prevalence and environmental persistence (Boczek & Rice, 2004; Osuolale & Okoh, 2018). Other pathogenic sequence types (ST), such as *E. coli* ST131(Banerjee & Johnson, 2014;

Nicolas-Chanoine *et al*., 2014), however, appear to cause infection in people exposed to treated sewage outfalls (Leonard et al., 2018).

The average size of enteric bacteria is about 1-5 µm, which allows microfiltration treatment of secondary effluent to effectively remove most (Ghayeni *et al*., 1999). Furthermore, bacteria are the group of waterborne pathogens most susceptible to all kinds of disinfection (ultraviolet [UV], chlorination, ozonation) and to all treatments in general, since the technologies were historically developed with bacterial pathogens in mind (National Research Council (US) Safe Drinking Water Committee., 1977).

1.2.2 Parasites present in wastewater

There are relatively few waterborne parasite species, specific to humans, likely to be problematic in treated wastewaters. Of particular note are *Cryptosporidium hominis* and *Enterobius vermicularis* due to their persistence (Rudko *et al*., 2017; Yang *et al*., 2013) and *Giardia intestinalis* due to its higher prevalence in society and cysts numbers in sewage (Hamilton *et al.,* 2018). Helminth ova are the largest-sized pathogens present in wastewater (ova average in size from 10-15 to 30-50 µm), but less frequent than the smaller cysts/oocysts (4-18 µm) of parasitic protozoa which partition less so to the sludge residuals (Fuhrimann *et al.,* 2016). Most of the parasitic protozoa are zoonotic species, of which *Cryptosporidium* and *Giardia* spp. are well known to cause large waterborne outbreaks via drinking water (Efstratiou *et al*., 2017). Saprozoic parasites (like free-living amoebae) are common natural inhabitants of aquatic environments, with *Naegleria fowleri* of particular concern in warm waters (Bartrand *et al*., 2014).

The removal of helminth ova begins in primary settling clarifiers by gravity force. Addition of coagulants (positively charged) further improves flocculation of mostly negatively charged ova. Ova of some helminth species, like *Enterobius vermicularis* have relatively low density and may bind less to suspended substances present in wastewater, that results in less than one log-reduction during primary and secondary wastewater treatment (Rudko *et al.*, 2017). Also, ova, are less susceptible to disinfection than bacteria with *Ascaris* eggs reported as the most UV-resistant water-related pathogen identified to date (Brownell and Nelson, 2006). If the reclaimed water has not undergone advanced treatment (filtration) and is intended for crop or urban irrigation, then helminth ova should be closely monitored, especially *Ascaris* due to its high prevalence in poorer demographic regions (up to 90% of all ova present in wastewater) and due to its low infectious dose (Jiménez, 2007).

For parasitic protozoa, like *Cryptosporidium* and *Giardia*, the likelihood of infection occurs with as few as 10 oocysts (Steiner *et al.*, 1997) and depends on the immune status of the person. However, the zoonotic *Giardia* species complex, results in 100-1000-fold more cysts in wastewater than *Cryptosporidium* oocysts and if not disinfected is the more problematic protozoa in wastewater (Schoen *et al*., 2017).

Overall, cyst of parasitic protozoa and helminth ova are at the lowest concentrations in treated wastewaters among water-relevant groups of pathogens due to lower concentrations in faeces and their large size resulting in deposition losses to solids/sediments.

1.2.3 Enteric viruses in wastewater

Viruses are the smallest of waterborne pathogens, being between 20 and 100 nm. Virus particles (virions) consist only of nucleic acid and a protein capsid (sometimes enveloped), and require a host cell to replicate (Mahy and van Regenmortel, 2008).

There are more than 120 enteric viruses from different phylogenetic groups known to cause a range of diseases in humans (*M48 Waterborne Pathogens*, 2006). The first virus to be linked to water was poliovirus (Baicus, 2012). But thanks to vaccination campaigns poliovirus is no longer a serious concern as a waterborne pathogen. However, other enteric viruses causing diarrhea and hepatitis (e.g. *Norovirus, Enterovirus, Rotavirus* & Hepatitis A virus) are of public health significance and have become key targets for detection and removal (WHO, 2017b).

A key feature of enteric viruses is that infected individuals shed extremely high numbers of virions. *Norovirus* and hepatitis A can reach 10^{11} virions per gram of stool (Bosch *et al.*, 2008; Costafreda *et al.*, 2006) and astrovirus up to 10^{13} virions/g (Caballero *et al.,* 2003). Therefore, enteric viruses are at least three orders of magnitude higher than bacteria and six orders of magnitude higher than protozoa in sewage. Taking into consideration their extremely low infectious doses - less than 10 virions for noroviruses (Yates, 2013); 15-50 virions leading to a tissue culture infection dose of 50% of cells ($TCID_{50}$) for coxsackieviruses (Couch *et al.*, 1965); about 5 virions for adenoviruses (Musher, 2003), prolonged shedding by either symptomatic or asymptomatic carriers, relatively high resistance to water treatment process and disinfectants, and high persistence in the environment (Xagoraraki *et al.*, 2014) - the WHO and other regulators

require the highest reductions of enteric viruses to minimize risks from waterborne exposures (Schoen *et al.,* 2017). In other words, to achieve the same tolerable disease burden (of 10^{-6} Disability-Adjusted Life Year (µDALY) per person per year as recommended by the WHO) the log reduction target for enteric viruses must be the highest among the three major groups of pathogens (WHO, 2006b).

The WHO guideline requires a total virus reduction of 2–3 $\log_{10}$ in reclaimed wastewater for restricted irrigation (the irrigation of all crops with the exception of the crops for fresh consumption) and a 6–7 $\log_{10}$ reduction for unrestricted irrigation (including the crops that may be consumed raw) or urban ornamental irrigation (WHO, 2006b). At the other extreme, potable reuse of wastewater in the State of California requires 12 $\log_{10}$ reduction (State of California, 2016). These high log-reductions currently necessitate spiking studies under controlled conditions to validate treatment system performance (Zimmerman *et al.,* 2016).

1.3 Risk management controls and targets for safe reuse (& hence rationale to identify an on-line virus reduction surrogate of treatment performance)

Managing risk of infection due to recycled water use is an absolute requirement for the water industry to assure public health protection through safe reclaimed water uses. Although it is impossible to achieve zero risk, effective risk management ensures the level of risk “so negligible that a reasonable well-informed individual need not to be concerned about it” (Hrudey *et al.*, 2006). Waterborne risk management is a part of the holistic framework known in many jurisdictions as a water safety plan (WSP, WHO 2006). It begins

with risk assessment and via acceptable risk–based health targets and environmental exposure assessment provides basis for risk control measures and interventions, critical control points, and analytical and public health verification (Bartram *et al.*, 2001). As a part of risk assessment, Quantitative Microbial Risk Assessment (QMRA) – based hazard analysis helps to identify hazardous microorganisms and events and provides focus on the most significant hazards in terms of public health outcomes. It also aids in pinpointing control measures (or "barriers") that reduce hazardous agent entry (source control), concentration reduction (by treatment barriers), and prevent pathogen proliferation in the water system (engineered control) (Medema and Ashbolt, 2006).

Historically faecal indicator bacteria (FIB) have been used to detect sewage contamination of natural waters or wastewater treatment performance to indicate possible presence of bacterial pathogens like *V. cholerae* and *S*. Typhi. But advances in wastewater treatment over the last century have changed the overall situation in public health. At present viral pathogens are considered to be the priority hazard in reclaimed wastewater and require special attention due to their high prevalence and low infectious doses (Gerba, 2007), relative resistance to some water disinfection treatment (WHO, 2011) and prolonged survival in water (Gerba *et al.*, 2017; Grabow, 2007; Rusinol & Girones, 2017).

Surrogates of human enteric viruses like F-specific coliphage MS2, murine noroviruses, or the sewage indigenous plant virus, pepper mild mottle virus (PMMoV), have been proposed to detect faecal contamination or to estimate virus removal during WW treatment (Ahmed *et al*., 2019; Boudaud *et al*., 2012; Hamza *et al*., 2011; Langlet *et al*.,

2009; Park *et al.*, 2011). Virus removal efficiency can be accurately determined by well-established electron microscopy- and molecular-based methods of virus detection/enumeration in water and the data used in system assessment for risk management planning. However, these methods are not suitable for operational monitoring due to time and labour requirements, low pathogen concentrations, or presence of amplification reaction inhibitors (Lucena & Jofre, 2014). Regular virus-spiking is not practical at large-scale plants. Recently, the attention of the research community has been drawn to indigenous viruses in wastewater, mostly bacteriophages and PMMoV, as possible surrogates of human enteric virus removal. The indigenous wastewater bacteriophages outnumber pathogenic viruses by several orders of magnitude, have similar morphological and biochemical properties, are randomly distributed in a sample, and do not require spiking of pure cultures. Moreover, the burst size of bacteriophages averages 10^2-10^3 virus particles per bacterium at the time of the cell rupture (Delbruck, 1945), which makes indigenous bacteriophages the most numerous group of microorganisms present in water (Clokie *et al.*, 2011). Therefore, they are good candidates for monitoring water treatment performance. Unfortunately, most bacteriophage assays are either time-consuming culture- or Polymerase Chain Reaction (PCR)-based and too slow and infrequent for timely process control (Medema & Ashbolt, 2006; Salter *et al.*,2010; US EPA, 2015). With the development of new sensor- or fluorescence-based techniques for online virus monitoring this drawback could be overcome. Unlike fluorometric methods for on-line bacterial monitoring of waters, there is currently no

method developed nor validated approach for on-line viral monitoring of water treatment performance (Rockey *et al.*, 2019).

1.4 Thesis scope, aims and chapter outlines

As described above, water reuse seems an inevitable part of the solution to supply source water to our increasing urbanized population. While there are validated advanced water treatment technologies and water safety management plans to provide safe reclaimed wastewater for various applications, one of the weakest links in this process is day-to-day validation of human enteric virus removal from wastewater. As an acute hazard, human enteric virus breakthrough of treatment for even short periods could result in an outbreak in the receiving population. Therefore, a real-time method of virus abundance estimation at critical control points is considered urgent and prudent to ensure safe use of reclaimed water, especially for unrestricted irrigation, non-potable, and potable reuse. Monitoring viral pathogens, especially after multi-process wastewater treatment processes, remains a challenge because currently employed methods usually have detection limits that are higher than virus concentrations considered to be safe (Chaudhry *et al*., 2017) and provide results with turn-around times unsuitable for many applications.

First attempts to enumerate viruses using flow cytometry were made by marine biologists. Initial sample preparation and analysis protocol were originally proposed by Marie *et al.* (1999) and these methods have not changed much since: the sample is first microfiltered to remove large debris and bacterial cells and then stained with deoxyribonucleic acid (DNA)-binding fluorescent dye (SYBR® Green I) at high temperature

to facilitate the stain access to viral DNA. Multiple studies of marine viruses (Marie *et al.*, 2001; Roudnew *et al*., 2014; Tomaru & Nagasaki, 2007) and some wastewater studies (Brown *et al.*, 2015; Brown *et al*., 2019) employed this particular method. Hence, initial experiments were set up based on these published results for easier comparison of our findings. It has long been assumed that marine and other water environments are dominated by bacteriophages with DNA genomes (Dion *et al*., 2020; Weinbauer, 2004; Wommack & Colwell, 2000). However, it has also been shown that picorna-like RNA viruses are present in marine environments and are largely not detected by common fluorescence-based methods (Steward et al., 2013). Of all sequenced bacteriophages less than 10% are RNA or single strand DNA viruses, and 90% are double stranded DNA bacteriophages (Hatfull & Hendrix, 2012). Analysis of sewage viromes has shown that 67% of assigned contigs belonged to bacteriophages, of which 92% had a DNA genome (Gim *et al*., 2014). Based on the above, it was assumed that the dominant fraction of DNA viruses in wastewater can be representative enough to reflect virus removal during the treatment process.

Therefore, the **long-term goal** of this research was to develop and validate a sensitive and accurate method for near real-time monitoring of indigenous viruses suited to verifying wastewater treatment prior to reuse.

The specific objectives of this research were to:

- Evaluate Flow Cytometric (FCM) methods for bacteriophage enumeration and describe an effective flow virometry approach for wastewater virus quantification;

- Identify fluorescent interference by SYBR® Green colloidal suspensions and chemical constituents of wastewater matrix in flow virometry; and
- Evaluate Fluorescence Excitation Emission Matrix Scanning (FEEMS) as an alternative method for bacteriophage estimation and develop an approach for wastewater analysis.

Chapter 2 reviews the scientific background for this thesis research.

My first research objective is addressed in Chapter 3, which presents the publication "Colloid chemistry pitfall for flow cytometric enumeration of viruses in water", in Water Research X, doi: 10.1016/j.wroa.2019.100025. This chapter explores the colloid chemistry mechanisms behind SYBR® Green I fluorescent signal formation and the emulsion-forming behaviour of fluorescent DNA-staining dyes in aqueous solutions. Additionally, the sensitivity and accuracy of FCM for enumeration of DNA-stained model bacteriophages λ, P1, and T4 were evaluated.

Chapter 4 addresses the second research objective and a manuscript, titled "Outer limits of flow cytometry to quantify viruses in water", submitted to ACS Environmental Science & Technology Water. Chapter 4 describes the current use of flow virometry as neither sensitive nor accurate enough to meet log-reduction targets described to manage water reuse. It focuses on the interference of organic carbon-rich wastewater background with the virus resolution and on the lower-than-expected virus estimates from wastewater.

The third research objective is presented in Chapter 5, the manuscript titled "Optimizing fluorescence-based assay for virus monitoring of wastewater", is prepared for

submission to ACS Environmental Science & Technology Water. Here we describe a procedure to increase the sensitivity and resolution of a fluorescence scanning-based assay for virus quantification suited to wastewaters. The protocol proposed in this chapter could be implemented for on-line virus monitoring to assess times of poorer virus removal performance.

Chapter 6 presents the conclusions, their significance to the water industry and public health, as well as future research directions.

Appendix 1 describes the attempt to label viruses using fluorescently labeled recombinant DNA- and RNA-binding proteins.

Chapter 2: Literature review

2.1 Municipal Wastewater Composition

Municipal, or domestic, wastewater includes wastewater discharged from residences, public buildings such as hospitals or schools, and commercial facilities such as restaurants, sports arenas, office buildings, *etc*. It is usually collected in sanitary sewers and largely gravity transported to wastewater treatment facilities. In some cities, for example in older areas of Edmonton, stormwater is deliberately mixed with sewage in what are termed combined sewer overflows (CSOs) that transport as much of the mix as possible for downstream sewage treatment.

The major contaminants of concern in municipal wastewater are suspended solids, biodegradable organics, nutrients and pathogens (Huang *et al*., 2010; Lakatos, 2018; Sima, 2015). However, over 99.8% of municipal wastewater is water (Salvato, 1992), an increasingly sought after resource in urban areas.

2.1.1 Chemical composition, solids particle size classification

All the contaminants present in wastewater, except dissolved gases, contribute to its total solids burden. Solids are typically defined in the water industry as filterable (dissolved) or non-filterable (suspended); by chemical characteristics – organic (volatilized) that get oxidized when heated at 550°C and inorganic (fixed) that does not; and by settleability – settleable are the solids that are able to settle within 1 hour without mixing (Von Sperling, 2015). More generically, solids can be defined as suspended, colloidal, and dissolved – all aspects relevant to the analysis of virus-size particles. The size-based

classification of wastewater solids is illustrated in Figure 2.1. In the context of the current study, it is important to note that viruses are positioned within the colloidal fraction of the solids.

Suspended solids consist of fixed matter such as fine sand and silt, as well as fibre, human and food waste. Biodegradable organics consists of 40-60% nitrogen-rich protein, 20-50% carbohydrates, and 10% lipids (Peavy *et al.*, 1985). Nitrogen in raw sewage is present mainly in the form of proteins, urea, and ammonium (as the first stage of organics decomposition). Phosphorus is present as a part of organic matter (*e.g.* nucleic acids within microorganisms) or laundry aides (*e.g.* trisodium phosphate). The organic matter present in wastewater is of particular importance as one of the most serious water pollution problems since, along with ammonium, its biodegradation (measured by the biochemical oxygen demand test) leads to oxygen depletion by microorganisms. On average humans produce 20-30 g biochemical oxygen demand over five-day test (BOD_5) *per capita* per day (Polprasert and Koottatep, 2017). BOD_5 in "strong" (organic-rich) wastewater may reach 400 mg/L, but municipal wastewater typically averages some 250 mg/L (Tchobanoglous *et al.*, 1991).

Organic matter analysis in terms of its chemistry is not practical for routine wastewater operations. Instead, indirect methods of organic matter quantification such as biochemical and chemical oxygen demand (BOD and COD), as well as direct total organic carbon (TOC) quantification are used. The COD/BOD proportion is used to characterize the total/biodegradable organic matter; low proportion shows high levels of biodegradable matter and is an indication for biological treatment. For raw domestic sewage the

COD/BOD proportion varies from 1.7 to 2.4 (Von Sperling, 2015). As the wastewater process progresses, the COD/BOD ratio increases due to removal of the biodegradable fraction, while the recalcitrant organic fraction remains relatively stable.

Besides traditional wastewater contaminants like excessive nutrients and biodegradable organics, many other minor (by the concentration, not the potential impact) pollutants like dioxins, endocrine disruptors, detergents, pharmaceutical residues, *etc.* might be present and are considered contaminants of emerging concern (Eriksson *et al.*, 2002; Huang *et al.*, 2010).

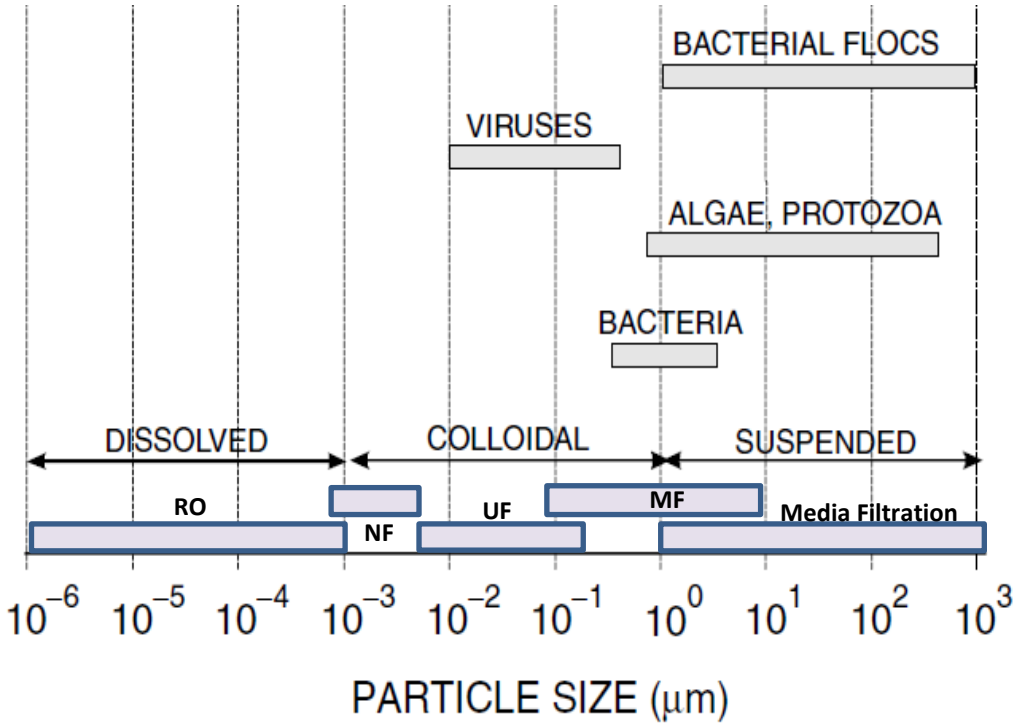

Figure 2.1 Classification of wastewater solids by particle size and membrane process classification. Adapted from: US EPA 2001 and von Sperling 2015.

2.1.2 Microorganisms in biological wastewater treatment

In Section 2.2, the various stages and purposes of municipal wastewater treatment are reviewed. Here, the roles microorganisms play are discussed, given their major roles in secondary wastewater treatment (following physical settling of solids and grease removal in primary treatment), and which have representation from all major groups of organisms (Table 2.1) (Ruggiero *et al*., 2015). In biological wastewater treatment, microorganisms decrease BOD, nutrients (P, N) to lessen eutrophication of the receiving water bodies and remove/inactivate pathogens. Biological secondary treatment occurs optimally at a ratio of carbon, nitrogen, and phosphorus of 100:5:1 (Bitton, 2010). Relevant to the current thesis, there are two main groups of viruses within sewage, those excreted by humans (including human-infectious enteric viruses and phages to the gut bacteria and archaea (Manrique *et al.,* 2016; Paez-Espino *et al.,* 2016)) and viruses that infect microorganisms active in wastewater treatment (Barr *et al*., 2010). The dynamics of these two groups is discussed in Section 2.1.3. Below is a general introduction to pathogens in raw sewage to highlight the critical need to identify enteric virus removal during operation of wastewater treatment plants.

Table 2.1 Major groups of microorganisms present in wastewater.

Major group [A]		Main characteristics	Role in wastewater
Prokaryota	Bacteria	Unicellular organisms of various shapes and sizes from 2-5 to 100 µm or more. Majority has peptidoglycan cell wall. Many species are pathogenic to humans.	Play leading role in aerobic and anoxic stages of wastewater treatment. Responsible for oxidation of organic matter and nutrient transformation (*e.g.* nitrification/denitrification). Produce exopolysaccharides that aid flocculation of microbial biomass during final clarification.
	Archaea	Resemble bacteria in morphology and size. Have no peptidoglycan. Some metabolic pathways are closer to eukaryota rather than bacteria. No human pathogens are known from this group.	Constitute a large portion of microorganisms present in anaerobic sludge digesters. Methane is produced exclusively by archaea.
Eukaryota	Protozoa	Unicellular organisms with membrane-bound nuclei. May form colonies. No rigid cell walls. Aerobic or facultative. Feed themselves on other microorganisms or detritus. Some species are human pathogens.	Protozoa mainly graze on bacteria, aiding in faecal bacteria removal. They are most often found in activated sludge. The composition of protozoan species indicates the BOD removal efficiency of the treatment process.
	Chromista	Unicellular or multicellular eukaryotic organisms. Possess plastids with chlorophyll C. This group includes organisms, formerly classified as Protozoa	Play the same role as protozoa and are mostly found in activated sludge.

	(some flagellates) and Algae (diatoms).	
Plantae	Autotrophic eukaryotes with rigid cellulose cell wall. In wastewater they are mostly represented by algae.	Algae are not present in sewage usually. They mostly develop in lagoons and settling ponds. [B]
Fungi	Heterotrophic uni- or multicellular organisms with chitin cell wall. Involved in decomposition of organic matter. Some species are pathogenic (allergenic) to humans or produce toxins	Fungi can be found in sewage, but mostly they are a part of activated sludge. Their biodiversity might be quite high. Some species are involved in sludge bulking and can negatively impact the treatment efficiency.[C,D]
Animalia	Multicellular heterotrophic eukaryotes. Some species are human parasites.	Rotifers are usually present at later stage of activated sludge treatment. They actively feed on low numbers of suspended bacteria due to strong ciliary action. Nematodes might also be present, but their role in activated sludge is questionable.[B]

A - (Ruggiero et al., 2015); B - (Bitton, 2010); C - (Assress et al., 2019); D - (Haihan Zhang et al., 2018)

2.1.2.1 Pathogens

Municipal wastewater is a major potential source of human pathogens released into the environment, and hence a core focus for treatment/management. Representatives of the major groups of pathogens present in wastewater are listed in Table 2.2. The species composition and the load of pathogens in wastewater depends on many factors: geographic region and climate, socio-economic conditions a human population lives in, contribution of agricultural wastewater, especially from animal husbandry, or the level of treatment the sewage was subjected to (Von Sperling, 2015).

A water-related disease can be defined as any significant or widespread adverse effects on human health, caused by inadequate water quality and/or availability (Bartram *et al.*, 2017; Stanwell-Smith, 2010). The elements of this definition are shown in Figure 2.2 and include the components of classical epidemiological triad: external agent (pathogen), susceptible host, and the environment that brings the agent and the host together (water). The water-related diseases are commonly divided into four categories (Table 2.3), depending on the source of infection and the mode of transmission.

Municipal wastewater also harbours a large number of antibiotic resistant bacteria and it is one of the major sources of antibiotic resistance genes released into the environment (Almakki *et al.*, 2019; Bouki *et al.*, 2013; Rizzo *et al.*, 2013). Horizontal gene transfer is known to occur in wastewater treatment plants (Kumar and Pal, 2018) and mainly takes place in aeration tanks (Tong *et al*., 2019). It occurs via direct uptake of naked DNA (transformation), conjugation and exchange of mobilfe genetic elements like plasmids and

transposons, or viral infection (transduction) via bacteriophages (Colavecchio *et al.*, 2017; Thomas & Nielsen, 2005). The latter will be addressed in more detail in the next section.

Table 2.2 Common pathogens in municipal wastewater [A]

Group of organisms	Species	Numbers in WW (per L)	Infectious dose
Viruses	Adenovirus 40,41	up to 10^6	unknown
	Norovirus	up to 10^6	<10
	Rotavirus	10^2-10^5	1-10
Protozoa	*Cryptosporidium parvum*	1-10^4	1-5
	Enthamoeba histolytica	1-10^2	~10^3
	Giardia lamblia/intestinalis	10^2-10^5	10
Helminths	*Ascaris lumbricoides*	up to 10^3	unknown, low
	Trichuris trichiura	1-10^2	unknown, low
	Ancylostoma sp.	up to 10^3	unknown, low
	Taenia sp.	1-10^2	unknown, low
Bacteria	*Campylobacter* spp.	10-10^4	10^4
	Salmonella enterica	1-10^5	10^3, S. Typhi - 10^5
	Shigella dysinteriae	10-10^4	10-200
	Vibrio cholerae	10^2-10^5	10^3-10^7

[A] Modified from: (Blaser & Newman, 1982; Chaoua *et al.*, 2018; Chen *et al.*, 2013; Fong *et al.*, 2010; Government of Canada, n.d.; Graham *et al.*, 1987; Jiménez, 2007; Steiner *et al.*, 1997; Stelzer *et al.*, 1988)

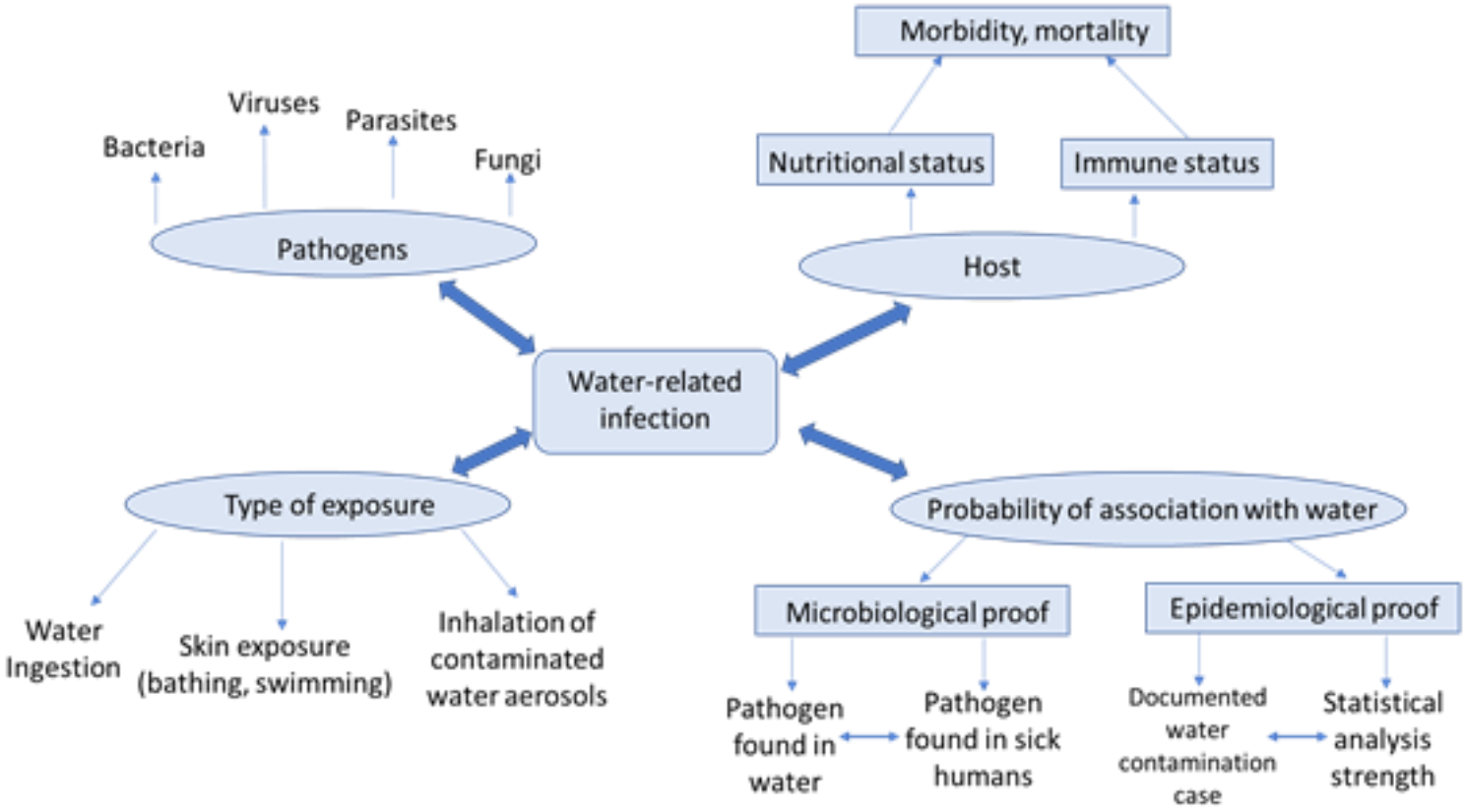

Figure 2.2 Major components of water-related disease. Adapted from: (Bitton, 2014; Stanwell-Smith, 2010; Von Sperling, 2015).

The direct detection of pathogenic microorganisms in wastewater requires time-consuming, costly, or high-detection limit methods, even today. For the same general reasons the US Public Health Service adopted coliform bacteria as an indicator of faecal contamination of drinking water in 1914 (Yates, 2007). Indicator microorganisms are utilized for three major purposes (Ashbolt *et al.*, 2001): as a treatment process performance surrogate (*e.g*. total coliforms & heterotrophic plate count for disinfection efficacy), as a faecal contamination indicator (*E. coli*, enterococci), and as index/model organisms to indicate pathogen presence or behavior. In 1966, Bonde outlined the criteria for an "ideal indicator", these being as follows: 1) must be present whenever a pathogen is present; 2) should not proliferate in water environment; 3) should outnumber the pathogen; 4) should be as resistant to disinfectants and survive longer in the environment; 5) readily grow on simple media and have characteristics for unambiguous identification; 6) be randomly distributed in the sample; and 7) grow independently of other organisms present. Other authors also required it to be a member of the warm-blooded animal intestinal microbiota (Bitton, 2010) and pose no risk to analyst (Payment *et al.*, 2003). The major groups of indicator bacteria are listed in Table 2.4. However, Lamendella *et al.* (2008) question the use of Bifidobacteria as pollution indicators due to their low persistence in the environment and stringent cultivation requirements. Recently, there is growing evidence of *E. coli* being an active member of environmental microbial communities and which challenges its use as an indicator of fecal pollution and wastewater treatment efficiency (Ishii & Sadowsky, 2008; Zhi et al., 2019)

Table 2.3. Categories of water-related diseases

Category	Route of transmission	Description	Examples
Water-born	Faecal-oral	Disease is related to ingestion of water that contains human or animal pathogens due to faecal water contamination.	Poliomyelitis, Hepatitis A, typhoid and paratyphoid fever, giardiasis, cryptosporidiosis, dysentery (amoebiasis and shigellosis), rotavirus, *etc*.
Water-based	Sapronotic, faecal-oral	Disease is caused by a pathogen, which naturally inhabits or spends part of its life cycle in water ecosystems.	Leptospirosis, cholera, yersiniosis (*Y. pseudotuberculosis*), schistosomiasis, guinea worm, *etc*.
Water-related	Vector	Vector insects breed in water or bite near water bodies.	Malaria, virus fevers (dengue, west Nile, yellow), African trypanosomiasis, filariasis
Water hygiene	Contact, faecal-oral, vector	Diseases occur due to low availability of water and resulting poor hygiene.	Trachoma, pediculosis, scabies, typhus (rickettsiosis), bacterial and fungal skin infections, and infections listed in the water-born section.

Modified from: (Grabow, 2010; Kulikalova *et al.*, 2014; Litvin *et al.*, 1997; Somov & Litvin, 1988; Von Sperling, 2015)

Table 2.4 Indicator microorganisms

Indicator	Description	Applications
Coliforms (total, thermotolerant, *E. coli*)	Historically first and most widely used group of indicator microorganisms. Easy to detect and identify. Sensitive to disinfection. Under favorable conditions might regrow in water or wastewater. Environmental growth unrelated to fecal pollution has also been reported.	Were used as indicators of faecal pollution by warm-blooded animals. Generally, do not correlate with protozoan or viral pollution or removal by water treatment interventions.
Enterococci	More resistant to water treatment process and persist longer in the environment than the coliforms. Rarely multiply in environmental waters. Easily cultivated.	Indicators of ground water and marine bathing waters faecal contamination.
Clostridium perfringens spores	Very resistant in the environment. Relatively resistant to many water and wastewater treatment processes. Easily cultivated.	Indicator of past faecal pollution. Surrogate for parasite and virus removal.
Bifidobacteria	Some species are uniquely associated with humans and do not proliferate outside the mammalian gut. They have stringent growth requirements that make them somewhat problematic to detect. In some forms of intestinal dysbiosis their numbers in the human intestine are severely reduced.	Might help to indicate fresh fecal pollution, as well as distinguish between human and animal faecal pollution. However, their wide use as indicator organisms is questionable.
Heterotrophic Plate Count Bacteria	A general test for heterotrophic bacteria enumeration as indicator for disinfection efficacy, not of potential faecal pathogens.	Might indicate problems with water treatment process, as well as potential for regrowth.
Bacteriophages	*Bacteroides fragilis* bacteriophages, somatic & F-specific coliphages, crAssphage. Specific parasites of obligate intestinal bacteria.	Indicators of faecal pollution. Surrogates for virus removal. Indicator of geospatial trends in untreated sewage.

Modified from: (Ashbolt *et al.*, 2001; Bartram, 2003; Bitton, 2010; Ishii & Sadowsky, 2008; Lamendella *et al.*, 2008; Payment & Franco, 1993; Resnick & Levin, 1981; Tsimmerman, 2017; Yates, 2007)

2.1.2.2 Nutrient removal during biological wastewater treatment and role of bacteriophages

Nutrient reduction is a critical step of the wastewater treatment process. Nitrogen and phosphorous must be reduced before the treated water is discharged into receiving waters to minimise eutrophication. It is particularly important if the treated wastewater is intended for potable reuse, as nitrate in drinking water may exert significant adverse health effects (Ward *et al.,* 2018), and nutrients may stimulate saprozoic pathogen growth in water conveyance systems (Ashbolt, 2015).

Nitrogen in domestic wastewater is mostly present in organic form or as ammonium and may reach 85 mg/L (Mara & Horan, 2003). Organic nitrogenous compounds are first transformed into ammonium by a wide variety of bacteria or fungi by means of proteolysis with further deamination of amino acids into ammonium (NH_4^+). The next steps have traditionally been seen as nitrification, a two-step aerobic conversion of reduced nitrogen compounds (ammonium, nitrosamines, *etc*.) to nitrite then nitrate, followed by anoxic conditions for denitrification to nitrogen gase. First, NH_4^+ is oxidized into nitrite by ammonium oxidizers like *Nitrosomonas*, *Nitrosospira*, and others. Then *Nitrobacter*, *Nitrospira*, *etc*., convert nitrite into nitrate. Despite some heterotrophic nitrifiers like *Arthrobacter, Pseudomonas, Alcaligenes*, and others present in wastewater, autotrophic nitrifiers dominate the process (Mara and Horan, 2003).

Denitrification is the reduction of nitrate into nitrogen gas and is the last step of traditional nitrogen removal during wastewater treatment. There are two major ways of biological denitrification: *assimilatory*, in which nitrate is used by microorganisms as a

nitrogen source for protein and nucleic acid synthesis, and *dissimilatory*, in which nitrate serves as an electron acceptor in anaerobic respiration (Bitton, 2010). For dissimilatory denitrification both organic and inorganic molecules might serve as electron donors. Virtually all facultatively-anaerobic heterotrophic microorganisms, typical for wastewater, are able to reduce nitrate. These include *Pseudomonas*, *Bacillus*, enterobacteria, and many others.

However, alternative pathways for nitrogen removal have also been described for wastewater treatment. The process of anaerobic ammonium oxidation (anammox) was first described in detail in 1990s (Van de Graaf *et al*., 1995). Ammonia is oxidized under anoxic conditions: $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$ and ammonium and nitrite contribute equally into this reaction. The microorganisms, responsible for this process belong to the phylum of bacteria, Planctomycetes (Jetten *et al.*, 2005; Zhang & Okabe, 2020).

For all of these bacteria-driven nitrogen reduction processes, bacteriophages are an active part of wastewater treatment systems (Ewert & Paynter, 1980; Khan *et al.*, 2006; Khan *et al.*, 2002; Withey *et al.*, 2005). Indeed, the majority of bacteriophages that are used in clinical applications to treat infections caused by *Pseudomonas, Klebsiella, Proteus, Bacillus* and many others, were originally isolated from wastewater treatment plants (Siatchikhina *et al.*, 2016; Tikunova *et al*., 2016). Viruses play important ecological roles, such as viral shunt (Suttle, 2005) that also has a detrimental impact on the biological treatment process via killing the dominant functional group of bacteria (“killing the winner”). Similar phage attacks are a known issue in dairy industry as they can completely halt the fermentation process (Marcó *et al.*, 2012). Withey (2005) noted that

failure of the nitrification process could be the result of a phage attack, first described for nitrifying bacteria by Wanner *et al*. (2000). To date, no Planctomycetes bacteriophages have been described (Dion *et a*l., 2020), but there is little doubt that finding these phages is just a matter of time. Therefore, the anammox process might also be affected by phage attacks as the anammox community matures and some bacterial species become dominant. Decreased biodiversity lessens the stability of microbials ecosystem, making them more prone to an external impact (Loreau & de Mazancourt, 2013; Marfenina, 2005).

Phosphorus is another nutrient, which is removed via microbial action during the wastewater treatment process. Phosphorous removal process, like nitrification, is also known to be unstable (Withey *et al.*, 2005)

The total phosphorus concentration in wastewater is around 20 mg/L. Around 50-70% come as orthophosphate, the rest is present as polyphosphates (from household detergents) and organic compounds (Bitton, 2010). There are two main ways of P-removal: chemical and biological. While chemical precipitation may be controlled by pH and metal cations like Ca^{2+}, biological removal of phosphorus is more complicated. Biological P-removal can be due to direct assimilation by the wastewater microorganisms and the resulting biomass production, as well as accumulation of polyphosphate (volutin) by certain microorganisms like some enterobacteria (*Klebsiella, Enterobacter*), pseudomonads, and filamentous bacteria (Kawakoshi *et al.*, 2012; Machnicka *et al.*, 2008); with the actual removal via physical separation of these P-accumulating bacteria as solids. As was mentioned above, wastewater harbors bacteriophages, which are likely also active

against P-accumulating bacteria. An activated sludge-isolated *Acinetobacter* spp. accumulates polyphosphate up to 20% of the cell dry weight and is a significant member of phosphate-removing community when assayed by culture (Deinema *et al.*, 1980). However, molecular methods suggest it is a minor player in P-accumulation in practice (Keating *et al.*, 2016; D. Wang *et al.*, 2012). These bacteria are no exception to bacteriophage predation (Jin *et al*., 2012; Li *et al*., 2012; Yang *et al*., 2019; Zhang *et al*., 2015).

Barr *et al*. (2010) reported an unexpected decline in the phosphorous removal performance of a laboratory scale bioreactor. Microscopic analyses revealed an abrupt decrease of a bacterium that dominated the process. The increased numbers of bacteriophage-like particles found in the reactor correlated with the decline in the reactor performance. Addition of the supernatant from the failed reactor to normally functioning reactors caused bacteria decline and process failure in those as well, hence supporting the idea of a phage attack. Satoh *et al.* (2013) using 16S rRNA approach for bioreactor microbial community analysis showed an abrupt selective killing of one of the operational taxonomic units (OUT), which might also be explained by a phage attack.

Therefore, on one hand indigenous bacteriophages in wastewater treatment process might effectively maintain ‘healthy’ biodiversity (Jover *et al*., 2014; Wilhelm & Suttle, 1999), but on the other hand they may reduce the effectiveness of pollutant removal.

2.1.3 Viruses in wastewater (WW): detection, abundance, variety, sources relevant to human health, surrogates (plant viruses, bacteriophages) and ecological roles

Viruses are not considered to be living entities but are obligate intracellular parasites (Moreira & López-García, 2009; Villarreal & Witzany, 2010). There are two major ecological groups of viruses present in wastewater: autochthonous (native) viruses, which propagate *in situ* and include mostly bacteriophages since bacteria are the dominating virus hosts in WW (Bitton, 2010; Foladori *et al.*, 2010), and allochthonous (extrinsic) viruses, which have human, animal, or higher plant origin. The autochthonous viruses (*sensu strictu* – bacteriophages) play a significant role modulating microbial processes of C/N/P cycling as well as with host numbers control in water environments (Jover *et al*., 2014; Wilhelm & Suttle, 1999), while the allochthonous (waterborne) viruses utilise their inherent persistence to aid in their dispersal and transmission (Atanasova *et al*., 2018; Verani *et al*., 2016).

Viruses can be detected using a variety of methods: culture-based, electron or fluorescent microscopy, immunological methods like enzyme linked immunosorbent assay (ELISA), molecular methods like PCR, or combination of these methods (*e.g.,* immunofluorescent microscopy, molecular beacons, or fluorescent protein-based reporters). Viruses as biological objects were discovered in culture by Ivanovsky, Bejerink and d'Herelle (D'Herelle, 2011; Lecoq, 2001) but remained abstract until the invention of transmission electron microscope (Ackermann, 2012). To this day electron microscopy remains the most informative method to study virus morphology, virus life cycle and its interactions with the host, as well as to identify the viral nature of an emerging infection

(Roingeard, 2008). In water research, transmission electron microscopy (TEM) unambiguously proves the identity of particles counted as virus and should serve as the "gold standard" for development of new methods of virus enumeration. But despite their obvious advantages for virus enumeration, direct microscopy (TEM or fluorescent microscopy) cannot be used for virus identification like molecular or immunology-based methods. Hence, for detection of specific pathogenic viruses of interest, one needs to use virus-specific molecular methods (Costafreda *et al.*, 2006; Eftim *et al.*, 2017; Kopecka *et al.*, 1993) or antibody-based methods (Fumagalli *et al*., 2018; Khamrin *et al.*, 2008; Qian *et al*., 2015). Some pathogenic viruses can be detected only by molecular techniques as there are no culture methods for them. Molecular techniques are also useful for genotyping and phylogenetic characterisation of viruses that might help to identify the source of fecal contamination in water (Girones *et al*., 2010). However, specific PCR conditions, primers and probes that are used, and presence of inhibitors can significantly affect sensitivity and accuracy of the method (Bofill-Mas et al., 2006). PCR-based techniques do not provide reliable information about virus infectivity (as will be discussed below). In case of common water treatment interventions like chlorination or UV the results of viral genome quantification do not always reflect the reduction in infective virus numbers (Girones et al., 2010). Both optical and molecular methods of virus detection have their niche in water research and analysis. There are pros and cons for each technique and the choice should be the result of careful consideration of many factors from study objectives to properties of an individual sample.

Total virus-like particle (VLP) counts obtained using epifluorescent microscopy are fairly consistent among many studies of sewage and amount to 10^8-10^9 VLP/mL (Cantalupo *et al.*, 2011; Otawa *et al.*, 2007; Pollard, 2012b; Wu & Liu, 2009a).

Raw sewage virus diversity is vast and largely remains to be explored (Cantalupo *et al.*, 2011; Ng *et al.*, 2012; Wu & Liu, 2009b). A recent culture-based study of somatic coliphages in raw sewage revealed 104 phages out of 48 Danish wastewater treatment plants (Olsen *et al.*, 2020). Ninety-one of those phages were described for the first time. Two groups (Cantalupo *et al.*, 2011; Wang *et al.*, 2018) reported that among all the deep-sequenced viral assemblages they analyzed, 66% to 99% of the sequences had no significant similarities in the databases – all highlighting our lack of knowledge in sewage virus diversity.

Human pathogenic viruses were reported by Cantalupo *et al.* (2011) to represent some 6% of the identified eukaryotic viruses and only 0.16% of the total assemblages analyzed. They belonged to eight families: *Adenoviridae, Astroviridae, Caliciviridae, Papillomaviridae, Parvoviridae, Picobirnaviridae, Picornaviridae*, and *Polyomaviridae* (Cantalupo *et al.*, 2011). The same group also showed that raw sewage virome is dominated by bacteriophages of the families *Microviridae, Siphoviridae, Myoviridae, Podoviridae, and Inoviridae*, thus complementing other authors data about the bacteriophage-dominated viromes of sewage (Luo *et al.*, 2017; Ogilvie & Jones, 2015; Weinbauer, 2004). There are some discrepancies between dominant bacteriophage families reported in different studies. Cantalupo *et al.* (2011) reported *Microviridae* to be the most dominant family and Wang *et al.* (2018) - *Siphoviridae*. At this point it is hard to

say if this is due to the objective differences in samples or due to the differences in the DNA processing, sequencing, and bio-informatics methodology the authors used.

There is only one source of human enteric viruses in water systems, which is widely accepted by the research community: human excreta (Allmann *et al.*, 2013; Grabow, 2007; Ji *et al.*, 2012; Rusinol & Girones, 2017), given the low likelihood of zoonotic viruses being present in raw municipal sewage (with exception of potentially zoonotic hepatitis E (Bayhan *et al*., 2016; Yugo & Meng, 2013)). The factors that impact human enteric virus occurrence in sewage include human population density, demographic and immune status structure of the population, and geographical location (Gerba *et al.*, 2013).

Traditionally most enteric viruses present in domestic wastewater are viewed as highly specific human pathogens (Ahmed & Harwood, 2019). When this is the case, a parasitic system is defined as a simple self-contained binary (Somov & Litvin, 1988) and it is of high value for human waste pollution source tracking. This definition implies that there are only two components: one pathogen and one host species. Human adenoviruses, noroviruses and human polyomaviruses have been shown to be highly specific to humans (Ahmed *et al*., 2010; Eftim *et al*., 2017; Hundesa *et al*., 2006; Li *et al*., 2015; McQuaig *et al*., 2006; Wolf *et al.*, 2010), though, qPCR methods that are used to quantify these viruses have rather low sensitivity (Wolf *et al.* and McQuaig *et al.* reported) and high uncertainties (Petterson *et al*., 2015). The relatively stable seasonal prevalence of adenovirus (Gerba, 2007; Lucena & Jofre, 2014; Rusinol & Girones, 2017), norovirus (Eftim *et al.*, 2017) and polyomavirus (Farkas *et al.*, 2018), as well as their ubiquitous distribution (WHO, 2005) make them convenient for wastewater monitoring and faecal pollution tracking. Another

proposed indicator of surface water faecal pollution is pepper mild mottle virus (PMMoV) (Hamza *et al*., 2011; Kitajima *et al*., 2018). It caused interest among water researchers after Zhang *et al.* (Zhang *et al.*, 2006) reported high prevalence and high concentration of this virus in human faeces (10^9 virions/g).

Another potential faecal pollution indicator virus reported in 2014 by *in silico* analysis of human faecal phageome (Dutilh *et al.*, 2014) is a bacteriophage, named crAssphage after the cross-assembly programme originally used to discover it. This phage is present in high numbers in all faecal samples analyzed by this group as well as in publicly available metagenomes and forms a common component of wastewater-impacted waters (Ahmed *et al.*, 2019). This 97 kbp DNA phage has been shown to infect various species of *Bacteroides*, one of the major bacterial taxon that inhabits human intestine, potentially contributing into ubiquitous presence of this phage in humans (Yutin *et al.*, 2018). Though the study of the crAss-like viruses is still *in statu nascendi* and more clarity is needed about animal-associated and environmental members of this group, first attempt to use human-associated crAssphages CPQ_056 and CPQ_064 showed high abundance of these viruses in untreated sewage – 4-7 $\log_{10}$ copies/mL (Korajkic *et al*., 2020). High copy number also makes crAssphages a potential surrogate for the wastewater treatment process monitoring.

Though allochthonous viruses might be present in wastewater in relatively high numbers, they still cannot outnumber the autochthonous viruses. As mentioned above, the vast majority of autochthonous viruses in wastewater are bacteriophages. This group of viruses was extensively studied as potential indicators of water qualities and

wastewater treatment processes (Grabow, 2001; Lucena & Jofre, 2014) since Guelin (1948) first suggested coliphages as potential indicators of enteric bacteria (i.e. their potential initially as allochthonous viruses of sewage). Somatic and male-specific F-RNA coliphages as well as phages of *Bacteroides fragilis* are commonly used as models and pathogen surrogates in water quality assessment (Jofre *et al.*, 1986; McMinn *et al.*, 2017; US EPA, 2015) due to their easy and rapid cultivation. There is some evidence, however, that under specific conditions like high densities of host *E. coli* and coliphages at optimal temperature somatic and F-RNA coliphages replicate outside of the human gut in fresh raw sewage (Grabow, 2001; McMinn *et al*., 2017; Nappier *et al*., 2019). Hence, technically, these could be considered as allochthonous. The results for somatic coliphages can be obtained within four hours (Lucena & Jofre, 2014) and they might outnumber pathogenic human viruses by a factor of 500 (Grabow, 2001). This heterogeneous group of coliphages has been found to be an adequate indicator of equally heterogeneous human viruses. Overall, their resistance to treatment interventions and persistence in the environment are much closer to those of human pathogenic viruses when compared with bacterial indicators (Lucena & Jofre, 2014).

F-Specific coliphages (naturally occurring or spiked) are of value as removal process efficiency indicators (surrogates) due to their morphological similarity to human pathogenic enteroviruses, astroviruses, and noroviruses (King *et al.*, 2012). When it comes to the recent coronavirus outbreak and the presence of SARS-CoV-2 in municipal sewage (Medema *et al.*, 2020), an enveloped *Pseudomonas* bacteriophage Phi6 might be an appropriate indicator of human respiratory coronavirus removal as F-specific coliphages

are for enteric viruses, due to its high morphological similarity to coronaviruses, but Phi6 is yet to be robustly evaluated (Silverman & Boehm, 2020). Despite the advantages and relatively low detection limit of coliphages, especially if high volume presence-absence testing is used (Yanko *et al.*, 1999), their indigenous numbers in sewage are still not high enough to ensure the 6 to 12 $\log_{10}$ reduction value (LRV), required by many legislations for water intended for reuse (Amarasiri *et al.*, 2017). Hence, methods of coliphage detection, though fast, are still far from *ex tempore*.

Monitoring total bacteriophage present in wastewater would be more useful for multiple-barrier system performance assessment than indigenous coliphages. Indigenous bacteriophage monitoring could also be cheaper than dosing the system with a cultured surrogate and could be used on continuing basis. The only stipulation is that a sensitive and accurate *ex tempore* method needs to be developed and validated.

2.2 The wastewater treatment process

The problem of human waste disposal has arisen since mankind switched from hunter-gatherer lifestyle to agrarian, and people started living in permanent settlements around ten thousand years ago. Ancient Minoans were the first nation documented to use sewers for waste removal some 3000 years ago (de Feo *et al.*, 2014). They were quickly followed by the civilizations of India, China, Greece and Rome. Romans especially put emphasis on sanitation of the city environment. After the decline of the Roman empire the “sanitation Dark age” lasted until early 19th century (Roccaro *et al.*, 2014). In the 19th century with the industrialization and associated population increase, the demand for sanitation led to the

development of waste collection and stabilization techniques like watertight pits and city-operated sewers. Regular pandemics of cholera (World Health Organization, 2019) and typhoid fever outbreaks added to the pressure. The newly understood role of pathogens in waterborne disease (Koch, 1893) led to the demand for water treatment. The beginning of the 20th century opened the era of the industrial-scale water treatment.

Today in Canada there are some 1,259 wastewater treatment facilities, 1,244 lagoon systems, 6,104 wastewater pump stations, 4,762 wastewater lift stations, and 685 wastewater storage tanks (Statistics Canada, 2018). They process almost 6 billion cubic metres of sewage annually. Less than 5% of the sewage is discharged as untreated overflow (Statistics Canada, 2019).

The following is a brief review of the three main objectives of wastewater treatment:

a. Reduce the content of total organic contaminants (TOC, BOD);

b. Remove excessive nutrients (nitrogen, phosphorous) to prevent eutrophication and saprozoic pathogen growth within receiving or reuse waters; and

c. Remove or inactivate pathogens.

2.2.1 Wastewater treatment steps (primary, secondary, tertiary, disinfection, advanced)

Wastewater treatment is a complex technology that is based upon mechanical, physical, chemical, biological, and physical-chemical methods (Table 2.5). The treatment interventions that rely on physical and mechanical methods are called *unit operations*. Chemical- and biological-based interventions are called *unit processes* (Bitton, 2010). Unit operations and processes might comprise several treatment methods. The integration of

treatment methods makes up the treatment systems. Unit operations and processes can occur simultaneously in the same treatment unit.

Wastewater treatment is usually classified as preliminary, primary, secondary, and tertiary stages (https://www.epcor.com/about/who-we-are/where-we-operate/edmonton/Documents/GoldbarTreatmentProcess.pdf, Fig. 2.3).

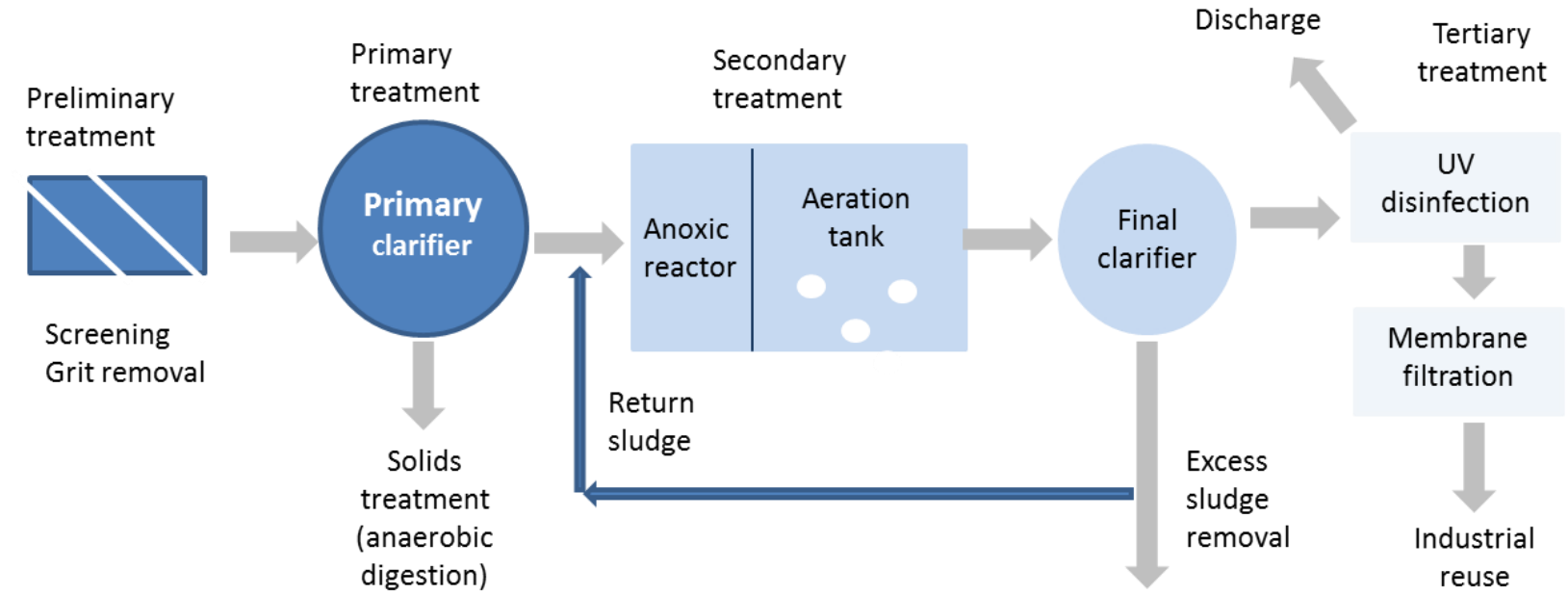

Figure 2.3 Schematic representation of wastewater treatment process at Gold Bar WWTP, Edmonton, Canada.

Table 2.5 Methods of wastewater treatment.

Group	Method
Mechanical	Separation
	Capture
	Settling
	Filtration
	Flotation
	Aeration (air scouring)
	Reverse osmosis
Physical	Evaporation
	Freezing
	Magnetic and electromagnetic treatment
Chemical	Oxidation
	Reduction
	Neutralization
	Complexation
	Precipitation
	Coagulation/flocculation
	Sorption
	Extraction
	Ion exchange
Biological	Aerobic or Anaerobic.
	Natural: Septic fields
	Lagoons
	Engineered: Activated sludge
	Attached microbial growth

Adapted from: (Bitton, 2010; Von Sperling, 2015)

The main objective of the **preliminary** cleaning stage is the removal of large debris and coarse particulate matter by mechanical methods (screening, grit removal, flotation) to reduce the load to subsequent stages (Figure 2.3).

Primary treatment removes non-coarse settleable solids of inorganic and organic nature. This treatment step largely employs physical methods. Chemically enhanced primary treatment includes the addition of coagulate and time/mixing for flocculation-sedimentation. Settling in primary clarifiers removes around 50% of wastewater solids. It reduces the organic load prior the secondary treatment stage, where the BOD removal is more expensive – being driven by costly aeration to stimulate aerobic bacterial activity. The solids accumulated at the bottom (primary sludge) is removed and piped to the anaerobic digesters for subsequent treatment. At this stage phosphorous may also be removed by precipitation/biomass (Muryanto & Bayuseno, 2012; Von Sperling, 2015). Basically, septic tanks, invented over a century ago and mostly used at the individual household level, are primary settling clarifiers. They also have some function as anaerobic digesters due to the long time (months) that the settled solids remain in the tank.

The main objective of the **secondary stage** of wastewater treatment is organic matter reduction by microbial activity, to reduce the loss of dissolved oxygen, which would occur if organic matter was released untreated to receiving waters. The controlled conditions of the process result in biodegradable pollutant removal within a smaller period through the enhanced activity of microorganisms by a limited number of biological wastewater treatment systems (as summarized in Table 2.6).

Biological wastewater treatment processes are driven by complex microbial communities that contain bacterial species with high proteolytic activity (*Bacillus* spp., *Pseudomonas* spp., non-faecal coliforms, *etc*.). As a by-product of this enhanced microbial activity, human enteric viruses are also degraded. For example, Cliver and Herrmann (1972) demonstrated a high antiviral effect of *P. aeruginosa* against Coxsackievirus and showed the uptake of viral protein components as a nutrient by the bacterial cells. Green (1976) and Lund (1983) reported temperature to be the determining factor of enterovirus inactivation with direct correlation of temperature and virus inactivation. Hurst *et al*. (1980) and Lund (1983) also described the positive effect of aerobic conditions on enteric virus inactivation. Overall, the most effective enteric virus removal seems to take place under conditions optimal for the growth of aerobic bacteria with high metabolic activity (see log-reductions in Table 2.6). In nature, high microbial activity occurs within the surface soil zone, hence soil application has long been used for sewage treatment/disinfection.

Table 2.6 Main biological wastewater treatment systems.

System	Brief description	Virus log_{10} reduction
Stabilization ponds	Facultative pond (relatively low depth, large area, oxygen is supplied by photosynthesis); Anaerobic pond (deeper than facultative pond and with smaller area); Facultative aerated lagoon (similar to facultative pond, but oxygen is supplied by aeration); Completely mixed aerated lagoon; High rate ponds (lowest depth that allows the sunlight to penetrate the entire liquid mass, leading to high oxygen levels by photosynthesis); Maturation ponds (high retention time, mainly designed for pathogen removal)	0.8 -2.9
Land disposal	Slow rate system (irrigation of agricultural crops with untreated sewage that provides water and nutrients to plants, it is one of the most ancient treatment methods in human history); Rapid infiltration (lower water evaporation than in slow systems, most commonly was used for ground water recharge); Subsurface infiltration (most commonly it is supernatant from septic tanks, that infiltrates into the soil through a bed of porous material like gravel or sand); Overland flow (Figure 2.4: intermittent application of wastewater into soil – plant root system with subsequent collection of water filtered through soil in drainage furrows); Constructed wetlands (shallow water-based system with aquatic plant growth, in which all the processes take place at soil-root system as well).	1.4 -2.2
Anaerobic systems	Upflow anaerobic sludge blanket reactor (methane-producing digester with three phase separation system, sludge also plays a role of a filter and its production is low); Anaerobic filter (water passes through gravel-filled filter under anaerobic conditions, BOD is removed by biofilm-based microorganisms)	1 – 4

Activated sludge	Conventional (consists of aerated reactor and secondary clarifier tank, very efficient BOD removal due to high microbial biomass concentration and long biomass retention time); Extended aeration sludge (similar to conventional process, but the aeration tank is bigger and biomass retention time longer); Intermittently operated sludge (aeration and clarification processes alternate and take place in the same vessel); Activated sludge with nitrogen removal (the biological reactor consists of anoxic and aerated zones); Activated sludge with nitrogen and phosphorous removal (the biological reactor includes anaerobic, anoxic, and aerated zones)	1.3 – 2.1
Aerobic biofilm-based reactors	Low rate trickling filter (reactor filled with gravel or plastic biofilm carrier, BOD availability to microorganisms is low due to the low rate of feed water, no excess sludge is formed); High rate trickling filter (high BOD availability, excess sludge needs to be further processed); Rotating disc biological contactor (can combine aerobic and anaerobic environment, 8 to 10 times more efficient as trickling filters due to high microbial load on the discs); Submerged aerated biofilter (biofilm is developed on the surface of the support medium (*e.g.* Pall rings) that floats in the liquid)	0.9 - 2.4

Adapted from: (Barrios-Hernández *et al.*, 2020; Cheremisinoff, 2019; Nicosia *et al.*, 2001; Pal, 2017; Rao *et al*., 1981; Soliman *et al*., 2020; Symonds *et al.*, 2014; Verbyla & Mihelcic, 2015; Von Sperling, 2015)

Post-secondary stage effluent generally has low levels of nutrients and other pollutants making it suitable for discharge into receiving waters. However, pathogens, including human viruses, still survive the treatment process (Qiu *et al*., 2015; Rose *et al*., 2004). Therefore, secondary effluent is usually disinfected prior to discharge to keep surface waters "fishable and swimmable" (Davis, 2010).

Also, depending on the type of secondary treatment and disinfection, prior to effluent discharge to receiving waters, it may be postaerated**.** That is to increase the dissolved oxygen (DO) from 0.5 to 2 mg/L to saturation (8-14 mg/L) (Agency, 1977). Cascade aeration is the simplest method to achieve desired DO levels (Davis, 2010).

Disinfection. Chlorination and UV irradiation are the two most used methods of wastewater disinfection. Chlorination became the standard method of wastewater disinfection by mid-20th century and at that time replaced original ozonation due to cost effectiveness and no need to produce the agent on-site (Gray, 2013a). Initially chlorine gas (Cl_2) was directly used, but for health and safety reasons is now replaced with salt forms (sodium and calcium hypochlorite - NaOCl and Ca $(OCl)_2$, as well as calcium hypochlorite chloride (also named chlorinated lime) - $CaOCl_2$). In bacteria and in parasitic protozoa (however to a much lesser extent), chlorination disrupts electron transport (respiratory) chain, arrests protein and nucleic acid synthesis, and causes physical damage to the cell membrane(Carey *et al.*, 2004; Haas & Engelbrecht, 1980). In viruses chlorination breaks capsid integrity and causes damage to nucleic acids (Wigginton *et al*., 2012) .

The mechanism of UV-mediated virus inactivation seems to be group-dependent. Polychromatic UV irradiation caused nucleic acid damage to the coliphage MS2 (Beck *et*

al., 2016). In human adenoviruses polychromatic UV irradiation caused extensive protein damage and maximum loss of protein signature at a wavelength of 240 nm (Beck *et al*., 2018).

Less than 1 mg/L of free chlorine inactivates 99.6 to 100% of poliovirus and *E. coli* in less than 10 minutes and in wide range of temperatures. However, protozoan parasite cysts might require more than 10 mg/L and more than two hours of contact time (Davis, 2010). The disadvantage of chlorination is that chlorine oxidizes any natural organic matter to form a number of potential carcinogenic by-products (trihalomethanes, chloroacetic acid, nitrosamines *etc*.) (Cotruvo & Amato, 2019). After the disinfection process residual chlorine must be neutralized to reduce toxic effects on aquatic biota and to reduce the production of disinfection by-products in natural waters with higher organics content (US EPA, 2000). For dechlorination, sulphur compounds like sulphur dioxide, sodium sulphite or metabisulphite are used. However, chemical dechlorination can be challenging to control when zero levels of free chlorine are required. Excessive sulphur dosing can lead to formation of sulphate with the resulting dissolved oxygen content decrease and lower pH of the finished effluent. In the receiving water bodies there is a risk that the sulphur compounds are reduced into sulphide, which is also toxic to aquatic life (King, 1976).

UV disinfection of treated effluent has none of the side effects associated with chlorination and it does not require any chemical additive. Discovery of the germicidal effect of UV irradiation in the treatment of a skin form of tuberculosis brought a Nobel Prize in medicine to Niels Finsen in 1904. By 1909 UV was used to disinfect drinking water

in a full scale treatment facility in Marseilles, France (Gray, 2013a). However, it was not common until the 1990s with the development of new lamps and ancillary equipment for reliable application in water disinfection (Asano *et al.*, 2007). With the proper dosage, UV irradiation is effective against bacteria, protozoa and viruses (Gray, 2013b). However, of the human enteric viruses, adenoviruses appear to be the most UV-resistant (Meng & Gerba, 1996). The efficiency of UV disinfection is very dependent on the transmittance quality parameters of the water being treated (*e.g.* turbidity and color) and in secondary treatment the usual dose is 50–140 mJ/cm^2 (Davis, 2010).

The main mode of action for UV irradiation (at 254 nm) is the damage of nucleic acids due to dimerization of thymine, which makes the replication impossible (Wang & Smith, 2008). UV irradiation is a safe and effective method of disinfection that produces few toxic by-products compared to chlorination (Zoschke *et al.*, 2012). However, UV disinfection does not have residual disinfection effect and there is a potential for photoreactivation of cellular microbial pathogens (Bitton, 2010; Silverman & Boehm, 2020).

The mechanism of polychromatic UV-mediated virus inactivation has increased the popularity in the use of so-called medium-pressure UV lamps (that in addition to 254 nm, have emission peaks at 265 & 286 nm). Polychromatic UV irradiation caused nucleic acid damage to bacteriophage MS2 (Beck *et al*., 2016) and for adenoviruses - extensive protein damage and maximum loss of protein signature (Beck *et al.*, 2018). Overall, polychromatic UV irradiation has been shown to be more effective in virus inactivation, and adenovirus inactivation in particular, than monochromatic irradiation at the 254 nm wavelength (Beck

et al., 2016, 2018), however examination of microorganism photoreactivation after polychromatic UV irradiation data would benefit from additional research.

Ozonation can also be used for effluent disinfection. This method causes destruction of the cell wall, damage to nucleic acid nitrogen bases, and breaks covalent carbon-nitrogen bonds of organic substances in the microorganisms (Asano *et al.*, 2007; Gray, 2013a). However, it is highly corrosive to the equipment and toxic to higher life forms, resulting in high safety concerns. Besides pathogen control, ozonation (advanced oxidation) is used in tertiary treatment of water, due to its' ability to oxidize inorganic compounds, especially reduced metals, oxidize synthetic organic compounds like pesticides, pharmaceuticals and hormone disruptors (Broséus *et al.*, 2009; Da Silva *et al.*, 2009; Gomes *et al.*, 2017; Tijani *et al.*, 2014), and remove natural organic compounds including precursors of chlorination by-products (Sadrnourmohammadi *et al.*, 2020). It also removes taste and odour, algal toxins, and colour (Gray, 2013a).

Tertiary treatment. The need for wastewater purification, more advanced than what secondary treatment technology could offer, was recognized in the 1970s during urban expansion that led to increased loads of organic matter and suspended solids. Also recognized was the need for more efficient nutrient removal and disinfection of effluents, particularly for water reuse (Davis, 2010). Since then, many of the "advanced" methods of water purification, like activated carbon adsorption, chemical phosphorous removal, and advanced oxidation, generally became conventional and were incorporated in different stages of the treatment process. In Alberta 81.4% of all discharged wastewater is tertiary-

treated (Statistics Canada, 2019). Technically speaking, all the disinfection methods are tertiary as well.

For advanced pathogen removal, various membrane filtration methods are preferred, which are based on the physical barrier through which water passes under pressure. The size distribution of wastewater contaminants and the corresponding membrane processes are shown in Figure 2.1. Granular (sand) filtration is a conventional method in drinking water treatment, and in wastewater treatment it is used to remove residual flocs after secondary settling (Davis, 2010). Membrane-based filtration is used in both drinking and wastewater treatment processes. The main difference between membrane filtration and granular media filtration is that most of all trapped substances accumulate on the membrane surface, thus forming an additional filtering layer of sediment, which has its own resistance (Bourgeous *et al.*, 2001). Depending on the pore size of the membrane, the following types of filtration process are distinguished: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO). Microfiltration (0.1–1.0 µm) removes bacteria, large viruses, and pathogen-associated solids. Ultrafiltration (0.01–0.1 µm) removes viruses and high molecular-weight substances (proteins, starch, fats, etc.). Molecules and ions of low molecular weight substances are extracted from solutions by nanofiltration and reverse osmosis (0.0001–0.001 µm) (US EPA, 2001).

In recent years various combinations of physical and physico-chemical methods have emerged as potential tertiary treatment interventions, which may be of special interest for treatment of the wastewater with intended reuse (Table 2.7).

Table 2.7 Prospective methods of tertiary wastewater treatment.

Methods	Description	Reference
Sonophotocatalysis	Combines the use of a photocatalyst TiO2 in the presence of ultrasonic and UV irradiation to give a synergistic effect which can enhance the degradation of pollutants due to the formation of highly reactive free radicals.	(Joseph *et al.*, 2009)
Multi-spark electric discharge	Disinfection by the combination of pulsed UV plasma, acoustic shock-waves, ozone, free radicals, and hydrated electrons.	(Anpilov *et al*., 2004)
Combined advanced oxidation and membrane filtration	Removal of residual pharmaceuticals	(Ganiyu *et al.*, 2015)
Combined ozonation and adsorption	Chemical pollutant removal	(Gu *et al.*, 2011; Reungoat *et al.*, 2012)
Insoluble organic polymer adsorption (cyclodextrin)	Pharmaceutical and endocrine disruptors removal	(Nagy *et al*., 2014)

2.2.2 Virus removal during each treatment process

As was described above, wastewater treatment is conducted by a train of unit operations, where each operation adds to total pathogen removal. Due to their size and capsid protein characteristics, viruses behave as colloid particles. Therefore, in wastewater they may be absorbed onto or ensnared into suspended solids by electrostatic and hydrophobic interactions (Gerba, 1984). These mechanisms allow co-removal of viruses with suspended solids. For human pathogenic viruses in wastewater they may also be taken up, protected from treatment steps, and remain infectious within free-living protozoa (Atanasova *et al*., 2018; Battistini *et al*., 2013; Folkins *et al*., 2020; Verani *et al.*, 2016). Therefore, human pathogenic viruses are not able to proliferate in wastewater but may persist as infectious virions for considerable time. Virions, however, will eventually decay naturally via potential inactivation by microbial metabolites and predation during biological stages of treatment. They can also be inactivated by disinfectants, by chemical (*e.g*. use of copolymer with antiviral activity during enhanced primary stage (Xue *et al.*, 2014)) and mechanical methods or some combination thereof. After secondary treatment, concentrations in effluent of up to 10^4 /L for adenoviruses and rotaviruses and up to 10^6 for noroviruses in 30-100% of samples have been reported (Rusinol & Girones, 2017).

Secondary wastewater treatment usually employs the activated sludge process, which is a train of unit processes including particulate settling, biological decomposition, and for protected waters, disinfection before discharge. Physical solids separation by filtration might be used for tertiary treatment if wastewater is being treated for subsequent reuse. A combined biological and filtration (microfiltration or ultrafiltration) reactors, also called

membrane bioreactors, have increase in popularity over recent years due to their efficiency in pathogen removal (Zhang *et al.*, 2016). In general, most conventional unit processes are designed for the removal of suspended solids and not for virus elimination specifically but co-eliminate viruses as well.

The log removal/inactivation of viruses varies noticeably among different processes, as summarized in the Table 2.8. Sedimentation- and filtration-based primary treatment processes rarely remove more than 1 log and only re-locate the virus from water to sludge, but do not inactivate them. Additional sludge treatment processes, like composting, heat treatment, or liming are needed for virus reduction and preventing it from further circulation of infectious virions in the environment (Dumontet *et al.*, 1999). The biologically based conventional secondary treatment processes cause more substantial reduction from the water phase due to enmeshment of viruses into activated sludge flocs and attachment to biofilms (Zhang *et al.*, 2016). Advanced tertiary treatment processes using coagulants, membrane filtration and advanced oxidation processes are the most efficient in virus removal/inactivation (Bielefeldt *et al*., 2010; Mamane *et al*., 2007; Shirasaki *et al*., 2016; Shirasaki *et al*., 2017; Soliman *et a*l., 2020; Wang *et al*., 2018). The membrane filtration efficiency is clearly dependent on the membrane pore size cut-off with the lowest removal by microfiltration and the highest removal by nanofiltration and reverse osmosis (Adham *et al.*. 1998; Madaeni *et al.*, 1995). The enhanced efficiency of membrane bioreactors in comparison to conventional activated sludge is mainly due to the membrane cut-off size (Purnell *et al.*, 2016). Disinfection is the final step of wastewater treatment that inactivates the residual viruses. The efficiency of different

disinfection methods varies depending on the virus. Adenoviruses are known to be more resistant to UV inactivation than other enteric viruses (Gerba *et al.*, 2002), but more sensitive to chlorination as only 0.04-0.15 mg·min/L of chlorine is required to achieve 4 $\log_{10}$ reduction (Cromeans *et al.*, 2010).

However, many authors consider ozone and UV disinfection to be more efficient than chlorination (Lazarova *et al.*, 1998; Lee *et al.* 2015; Wigginton *et al.*, 2012) despite no residual effect. To ensure high efficiency virus inactivation, combination of disinfection methods with different molecular mechanisms of virus damaging should be a priority (Giannakis *et al.* 2018; Giannakis *et al.* 2017; Mamane *et al.* 2007).

Table 2.8. Removal/inactivation of viruses by various treatment processes.

Process	Log_{10} reduction of viruses	Reference
Primary treatment		
Conventional settling	0 – 1.8	(Bosch *et al.* 1986; Yasunori *et al.*, 2002)
Chemically enhanced settling	0 – 2.6	(Lucena *et al.*, 2004; Payment *et al.* 2001)
Secondary treatment		
Activated sludge:		
F-coliphage	0.83 – 1.65	(Hata *et al*., 2013; Prado *et al*., 2019)
Norovirus	0.95 – 2.32	(Montazeri *et al.*, 2015)
Enterovirus	1.4 – 2.2	(Hata *et al*., 2013; Montazeri *et al*., 2015)
Adenovirus	0.59 – 1.88	(Prado *et al*., 2019)
Rotavirus	0.37 – 1.39	(Hata *et al*., 2013; Montazeri *et al*., 2015)
Reovirus	1.23 – 1.75	(Hata *et al*., 2013)
Trickling filter:	0 – 0.82	(Prakash & Chaudhuri, 1982)
Pepper mild mottle virus	0.8	
Aichi virus	0.8	(Oakley and von Sperling, 2017)
Norovirus	1.7	
Enterovirus	2.0	
Adenovirus	1.0	(Schmitz *et al.* 2016)
Membrane bioreactor:		
Norovirus	1.3 – 5.2	(Sima *et al*., 2011)
Enterovirus	1.8	(Ottoson *et al.* 2006)
Adenovirus	5.0	(Kuo *et al*., 2010)

Tertiary (advanced) treatment		
Chemically enhanced settling of secondary effluent	>4 (dosed MS2)	(Zhu *et al.* 2005)
	2-3	(Dittrich *et al.* 1996)
Microfiltration	2.83	
Ultrafiltration	0.5 – 2.5 (norovirus)	(Gentile *et al.* 2018)
	2.8 (pepper mottle virus)	(Lee *et al.* 2017)
Nanofiltration	5 – 9	(Singh *et al*., 2020)
Reverse osmosis	>6.5	(Adham *et al*., 1998)
Disinfection:		
Ozonation	2.5 – 6	(Gomes *et al.* 2019)
UV	1.46 – 1.67	(Yuanyuan Qiu *et al*., 2018)

2.2.2.1 Virus surrogates and their field performance

As discussed in the introduction, increased water shortages are driving the reuse of wastewater to address potable and non-potable water uses. Whereas it is feasible to produce potable quality water from wastewater, the short-duration failure of adequate treatment poses the highest human health risk (Schoen *et al.*, 2020). Wastewater must pass multiple barriers before it can be used as potable water (WHO, 2017b). Therefore, every project for water recycling, and drinking water supply augmentation specifically, must be evaluated for potential risks and include a robust monitoring program for performance and compliance (Drewes & Horstmeyer, 2016; Tchobanoglous & Leverenz, 2019). In the case of treatment failure, it has long being known that the risk of infection caused by viral pathogens is 10 to 1000 times higher than bacterial infection (Haas *et al.* 1993).

Hence, wastewater treatment processes must be evaluated, validated, and constantly monitored to provide safe produce water. There are four types of monitoring for contaminants: start-up (baseline) – collection of the information needed for risk assessment; validation – to prove the effectiveness of each barrier; operational – to monitor that the treatment process performs as designed; and verification – a final assessment of the treatment effectiveness and compliance with the health-based targets (Law, 2017; WHO, 2017b). In wastewater reuse scenarios it is critical to ensure human enteric virus removal to meet the health-based targets (WHO, 2001a). However, monitoring for the diversity of pathogenic viruses that might be present in wastewater is impractical. It is costly, time consuming, and in many cases pathogenic viruses may still be

problematic when below current detection limits, or the detection of infective viruses might be problematic (Gerba & Betancourt, 2019). Therefore, indicators for enteric viruses are used. Ahmed *et al.* (2020) describe what the ideal indicator should be:

a. be unable to replicate in water;
b. be specific for contamination by human feces and human pathogenic viruses;
c. be non-pathogenic for humans;
d. have physical characteristics similar to pathogenic viruses;
e. be as resistant or more resistant to treatment processes as pathogenic viruses;
f. be a member of warm-blooded animals intestinal virome;
g. easy to detect; and
h. be applicable to all types of waters.

There is no ideal indicator that fulfills all of the above requirements. Rather indicator/surrogate should be chosen for their intended purpose: surrogate viruses for validation of a barrier process must have morphology (shape & size) and nucleic acid composition similar to pathogenic viruses (*e.g.* F-specific coliphages); faecal indicator viruses must originate in human intestine and not proliferate outside of it (*e.g.* crAssphage).

Indicator viruses must be present in wastewater in high prevalence and substantial concentrations, so they will be easier to detect in finished water. To date human polyoma virus (Hewitt *et al.* 2013), crAssphage (García-Aljaro *et al.* 2017; Korajkic *et al.*, 2020), PMMoV (Hamza *et al.*, 2011a), and human adenovirus (La Rosa *et al.* 2010; Qiu *et al.*, 2018) have been reported in highest concentrations (7-10 $\log_{10}$/L) in sewage.

Unfortunately, at this time little is known about removal of crAssphage through the treatment process (Ahmed *et al.*, 2020). Polyoma and adenoviruses are human pathogens. PMMoV is of questionable geographic distribution plus its morphology does not resemble enteric viruses (Kitajima *et al.* 2018). PMMoV is more similar to enveloped, respiratory viruses and more poorly removed by membrane processes (Papp *et al*., 2020). However, PMMoV has been shown to be equally or more resistant to many forms of wastewater treatment then enteric viruses (Papp *et al*., 2020; Symonds *et al*., 2018). Coliphages, although present in sewage in lower numbers than aforementioned viruses, are well studied surrogates for human adenovirus, rotavirus and enterovirus (McMinn et al., 2017a; US EPA, 2015a). The $\log_{10}$ reductions of F-specific coliphages and Norovirus GII strongly correlated (Pouillot *et al*., 2015). Based on meta-analysis of coliphage MS2 data, it has been proposed as a validation and operational monitoring indicator in membrane bioreactor treatment (Amarasiri *et al*., 2017). However, PMMoV and coliphage PhiX174 appeared to be better surrogates for pathogenic viruses for coagulation and membrane filtration processes than MS2 (Shirasaki *et al.* 2016; 2017).

The $\log_{10}$ reduction of crAssphage, polyoma and PMMoV viruses through wastewater treatment processes data are summarized in a recent review (Ahmed *et al.*, 2020). Overall these viruses demonstrate similar or lower $\log_{10}$ reduction than pathogenic viruses.

To date, fluorescent particles of various diameters, depending on the target organism size, are used as viral surrogates for the treatment systems validation and verification (Bielefeldt *et al*., 2010; Li *et al*., 2020; Mi *et al*., 2004). These particles are readily available, generally nontoxic, can be added in sufficient concentration (*e.g.,* 1 mL of 20 nanometer Ø

FluoSpheres® contains ~10^{16} beads), and are easy to detect. They are mostly applicable for physical removal operations like filtration. However, particle surrogates should be used with caution as they can cause membrane fouling. Potentially, co-precipitation of these microspheres with suspended solids could also be evaluated if the chemical characteristics of the chosen microspheres surface coating (like hydrophobicity, surface charge, *etc.*) are close to those of real virus of interest. However, no study was identified that used fluorescent microspheres to evaluate virus removal by suspended solids settling.

Fluorescently labeled bacteriophages, especially green fluorescent protein (GFP) - displaying constructs (Kaźmierczak *et al*., 2014), could be dosed as surrogate organisms to estimate virus inactivation. But this approach has more disadvantages at this point than using synthetic fluorescent particles: they are much more costly to produce and the labeled viable bacteriophage preparations are hard to standardise for routine use.

2.3 Wastewater reuse

In last three decades water scarcity has become an issue not only in arid regions, but also in temperate climate areas where growing population, increased living standards, and urbanization increased safe drinking water demand. At the same time increased population and high *per capita* water consumption creates larger volumes of wastewater produced (Huertas *et al.*, 2008; Wu *et al.* 2013). Hence, reclaimed water can be apt for many urban applications as non-potable or potable (Schoen et al., 2020). Water reuse includes agricultural, industrial, urban, environmental and recreational, ground water recharge, and potable source augmentation (US EPA, 2004). Each application also requires some nonmicrobial parameters to be met (Table 2.9). Besides those, microbial quality parameters are applicable to all types of water reuse.

2.3.1 Drinking water: potable reuse vs unintended reuse

There are two major categories of wastewater reuse: unintended and intended. Unintended reuse, when a drinking water system uses a surface water source that receives upstream wastewater discharges, is common in many countries and is a simple result of the geographical setting. It was recognized more than hundred years ago that "London's water might have been through seven kidneys" prior reaching a tap (Sharma & Sanghi, 2013). The volume percentage of discharged wastewater is quite low, but it might substantially increase during a low flow period (WHO, 2017b) resulting in a high risk event or scenario (Havelaar & Melse, 2003; Medema & Ashbolt, 2006).

Intended (planned) wastewater reuse, on the other hand, is a deliberate and controlled use of wastewater that passes multiple treatment barriers according to certain standards. Potable reuse might be direct (DPR) or indirect (IPR), i.e., without further treatment or with, respectively. In all these cases, some expected LRV for human enteric viruses is specified and needs to be validated (see Section 2.3.4).

Potable reuse typically involves blending of highly treated municipal wastewater with the raw sourced water either prior to drinking water treatment process (*e.g.,* the city of Windhoek, Namibia and the village of Cloudcroft, USA), or the treated water inflows directly into the drinking water distribution system (Advanced Water Purification Facility in El Paso, Texas). To increase security, wastewater is increasingly required to pass through an environmental buffer like a surface water reservoir, groundwater aquifer or engineered reservoir prior to being withdrawn for drinking water treatment.

Potable reuse requires implementation of a safe drinking water framework to ensure public health and safety. This framework is based on three major components: health-based performance targets to achieve microbial, chemical, and radiological safety; water safety plan which includes system assessment, monitoring, and management; and independent surveillance to ensure that water safety plan is implemented effectively and health-based targets are met (WHO, 2017b).

Table 2.9 Nonmicrobial parameters of Concern in Water Reuse.

Type of reuse	Parameters	Concerns
Agricultural and urban irrigation reuse:	Salinity Sodium ion Trace elements Excessive Cl_2 residual Nutrients	Excessive salinity is toxic to some crops Sodium affects soil adsorption complex Residual chlorine is toxic in aquaculture
Industrial and municipal (firefighting, public transit vehicles wash) reuse:	Ca hardness Total alkalinity Chlorides, phosphate ions, ammonia Suspended solids Biocorrosion-causing microorganisms	Equipment scaling Corrosion Biofilm growth Clogging/fouling
Environmental & recreational:	Chemicals toxic to aquatic life Nutrients Heavy metals	Toxic effect on aquatic life Eutrophication and low dissolved oxygen levels
Ground water recharge:	Recalcitrant organics Nitrate	Might not be eliminated in the aquifer. Cause adverse human health effects

Adapted from: (Asano *et al*., 2007; US EPA, 2004)

2.3.2 Non-potable reuse: Agricultural and municipal

The majority of the reclaimed wastewater is used for irrigation (Lazarova & Bahri, 2008). The treated wastewater might be used for both landscape and agricultural irrigation. The need for water reuse in agriculture is driven by a combination of factors like growing proportion of intensive agriculture practices in the world and droughts that now occur not only in traditionally arid areas but in some temperate zones where they were not so common before (Australian Government Bureau of Meteorology, n.d.; Government of Canada Natural Resources, n.d.). Water quality requirements for agricultural reuse are somewhat less stringent than for urban, industrial or potable reuse (Guidelines for the Safe Use of Wastewater, Excreta and Greywater, 2006). This also favors the use of the reclaimed water in agriculture (Lazarova & Bahri, 2008). Nevertheless, reclaimed wastewater still has to meet specific requirements to avoid public health and environmental issues. Outbreaks of waterborne viral infections caused by inadequately treated irrigation water have been reported (Barrimah *et al*., 1999; Wei & Kniel, 2010). When the reclaimed water quality is adapted to final use, almost all agricultural irrigation demand may be satisfied with it (Lazarova & Bahri, 2005).

Similar to potable reuse, direct and indirect are also the two main types of wastewater reuse for agricultural irrigation. Direct reuse in agriculture is the use of treated water immediately after treatment, or after a short storage in an engineered reservoir. In case of the indirect reuse, treated wastewater is stored for prolonged periods of time in soil aquifers, or in open reservoirs either natural (lakes, wetlands) or engineered.

Quality parameters of reclaimed water that is intended for irrigation must include salinity/sodicity, TSS, and pH besides human health-based parameters (Ayers & Westcot, 1985). They are to protect soil quality and ensure sustainable agriculture. Nutrient removal during pre-treatment might not be necessary as the reclaimed water can also play a dual role of a source of moisture essential for plant growth and a fertilizer.

Reclaimed wastewater is used by many countries to irrigate a variety of crops. However, fodder, orchard, and industrial crops seem to be the leading crops irrigated (Lazarova & Bahri, 2008) as they pose lower health risk to consumers than wastewater irrigated vegetables intended for minimal processing or raw consumption.

Urban development in recent years has also put additional demand for recycled wastewater. Municipal reuse might include public facility applications like irrigation in municipal landscaping, public parks, and sporting fields (Anderson, 2008), as well as urban agriculture irrigation (Redwood & Huibers, 2008).

The main difference between agricultural and urban irrigation is the type of public exposure to pathogenic organisms. In the case of agricultural irrigation most human infections occur due to consumption of wastewater irrigated crops, while the risk of infections acquired by direct contact or aerosol is mainly limited to farm workers. In the case of urban (ornamental) irrigation direct contact and aerosol inhalation pose the highest risk for public health. Wastewater reuse in urban agriculture combines these risks. However, with well thought-through health protection measures many reuse constraints may be overcome.

The risk of pathogen transmission to the general public by wastewater irrigation is reduced by a number of protective measures: a) engineering practices like wastewater treatment, storage, and irrigation methods; b) policy and regulations like crop restriction, human exposure control, and immunisation; and c) agronomic practices like crop selection, timing of irrigation, and harvesting measures (Lazarova & Bahri, 2008).

In most countries, wastewater must be tertiary-treated to be used for unrestricted agricultural irrigation. US EPA guidelines require disinfection as well (2012). In general, wastewater treatment scheme must be based upon water quality requirements, control of water application, type of irrigated crops, soil characteristics, and public access (Huertas *et al.*, 2008; Salgot *et al.* 2018).

The choice of irrigation method might become an efficient barrier for pathogen exposure. In terms of health risk there are three levels of protection: low, medium, and high. Surge flooding and sprinkler types of irrigation provide low level of protection as they contaminate plants and have the highest risk of exposure to humans. Furrow irrigation provides medium level of protection. And drip and subsurface irrigation have the highest level of health protection by minimizing aerosolization and contact with pathogens (Lazarova & Bahri, 2008; USDA, 1997).

Crop restrictions specify the type of crops that is allowed to be irrigated with the wastewater of a certain quality. For example, industrial crops that are intended for further processing, like cotton or oil-bearing plants, or vegetables that are consumed cooked like potatoes, might be irrigated with reclaimed water of relatively lower quality.

Human exposure control measures include mandatory personal protective equipment for farm workers, following food hygiene standards for consumers, timing of irrigation, avoiding sprinkler irrigation in proximity of settlements and roads, public information about wastewater-based irrigation.

Besides irrigation, municipal wastewater reuse also includes public facility applications other than irrigation: toilet flushing in public facilities, cooling towers, street cleaning and dust control, washing public transit vehicles, firefighting, use in urban water features and natural stream flow restoration, snow making in ski facilities, *etc.* (Anderson, 2008). For these applications the use of tertiary-treated filtered and disinfected (preferably advanced-oxidized) municipal wastewater is preferred to ensure public health and safety.

In general, recycled wastewater for agricultural or urban applications should be treated and distributed according to its intended use, in other words, “fit for purpose”.

2.3.3 Reclaimed wastewater quality requirements. Health-based Log_{10}-reduction targets to deliver fit-for-purpose water for reuse

There are ecological and economic reasons that drive interest in the use of reclaimed water. The safe and sustainable use of recycled water means that public health and confidence in reuse is protected by established regulations.

The WHO has been providing guidance for safe reuse of wastewater since it first recommended health criteria and treatment processes for various wastewater applications (WHO, 1973). In 2006 the second revision of the guidelines, based on the results of the expert meeting that took place in Stockholm in 2001, was published

(Guidelines for the Safe Use of Wastewater, Excreta and Greywater, 2006). The Stockholm framework as it was known by, is an integrated approach based upon risk assessment and risk management to control water-related illness. It requires risk assessment prior to setting of health-based targets and risk management key points and also includes the evaluation of this approach in terms of public health outcome. This framework allows countries to adjust the WHO guidelines to their local epidemiological and environmental circumstances and set their health-based targets accordingly.

The public health outcomes need to be compared using a common measure that allows comparing different exposure pathways and illnesses. The disability adjusted life years (DALY) provides a unified summary of population health which allows to estimate the burden of disease caused by a given risk factor and compare risks to identify the priority (WHO, 2011). The DALY is a sum of years of life lost (YLL) and years lived with disability or illness (YLD).

The tolerable risk, defined by the WHO, is 10^{-6} DALYs (Havelaar & Melse, 2003). It is equivalent to a mild illness with low mortality rate (1 death per 100,000 population in a life-time) and annual morbidity of 1 per 1,000. The tolerable risk is usually considered as a sum of risk from key hazards (e.g. reference pathogens), and the risk management decisions are made to address the highest risk first (Asano *et al*., 2007). The WHO guidelines (Guidelines for the Safe Use of Wastewater, Excreta and Greywater, 2006) suggest three types of risk evaluation: laboratory testing, epidemiological investigations, and QMRA. Based on these evaluations, health-based performance targets that would bring hazard risk levels below the arbitrarily defined tolerable risk can be set. In general,

the required quality of recycled wastewater is to pose lower levels of risk than defined tolerable risk for a given reclaimed wastewater application. The Log_{10} reduction targets are calculated using the following formula: Log_{10} reduction = log (concentration in source water × Volume of water a person is exposed to × N exposure days in a year ÷ DALYd); expressed as μDALYd, being the pathogen dose equivalent to 10^{-6} DALY, and it is calculated for reference pathogens by QMRA.

The WHO guidelines are the basis for wastewater treatment regulations that are adopted by many countries (European Commission, 2018; Marecos do Monte, 2007; National Water Quality Management Strategy, 2006; US EPA, 2004, 2017; Zaidi,2007). The treated effluent standards and targeted reductions vary substantially in different countries and even between states in the USA (US EPA, 2004). For example, for urban non-potable reuse Australia requires wastewater to be treated to Class A standard, which means average densities of: 10 *E. coli* per 100 mL, 1 helminth ovum per liter, 1 protozoon per 50 liters, and 1 virus per 50 liters. However, the feasibility of these requirements looks questionable. Considering higher virus load in raw sewage and higher resistance of viruses to treatment processes comparing to bacteria, it is hard to imagine $5x10^3$ *E. coli* and only one virus in 50 L of treated wastewater. To produce drinking water from sewage, the Australian National Health and Medical Research Council recommends 8 log *Cryptosporidium*, 9.5 log enteric viruses, and 8.1 log *Campylobacter* reduction (NHMRC, 2008). The guidelines by US EPA and regulations by individual US states are also based on the type of water reuse (Table 2.10).

Canadian Guidelines for Domestic Reclaimed Water for Use in Toilet and Urinal Flushing require 2.6 log reduction for *C. parvum*, 4.2 for Rotavirus, and 5.3 for *E. coli* O157:H7 based on aerosol exposure calculations, and for pipeline cross-connection scenarios required Log10 reductions are 4.1, 5.7, and 6.8 respectively (*Canadian Guidelines for Domestic Reclaimed Water for Use in Toilet and Urinal Flushing*, 2010).

Table 2.10 Wastewater reuse regulations by some USA states.

State	Indirect potable reuse	Unrestricted urban reuse
California	12 log virus reduction 10 log *Cryptosporidium* and 10 log *Giardia* reduction	2.2 total coliform/ 100 mL 30 day geometric mean
Florida	No detectable coliforms in 100 mL	Coliforms below detection limit in 75% samples over 30 days
Nevada	12 log enteric virus reduction 10 log *Cryptosporidium* and *Giardia* reduction	2.2 faecal coliform/ 100 mL 30 day geometric mean
North Carolina	6 log reduction *E.coli*, $\leqslant$ 3/100 mL 5 log reduction coliphages, $\leqslant$ 5/ 100 mL	14 faecal coliform/ 100 mL monthly geometric mean

Adapted from (US EPA, 2004)

2.4 Fluorescence-based methods for wastewater analysis

Fluorescence-based methods like spectrophotometry can be used to rapidly detect contamination of potable water in "third pipe" systems (Hambly *et al.*, 2015a), or evaluate the microbial quality of drinking water in real time (Sorensen *et al.*, 2018). But for this thesis research the focus was on the use of fluorescent labels to enhance the detection of microorganisms, including viruses in water matrices (Pollard, 2012b). This thesis focused specifically on the applicability of flow cytometry (FCM) for virus enumeration, hence general principles and FCM are discussed next.

2.4.1 The mechanism and characteristics of fluorescence

The emission of light that occurs from a chemical substance in the electronically excited state is called luminescence. Fluorescence along with phosphorescence are the two subcategories of luminescence (Lakowicz, 2006). Fluorescence is the phenomenon of light emission following light absorption as shown on the simplified Jablonski diagram (Figure 2.4).

Fluorescence starts when a molecule with an electron in the ground state (on σ – orbital) absorbs a photon. This sends the electron to higher energy π orbitals. The higher the extent of the π-electron system – the longer the wavelength of emitted fluorescence (for example, anthracene with three benzene rings emits light in green, and pentacene with five benzene rings – in red). In general, the higher the wavelength, the lower the energy of the emitted photon: $E=hc/\lambda$, where h is Plank's constant, c – the speed of light, and λ is the wavelength.

Stokes' shift. Stokes' shift was named after the Irish physicist George Gabriel Stokes, who first described that fluorescence emission occurs at a longer wavelength than the excitation light in his 1852 manuscript "On the Change of Refrangibility of Light" (Stokes, 1852). The Stokes' shift is the difference between the wavelength of the emission maximum and the wavelength of the fluorescence absorption maximum: $\Delta\lambda$ = iemmax - λexmax.

The larger the Stokes' shift, the less a given fluorophore is prone to self-quenching due to high concentration and the easier it is to observe the emission with waveguides/filters used in flow cytometers and fluorescent microscopes (Vesey *et al.*, 1994). Figure 2.5 demonstrates the excitation/emission spectra and the Stokes' shift of the SYBR® Green I fluorophore, used in this thesis study.

Fluorescence quantum yields and fluorescence lifetimes. The quantum yield of a fluorophore is a ratio of emitted photons to the number of absorbed photons. The fluorescence lifetime is an average time the fluorophore molecules spend in the excited electron state. The higher the quantum yield and the lifetime, the brighter and more stable, hence, more functional for biological and environmental applications, the fluorophore is (Lakowicz, 2006).

Fluorescence quenching. The intensity of emitted light can be decreased by several different processes. The collisional quenching occurs by the deactivation of the excited fluorophore by another molecule, the quencher, with or without chemical interaction of the two molecules. In water analysis, TOC, oxygen and chlorine (Crump *et al.*1999; Henderson *et al.*, 2009; Korshin *et al.* 2018) can considerably affect the results either

increasing or decreasing fluorescence. Self-quenching due to the inner filtering effect usually occurs as a result of the absorption of excited radiation and/or emitted fluorescence by the sample background. Humic-like substances, present in water are known to be prone to self-quenching (Mobed *et al.*1996; Ohno, 2002; Zipper, 2003). To reduce this effect, the sample absorbance should be minimized (Gore, 2000; Spencer *et al.* 2007). Lower absorbance is achieved by using small pass length cuvette or diluting the sample.

Förster (1959) resonance energy transfer (FRET). This is the phenomenon of energy transfer from the excited fluorophore donor to an acceptor molecule through dipole-dipole non-radiative process (Figure 2.6). This process occurs whenever the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor and the energy emitted by the donor excites the acceptor. The donor must have adequate fluorescence lifetime and proximity (usually between 1 and 10 nm) for energy transfer to take place.

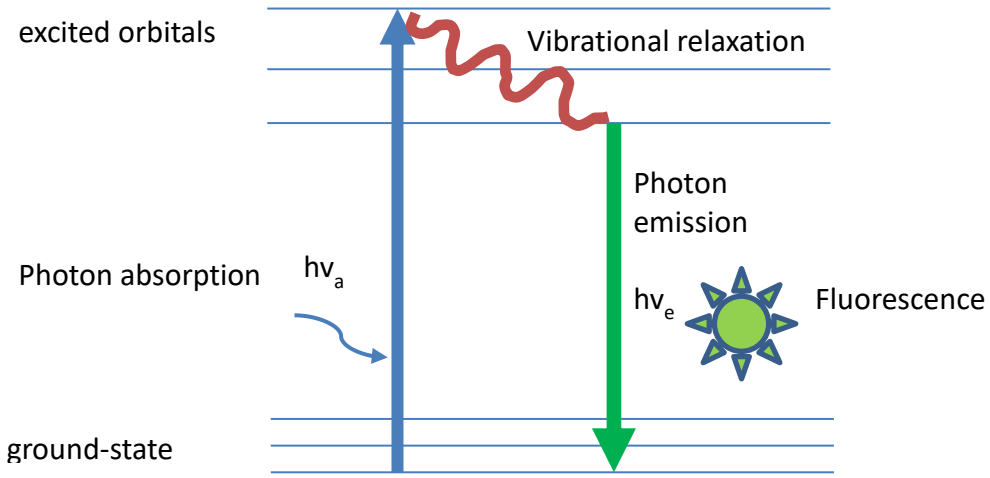

Figure 2.4 Mechanism of fluorescence. Jablonski diagram illustrates how light absorption creates the excited electronic singlet state, and as the electrons in the fluorophore return to the ground state a photon of lower energy is emitted.

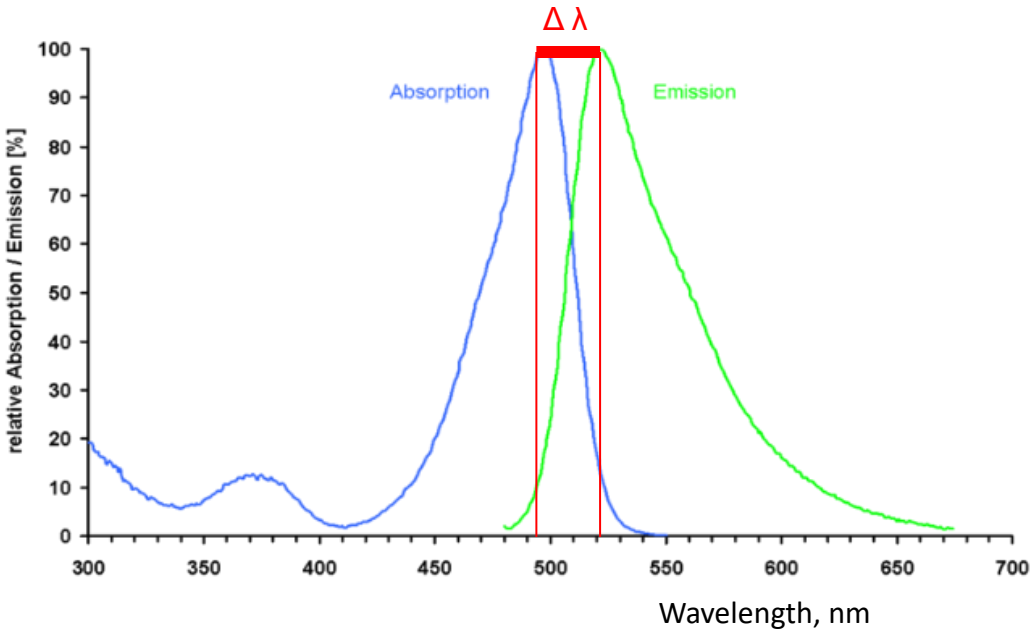

Figure 2.5. Excitation/emission spectra and the Stokes' shift of the SYBR® Green I.

Adapted from: https://commons.wikimedia.org/wiki/File:SYBR_Green_I_Spektrum.png

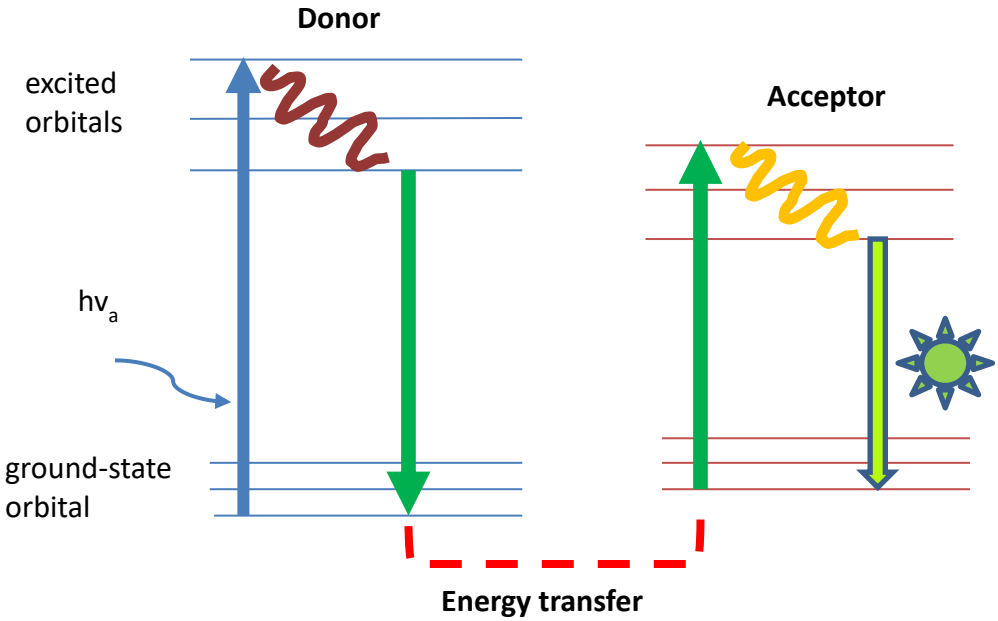

Figure 2.6 Resonance energy transfer (FRET) mechanism.

2.4.2 Fluorescent labelling

Fluorophores can be grouped from many points of view: chemical composition (for example, coumarins, rhodamines or fluoresceins), excitation/emission wavelength (from blue to red), quantum yield, binding affinity (NA, proteins, fatty acids, etc.), application methods (direct object staining or specific antibody labeling), *etc*.

Chemically, most fluorophores are aromatic since fluorescence is based on the π electron system. Some highly unsaturated aliphatic hydrocarbons are also fluorescent, but they are not usually used in biological research and, therefore, are out of this discussion.

In the microbial analysis, preference should be given to "bright" fluorophores – the smaller the target organism, the higher the quantum yield and photon energies should be. The molar extinction coefficient, which is the light-absorbing capacity of a dye, should be low. Also, small Stokes' shift (the difference between excitation and emission wavelength) makes fluorophore susceptible to self-quenching when its concentration is high. This will limit the number of fluorophore molecules attached to the target and types of molecules that may "pull" electrons from the fluorophore.

There are two major groups of fluorescent agents: endogenous fluorophores and external fluorescent agents. Endogenous fluorophores occur naturally in living organisms/cells. These include aromatic amino acids, of which tryptophan is the most fluorescent and the most abundant (Gore, 2000). Fluorescent properties of tryptophan depend on the environment and emission shifts from blue to red with a decrease in hydrophobicity (Vivian & Callis, 2001). Other endogenous fluorophores include NADH (Ince *et al.*1992), flavins (Kotaki & Yagi, 1970), porphyrins (Seybold & Gouterman, 1969),

cellulose (Khalid *et al.*, 2019) and some other compounds. In water environments, self-fluorescent humic substances are common (Baker, 2001; Henderson *et al*., 2009; Mobed *et al*., 1996).

Though a useful fluorophore in protein research, tryptophan can create substantial background noise in water samples with high DOC (residual proteins in particular) (Baker, 2001; Baker & Spencer, 2004) and obscure the target signal if a fluorophore with similar emission wavelength (of 350 nm) is used for antibody-labelled FCM pathogen detection. Self-fluorescence of naturally occurring humic substances in water overlaps with the SYBR®-stained DNA emission spectra (Pollard, 2012b). Preliminary fluorescence excitation and emission matrices (FEEMS) scanning for each type of water samples might be a useful tool to avoid overlapping of specific and non-specific signals and help choosing the right fluorophore to label the target (Chapter 5). Contrary to this, the red self-fluorescence of chlorophyll (a porphyrin-based molecule) can be particularly useful for water research applications. Enumeration of microscopic algae will not require any additional staining of the sample (Holm-Hansen *et al.* 1965; Trask *et al.* 1982).

External fluorescent agents can be divided into three subgroups: biological fluorophores (fluorescent proteins like GFP and its derivatives), organic fluorescent dyes (a broad range of chemicals), and fluorescent nanoparticles (quantum dots). The best-known biological fluorophore is GFP , originally derived in the 1960s from a jellyfish *Aequorea victoria* (Shimomura *et al.* 1962). It has an emission wavelength of 510 nm using a 480 nm excitation light source. Numerous variants of GFP have been developed to provide a broad emission spectrum. The gene for GFP protein has also been cloned into a wide variety of

organisms, including water-related pathogens (Köhler *et al*., 2000; Leff & Leff, 1996; Tolker-Nielsen *et al*., 2000) and the bacteriophage T4 (Kaźmierczak *et al*., 2014). The advantages of GFP include heritability once introduced into the genome, it has extremely low cell toxicity, it is highly stable, does not require any special substrates or co-factors, and can be detected by non-invasive visualizing by 480 nm excitation light. The main disadvantage of these applications is their limitation to laboratory settings, as genetically modified organisms should not be released into the environment. But the recent report of GFP-based biosensor chimeric protein for the detection of *E. coli* in drinking water (Gutiérrez-del-Río *et al*., 2018) opens new possibilities that do not require organism modification.

Organic fluorescent dyes are a broad group of chemical compounds with quite different optical and chemical properties. Microorganisms in water research can be stained with either fluorescently labelled antibodies, or with nucleic acid (NA)-specific dyes like FITC, Propidium iodide, Acridine orange, SYBR® Green. Fungi are usually labelled with chitin/cellulose specific Calcofluor White. The great advantage of protein- and NA-specific organic fluorescent dyes for virus labeling is their affordability and ease of use. However, these fluorophores are hydrophobic compounds and as such are not fully soluble in water. As a result, in aqueous solvents they produce colloid-sized droplets that might compromise the accuracy of virus detection and enumeration (discussed further in Chapter 3). In the case of an antibody of other signal protein labelling, the excess dye is removed and does not create any artifacts in further antibody applications. On the other hand, labelled proteins can potentially aggregate together (Fink, 1998) and form artificial

signals as well. Controls to verify the absence of such aggregates should be included in the experimental design in addition to the controls for dye colloid.

Staining microorganisms of interest with fluorescent antibodies is widely used for protozoa and bacteria. It produces an easily detectable signal due to the large size of the organism and many binding sites on the cell. With viruses it is not as easy: as virions are small with a smaller number of binding epitopes, plus, a labelled antibody can be of comparable size to a virus (Porter *et al*., 2008; Porter *et al*., 2006) and add to artifact signal or be mistaken for the target virus. All immunology-based methods of virus detection, including fluorescent antibody labeling, depend on antigenic properties of the virus, and the used antibody might miss some strains of the target virus. Also, fluorescent antibodies, especially custom ones, might be costly (Hamza *et al*., 2011b). And, unfortunately, this method is not applicable for detection of all the viruses present in the sample.

If the target organism size is large enough for a flow cytometer (FCM), self-fluorescent colloid dye particles or labeled antibodies can be easily resolved from the target population. But, for smaller targets (*e.g*. virions) the closer in size they are to the instrument detection limit, the harder it is to resolve artificial colloid dye particles from the target population (Dlusskaya *et al*., 2019).

Fluorescent quantum dots might be of use for labelling larger organisms like protozoa. But the applicability of quantum dots for virus labelling is questionable, especially for virus enumeration applications, unless only one binding site per quantum dot is guaranteed by the impregnation protocol. To date, all the protocols for quantum dot labelling allow more

than one binding site for a dot. This will allow one quantum dot to bind to more than one virus particle. Another quantum dot problem for virus labelling is that their size is very comparable to the size of viruses, making virus and quantum dot populations extremely hard, if not impossible, to resolve.

In conclusion, protozoa and bacteria have more labelling options and fewer technical issues than viruses: they can be stained with any of the above methods. For virus labelling one should choose fluorophores with higher emission photon energy (shorter emission wavelength), larger Stokes' shift, higher quantum yield, and low extinction coefficient. Photo-stability is not as important for FCM as for epifluorescent microscopy since the particles pass the laser beam quickly. Fluorophores like SYBR® Green or Gold, Hoechst 33342, fluorescein, DAPI, FITC, Alexa Fluor™ 488 and some others satisfy these criteria. Though NA-binding dyes protocol for virus staining is easier than of protein-binding dyes, there have been reports of successful capsid staining (Perez-Andino *et al.* 2009; Woda & Mathew, 2015; Zhang *et al.* 2010; Zhang *et al.* 2011).

2.4.3 Flow cytometry

2.4.3.1 Principles of the method

The history of flow cytometry began in 1956 when Wallace Coulter, an electric engineer, reported a new system for counting blood cells (Coulter, 1956) that was based upon the principle he discovered in the 1940s: changes in electrical impedance non-conductive particles create when they pass the opening between two electrodes. The oscillography recorded changes in conductivity and obtained cell size distribution data as

conductivity was proportionate to the cell volume. It was a particle counter, as we know it today.

The first multi-parameter instrument, based on spectrophotometry (absorption at 253 nm and scattering at 410 nm) was reported in 1965 (Kamentsky *et al.,* 1965). This instrument estimated the amount of nucleic acid per cell volume and became a major break-through in cancer research. In fact, the design principles, utilized in that instrument, have not changed much since: a suspended particle gets stabilized with sheath fluid and passes (fluidics system) through a laser, the signal goes onto/through a dichroic mirror (optics system) onto photomultiplier/amplifier and is recorded (electronics system). The FCM results are typically presented as two- or three-dimensional dot plots or as histograms. The FCM histograms provide a visualization of the number of signals and the intensity of each chosen parameter. The populations of the FCM signal events might be resolved either based on the signal intensity, signal duration time, or on combination of both (signal area) (Figure 2.7). Larger objects, like mammalian cells, can be resolved only in forward scatter signal, which reflects the particle size, and the side scatter, reflective of inner complexity and granularity of the particle. Smaller objects like bacteria and virions must be stained with fluorescent dye in order to generate enough signal intensity while passing the laser beam. Modern FCM instruments, equipped with multiple lasers, can analyze up to fourteen fluorochromes that are used for sample staining (like 2019 Attune NxT instrument by Thermofisher).

The flow cytometric analysis is a high-speed process that allows the analysis of hundreds to thousands of events per second, depending on the instruments fluidics

system setup. This provides cell analysis and if added, sorting of large number of samples in minutes. In comparison, manual handling the equivalent number of samples would take days to complete. Another advantage of the FCM is its high precision, with less than 5% machine error (Hammes *et al.*, 2008; Wang *et al.* 2010). Hence, FCM is sensitive, with as low as 100 bacterial cells/mL detection limit (Hammes & Egli, 2010), and compatible with various methods of cell staining.

Nevertheless, FCM also has its limitations. First, it is limited to liquid samples (Shapiro, 2003). The data analysis and interpretation are sophisticated, but often subjective (Herzenberg *et al.* 2006). For example, scatter does not indicate absolute particle size or complexity. The scatter signal intensity depends on various factors from cell surface morphology/texture to laser wavelength to refractive index of the particle itself or the sheath fluid.

Most particles analyzed are not of globular shape, therefore the signal intensity depends on the orientation of a particle when it starts crossing the laser beam (Shapiro, 2003). FCM also registers signals from abiotic particles like crystals, dust, etc., along with the target cells. Therefore, it requires multiple controls and elaborate standardization in order to identify and gate the signal of interest correctly (Macey, 2007).

Despite some current limitations, with appropriate selection of fluorochromes, FCM remains a powerful tool with potential applications in microbial ecology and water research (Buysschaert *et al*., 2019; Vesey *et al*., 1997). With further development of the methodology, instrumentation, and software, an on-line flow cytometer might facilitate

accurate real-life monitoring of engineered aquatic environments (Buysschaert et al., 2018).

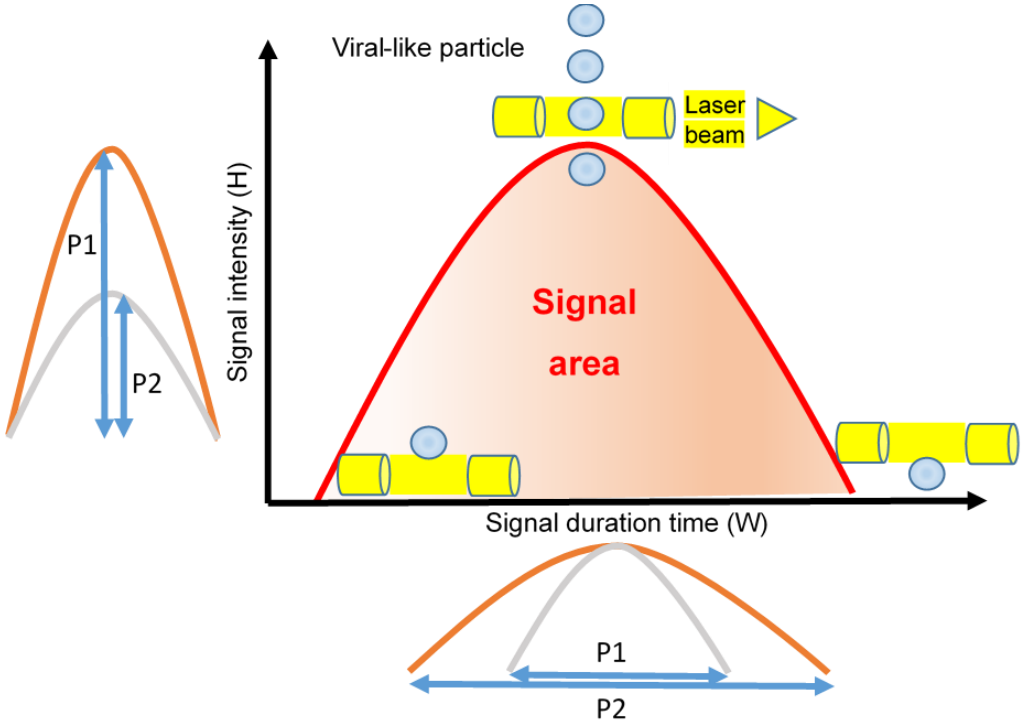

Figure 2.7. The flow cytometric signal. The signal intensifies as a particle starts crossing the laser beam, reaches its maximum when the particle is in the middle of the laser beam, and diminishes as the particle passes through. The populations of viral-like particles (P1 and P2) can be discriminated based on signal fluorescence intensity, on signal duration time, or integrated signal (area).

2.4.3.2 FCM applications in ecological studies

The first applications of FCM in ecological research were studies of marine phytoplankton (Paau *et al.* 1978). Due to chlorophyll auto-fluorescence and relatively large size of the target organisms, the phytoplankton component could be easily discriminated. All phytoplankton species contain Chlorophyll-a as the major pigment and the predominant source of red fluorescence with the emission > 610 nm in marine waters. This characteristic allows easy discrimination of phytoplankton cells from other particles in the sample (Yentsch & Yentsch, 1979). The orange fluorescence of less ubiquitous phycoerythrin has been exploited to analyse specific groups of algae like *Synechococcus* spp. and Cryptophytes in the open ocean (Wood *et al*., 1985). Chekalyuk and Hafez (2013) analyzed spectral excitation to measure various fluorescence constituents in natural waters for better structural characterization of phytoplankton communities. Some calcifying algae like *Emiliania huxleyi*, can also be easily discriminated by signal scatter only due to their high scatter signature (Veldhuis & Kraay, 2000). Protozoan cells and clump- and microcolony-forming algae can pose the difficulty of being too big for the FCM analysis. This issue has been circumvented by the design of the Optical Plankton Analyzer (OPA) (Peeters *et al.* 1989) and a sophisticated real-time CytoBuoy flow cytometer, which samples, analyses and transmits the data automatically (Dubelaar & Gerritzen, 2000; Dubelaar *et al.* 1999). Protozoan cells were successfully FCM enumerated as early as 1993. Dvorak (1993) reported *Trypanosoma, Leishmania, Giardia* and *Toxoplasma* enumerated successfully when stained with DNA-binding fluorophores, though, amoebae (*Acanthamoeba* sp. and *Enthamoeba*) displayed high fluorescent signal and a large

amount of cell debris that resulted in low resolution of the amoeba population from the background and failure of reliable enumeration.

Microbiologists started using flow cytometry since late 1970s in attempts to characterize the stages of bacterial growth cycle (Bailey *et al.*, 1977; Paau *et al.*, 1977). In 1982 Ingram *et al.* reported successful enumeration of *Legionella pneumophila* and Mansour *et al.* (1985) – of *E. coli* in blood, stained with fluorophore-labelled antibodies. A few years later Pillips and Martin (1988) questioned the FCM method reliability for bacteria analysis, pointing out high background noise and poor resolution of the target populations, the same issues we see today with virus detection (Dlusskaya *et al*., 2019). But by mid-1990s the technical limitations of the method that arise from smaller size of bacterial cells were overcome with the development of new fluorescent dyes and improved sensitivity and resolution of the FCM instruments. Since then, the FCM method has been widely used for a variety of applications, including claims for FCM enumeration of viruses, also known as “flow virometry” or just “virometry” (Rockey *et al*., 2019) [further discussed below]. The names of the FCM-based virus enumeration methods have not yet settled down and therefore are still used interchangeably.

FCM total cell counting

The total cell count is one of the most useful and straightforward applications of FCM in routine monitoring, quality control, and fundamental research. The speed, accuracy, and automation bring FCM ahead of other detection methods like plating, imaging, and even q-PCR (Wang *et al.*, 2010). The “Great plate count anomaly” estimates that less than 1% of organisms, present in natural habitats can be cultured (Hug, 2018). Considering that

for some uncultured microorganisms traditional isolation techniques may never succeed, microscopy has been routinely used for total cell counting in sediments and aquatic environments. However, microscopy has relatively low accuracy and is time-consuming, and for sediment FCM analysis the method still remains a challenge (Frossard *et al.* 2016). Where FCM excels is for aquatic environments, where fluorescently labeled bacteria are readily enumerated (Czechowska *et al.* 2008; Diaz *et al.* 2010; Hammes & Egli, 2010; Wang *et al.*, 2010). The challenge for the operator to discriminate between small bacterial cells and the background, as stated by Hammes and Egli (2010), has largely been minimized by recent development of bright fluorophores and digital FCM instruments with improved sensitivity and resolution.

Cell size estimation by FCM

Low angle forward scatter (FSC) is indicative of cell size (Macey, 2007; Shapiro, 2003). There were attempts to estimate bio-volumes of bacteria from FCM scatter data. Robertson and Button (1989) reported a linear correlation between FSC and cell volume in bacteria between 1.3 and 0.25 μm^3 when calibrated by Coulter impedance. However, others reported low sensitivity of FSC for small-sized cells and opted for side scatter (SSC) (Felip *et al.* 2007; Wang *et al.* 2009). Foladori *et al.* (2008) emphasized that the instrument sensitivity might vary and proposed silica beads with a similar refractive index to bacteria as a calibration standard.

Measuring SSC might be highly valuable for bacterial growth and life cycle studies, but questionable for microbial community analysis. As was mentioned above, the intensity of scatter signals depends on many factors, including the cell surface roughness. The cell

surface roughness varies among species and even strains and life-cycle stage. Therefore, twice as intense signal does not necessarily mean twice as large cell (Safford & Bischel, 2019).

FCM for active or non-active/dead cell estimations

Characterization of physiological state of the bacteria at single-cell level provides important information about the dynamics and functioning of a microbial consortium. Culture-independent viability analysis has the advantage of providing fast and accurate estimates of viable but non-culturable (VBNC) bacteria (Falcioni *et al.*, 2008; Santander *et al.*, 2010; Taimur Khan *et al.*, 2010). The use of multiple fluorophores with different binding characteristics allows evaluating such physiological parameters like membrane integrity, respiration, or intracellular enzyme activity. The most widely used method to estimate cell viability is staining the sample with nucleic acid binding dyes: membrane-permeable SYTO® or SYBR® that emit green fluorescence and membrane-impermeable PI with red fluorescence. But use of PI as an indicator for membrane integrity and cell viability needs to be carefully controlled and is not absolute. Shi *et al.* (2007) reported that at early logarithmic stage of bacterial (*Sphingomonas* sp. and *Mycobacterium frederiksbergense*) growth on glucose medium about 40% of the population was stained with the PI, yet these cells were viable and had normally charged membranes. The authors suggested that at this growth stage the cell envelope is somewhat loose and therefore permeable for the PI. There have been no attempts to estimate virus capsid integrity with flow cytometry using PI. This is most likely due to combination of virus and dye characteristics: small genome size of the virus and relatively low brightness of the PI

(its fluorescence is enhanced 20- to 30-fold upon binding to DNA (*Propidium Iodide | Thermo Fisher Scientific - CA*, n.d.)). Attempts to estimate capsid integrity and differentiate between infectious and damaged virions using ethidium or propidium monoazides (EMA and PMA) and subsequent DNA amplification generated some ambiguous results (Hamza *et al.*, 2011b; Leifels *et al*., 2016). Fittipaldi *et al*. (2010) reported "discrimination of infectious bacteriophage T4" using PMA-qPCR technique. However, according to their findings this method discriminated between infectious and non-infectious viruses only when virus was inactivated at 110°C and failed to do so when virus was inactivated at 85°C. Recent report of successful norovirus infectivity evaluation in shellfish using PMA-qPCR (Sarmento *et al*., 2020) lacks an important control (PMA treatment of pure RNA), thus questioning the authors' conclusion. Overall, this method of viral capsid integrity estimation seemingly has the same issues that FCM virus enumeration does. It needs the same attention to controls and better understanding of the system and its' mechanisms before this method can be applied to virus analysis in water.

Another environmental parameter that microbiologists are interested in is the nucleic acid content of bacterial cells. It has been used to characterize different populations in natural aquatic systems (Servais *et al.*, 2003; Wang *et al.*, 2009) and sub-populations with different chromosome number in pure cultures (Müller *et al.*, 2002). Analysis of natural populations invariably results in two major subgroups: High Nucleic Acid (HNA) and Low Nucleic acid (LNA). There is still a debate about physiological meaning of this phenomenon (Hammes & Egli, 2010). The major issue in this debate is relation of NA content and bacterial cell viability and metabolic activity (Longnecker *et al.*, 2005). Most

authors report that HNA bacteria provide the most metabolic activity, while the LNA bacteria functions are unknown (Wang *et al.*, 2010). Servais *et al.* (2003) argued that LNA bacteria are metabolically inactive and Falcioni *et al.* (2008) suggested that most likely they are in the VBNC state, but Wang *et al.* (2009) managed to isolate and maintain a culture of LNA bacteria in the laboratory.

Flow cytometric analysis of aquatic viruses

In 1979 Hercher *et al.* reported the first successful flow cytometric *detection* of reovirus and bacteriophage T2 (not enumerated) using forward scatter / side scatter parameters. It took twenty more years and the development of bright fluorescent dyes before marine biologists attempted to enumerate algal viruses (Marie *et al.*, 1999). Since then numerous studies of marine virus enumerations have been published (Brussaard, 2009; 2004; 2000; 2010; Chen *et al.*, 2001; Magiopoulos & Pitta, 2012; Tomaru & Nagasaki, 2007). Most studies estimate viral abundance in marine environments between 10^5 and 10^7 virus-like particles (VLP)/mL. However, Steward *et al.* (2013) questioned these estimations in their article "Are we missing half of the viruses in the ocean?". Their results of total NA measurements and the fact that marine environments are dominated by small genome-sized RNA picorna-like viruses (Steward *et al.*, 2000) let them conclude that the virus counts, and therefore the rates of virus-mediated biological processes are significantly underestimated by currently used fluorescence-based methods. Marine environments also contain algal and prokaryotic DNA viruses with genome size above 200-300 kbp (Steward *et al*., 2000; Suttle, 2005). Most of the FCM-based enumerations of marine viruses were obtained with analog BD FACS® instruments with relatively low

sensitivity/resolution. Hence, most of the viruses detected by those instruments would belong to the large-size genome group, which amounts to less than 10% of all viruses (Steward *et al*., 2000). Moreover, Hammes and Egli (2010) showed that even LNA bacteria resolution using these instruments might pose challenges.

Flow cytometry-based virus sorting was first reported by Allen *et al.* (2011). The group attempted to sort individual T4 and λ virus particles. Yet their lack of controls to prove the virus identity of the population throws doubt on the results published. The group used an unstained virus suspension as a control for the dye. However, the presence-absence of the dye facilitates interactions of this dye with ALL constituents of the sample, not just the virus. Unfortunately, the published protocols did not provide clarity regarding the composition of the bacteriophage suspensions and the possible interaction of these components with fluorescent stains. As a result, 98% of the wells of the sorting plates were empty. An assumption that the SYBR® Green dye signal population was gated as virus and that individual fluorescent dye colloid particles were sorted, would explain the low fraction of virus in sorted material. Nevertheless for larger, giant viruses (~200-400 nm Ø) sorting was demonstrated by Khalil *et al.* (2017) using a pure culture, and confirmed by transmission electron microscopy (TEM) and polymerase chain reaction (PCR).

In most environmental studies researchers use DNA-binding fluorophores to stain viruses prior the FCM analysis. Staining the virus capsid prior to FCM analysis might be more stable than the DNA staining because many protein-binding dyes form covalent bonds with the amine groups of proteins and the stained virus can be easily rinsed from the excess dye. Dengue virus remained viable after capsid staining (Woda & Mathew,

2015). This staining method might also be useful for amoeba-virus interaction experiments.

However, one has to keep in mind the potential existence of other confounding particles that might be present in preparations, like membrane-derived vesicles or so called “gene transfer agents” (Forterre *et al.*, 2013) that might interfere with fluorescence-based methods of virus enumeration including flow virometry. Recently discovered, small-sized bacteria like *Microbacterium* might also have the capability to go through the filter but in general should not affect total virus counts substantially due to their relatively low abundance compared to viruses.

2.4.3.3 FCM applications for engineered water systems analysis

In wastewater analysis FCM has been used for two major purposes: microbial community characterization and viability assessment. Multiple studies applied FCM to characterize microbial communities at various stages of water treatment processes using various staining techniques. First, the total cell enumeration by FCM has been proven to be more sensitive and time-efficient than traditional Heterotrophic Plate Count (HPC) method (Hammes *et al.*, 2008; Hug, 2018; Van Nevel *et al.*, 2017). Though, some attempts to enumerate and characterize bacterial consortium of activated sludge or waste stabilization ponds water produced FCM dot plots that were somewhat challenging to interpret (Coggins *et al.*, 2020; Günther *et al.*, 2008), or the bacterial counts were too high (Manti et al., 2008): treated wastewater effluent with 10^8 cells/mL would fail turbidity requirements. Foladori *et al.* (2010) convincingly demonstrated bacterial cell viability and

physiological activity at various wastewater treatment stages by staining bacteria with SYBR® Green I and PI, and with the fluorogenic substrate 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein Acetoxymethyl Ester (BCESF-AM), respectively.

Multiple studies focused on polyphosphate-accumulating sub-populations of bacteria that play a role in phosphorus removal during the wastewater treatment process. These studies employed various staining techniques including green fluorescence of tetracycline (Gunther *et al.*, 2009) and yellow fluorescence of DAPI (Hung *et al.*, 2002) upon binding to polyphosphate granules. McIlroy (2008) and Li *et al.* (2019) used fluorescent *in situ* hybridization (FISH) to target specific DNA sequences in glycogen-accumulating and phosphate-accumulating organisms and Tay *et al.* (2002) enumerated *Bacteroides* species in activated sludge, both utilising FCM.

Viability FCM analysis at a single-cell level allows rapid quantitative measurement of viable bacteria including VBNC cell forms (Hammes & Egli, 2010; Safford & Bischel, 2019). The use of multiple fluorophores with different binding sites allow researchers to evaluate the mode and the extent of the cell damage (Foladori *et al.*, 2010; Pianetti *et al.*, 2005). The fluorophores that assess the membrane integrity were employed by Giao *et al.* (2009) to aid in identifying the effect of chlorine disinfection on *L. pneumophila*; Ssemakalu *et al.* (2012) studied the solar inactivation of *Vibrio cholerae*; and Foladori *et al.* (2007) investigated the effects of sonication on pure cultures of *E. coli* and *Enterococcus faecalis* as well as microorganisms present in raw wastewater and activated sludge. FCM analysis also shows intermediate fluorescence of sub-lethally injured cells during cell viability assessment. Overall, FCM in combinations with staining provided additional and more

extensive information about cell damage in an efficient and reproducible manner (Falcioni *et al.*, 2006; Hewitt *et al.*, 2000; Nebe-Von-Caron *et al.*, 2000).

Despite the advantages of FCM multi-parametric analysis of cell viability, Hammes and Egli (2010) warned that "the tendency exists to view viability-staining methods as off-the-shelf and from-the-manuscript applications, with the danger of erroneous applications and interpretations". Different water treatment conditions, different disinfectants used, different microorganisms targeted, all require rigorous validation and standardization of the viability staining protocols. Nie *et al.* (2016) compared effects of chloramine, free chlorine and ultraviolet (UV) irradiation on *S. aureus* and *E. coli* and demonstrated inactivation but no damage to the cell membrane by the UV irradiation. On the other hand, chlorine demonstrated strong and fast damaging effect on the cell membrane, but it also caused nucleic acids to decay. Phe *et al.* (2005) also showed that with increasing chlorination dose, the fluorescence of PI-stained DNA and RNA in *E. coli* rapidly decreased. The authors also attributed this effect to severe damage of NA by chlorine after which PI could no longer bind. These effects need to be taken into consideration in UV- and oxidant-disinfection experiments as they can lead to underestimation of actually dead bacteria, realising that maybe definition dependant (Flemming *et al*., 2016; Königs *et al*., 2015). To clarify these challenges, Berney *et al.* (2008) used combination of fluorophores to estimate six cellular functions of *E. coli* after sun- and UV-treatment: efflux pump activity, membrane potential, membrane integrity, glucose uptake activity, total ATP concentration and cultivability. This study proved the efficiency of sun-inactivation of *E. coli* and gave a detailed picture of cells "agony".

Direct FCM enumeration of total bacterial cells stained with SYBR® or SYTO™ fluorophores allows quantification of bacterial removal during multiple stages of water treatment process. Multiple studies applied this method to enumerate bacteria in wastewater (Brown *et al.*, 2019; Foladori *et al.*, 2015; Foladori *et al.*, 2010; Ma *et al.*, 2013) and quantify bacteria removal in drinking water treatment process (Berney *et al.*, 2008; Hammes *et al.*, 2007 and 2008; Helmi *et al.*, 2015; Ho *et al.*, 2012; Liu *et al.*, 2014; Wang *et al.*, 2017).

Microbial communities in engineered water systems respond to various influences related to source water quality and the treatment processes. These changes might continue over long periods of time (seasonal dynamics) (Flowers *et al.*, 2013; Pinto *et al.*, 2014; Prest *et al.*, 2016) or might be caused by the short-time operational disturbances like hydraulic regime changes (Douterelo *et al.*, 2013), short-term periodic fluctuations (Hashimoto *et al.*, 2014; Shade *et al.*, 2013;Wang *et al.*, 2011), aperiodic fluctuations caused by rainfall (Besmer & Hammes, 2016), or combination of both (Besmer *et al*., 2016). These increases in the microbial loads might be short-term events but they pose the highest risk to public health. And a common practice of infrequent direct pathogen monitoring provides a negligible risk barrier (Signor & Ashbolt, 2006). Traditional methods of microbial water quality monitoring are simply not able to detect such short events (Hammes & Egli, 2010), as they take several hours to several days to complete, depending on the method. The automated online flow cytometric bacterial enumeration solves this problem to a degree. In 2012 Hammes *et al.* published the conceptual design of an automated flow cytometer for online bacterial monitoring. The instrument they

developed demonstrated less than 5% standard deviation in enumeration of bacterial cells within 10^3-10^6 cells/mL range and was able to detect at least two fluorescent stains. Further applications of this instrument (Van Nevel *et al.*, 2013) allowed to accurately measure cell concentrations and assess their viability. Though, the authors emphasized that a standardized staining protocol and a short measurement time should be stringently followed. Considering the progress of the FCM monitoring of bacterial populations in various engineered water systems and appreciable amount of full-scale studies (De Roy *et al.*, 2012; Gillespie *et al.*, 2014; Van Nevel *et al.*, 2017) bacterial enumeration and characterization in engineered water environments is relatively well established for FCM. Virus enumeration poses more challenges to the FCM method due to small diameter and genome size of virus particles (virions). There are few studies that addressed virus enumeration in the source water and wastewater (Brown *et al.*, 2019 and 2015; Huang *et al.*, 2015; Ma *et al.*, 2013; Roudnew *et al.*, 2014, 2013, and 2012). Unfortunately, these publications exhibit the problem Hammes and Egli (2010) warned about in their discussion of the live-dead bacteria staining method: the researchers view the FCM marine virus enumeration protocol by Brussaard (2004 and 2010) as the “from-the-manuscript” and ready-to-use without any critical evaluation of its applicability for engineered water systems. Moreover, in different studies Green fluorescence/Side Scatter Signal of similar intensity might be gated as noise or as VLPs depending on the focus and the objectives of the study. For example, Roudnew *et al.* (2012) aimed to assess abundance of both bacteria and virus-like particles and Brown *et al.* (2015) described “a rapid FCM protocol to enumerate planktonic and floc-associated extracellular viruses in

activated sludge". In both publications the populations with low-intensity Green fluorescence/Side Scatter signal were defined as "virus". On the other hand, Nishimura *et al.* (2005) focused on bacterioplankton subgroups rather than virus, and similar low intensity FCM signal was defined as "noise".

The FCM count estimates reported as viruses vary from 10^5 VLP/mL in groundwater (Roudnew *et al*., 2012) to 10^8 VLP/mL in microfiltration influent (Huang *et al.*, 2015), and to 10^9 VLP/mL in activated sludge (Brown *et al*., 2015). Safford and Bischel (2019) admitted the need for the robust validation system to prove that the populations detected by FCM are indeed virus rather than other fluorescent particles. To date, no published work by the authors just referenced had adequate controls to validate their estimates as truly generated by virions.

2.4.4 Fluorescent spectroscopy for water analysis

2.4.4.1 Organic matter composition analysis

A range of well-established methods for routine monitoring the chemical, microbiological and physical parameters like biochemical and chemical oxygen demand (BOD and COD), total organic carbon (TOC), pH, turbidity, total suspended solids (TSS), *etc*., is used to ensure the quality of processed and natural waters (Carstea *et al.*2016; Hambly *et al.*, 2015a; Huang *et al.*, 2010; Maimon & Gross, 2018). However, the organic substance monitoring methods like BOD and COD are time-consuming, utilize foreign bacterial cultures or harsh chemicals, which makes them unsuitable for online monitoring (Bourgeois *et al.*, 2001). The TOC monitoring can be used in real-time settings (Assmann *et*

al., 2017), but it requires expensive equipment and still is not able to differentiate between biodegradable and non-biodegradable organic matter. It also provides no information regarding the oxidation state of the carbon and, therefore, does not reflect the oxygen demand (Vanrolleghem & Lee, 2003).

The potential for fluorescence spectroscopy to provide real-time monitoring of sewage "strength" and for treatment process control was first recognized by Reynolds and Ahmad (Ahmad & Reynolds, 1995, 1999; Reynolds & Ahmad, 1997). In their experiments synchronous fluorescence spectra (SFS) of sewage samples showed well-defined and reproducible structure with a peak at about 280 nm, which they attributed to the "biodegradable aromatic hydrocarbon constituent". In subsequent publications this peak has been referred to as tryptophan-like (T) (Baker & Spencer, 2004; Henderson *et al*., 2009).

There are two major optical methods of wastewater process monitoring: UV-vis absorption and fluorescence spectroscopy. Both methods are fast, sensitive, require neither sample pre-treatment nor reagent, and, therefore, are non-invasive (Bourgeois *et al.*, 2001; Henderson *et al.*, 2009; Park & Snyder, 2018). They can provide high temporal and spatial resolution and quickly reflect dynamic changes, which is crucial for early detection of pollution or sewage/drinking water cross contamination events and for the water treatment process control (Bourgeois *et al*., 2001).

Though UV-vis absorption has been widely researched and commercially applied for online monitoring (Broeke *et al.*, 2006; Jeong *et al.*, 2007), the fluorescence spectroscopy has been found to be 10-1000 times more sensitive (Henderson *et al*., 2009) and it is able

to discriminate between different organic substances with similar absorbance and different emission wavelength (Carstea *et al.*, 2016; Knapik *et al.* 2014).

The fluorescent excitation and emission matrices (FEEMs) are three-dimensional (excitation x emission x intensity) “maps of sample” that characterize the specific excitation and emission wavelength unique to each chromophoric substance present in the sample. At low concentrations of chromophoric substance the “peak” signal intensity is proportionate to its concentration (Henderson *et al*., 2009). The spectral signature of each sample offers large amount of data, suitable for multiple methods of data analysis. Simple peak-picking was the first tool used by Coble (1996) to identify humic-like, tryptophan-like and tyrosine-like fluorescent signals. Today eight peaks are commonly identified in water systems: tyrosine-like peak B – 275/305 nm; tryptophan-like (protein-like) T1/T2 – 275/340; humic-like peak A – 260/400-460 nm; humic-like peak C (sometimes subdivided into two peaks C1 and C2 (Henderson *et al*., 2009)) - 320-360/420-460 nm; soil fulvic acid D and E – 390/509 and 455-521 nm respectively; plankton-derived N – 280/370 nm; and pigment-like P - 398-660 nm (Coble, 2007). This detailed peak substantiation allowed Baker *et al.* (Baker, 2001 and 2005; Baker *et al.*, 2003) to demonstrate river water contamination with sewage discharge and landfill leachate. Clean environmental waters are dominated by humic-like fluorescence peaks A and C since the dissolved organic matter mostly originates from soil and plant material, while the sewage-origin organic matter is dominated by protein-rich peak T due to high microbial activity. The recycled wastewater peak T intensity is higher than the potable water peak T. The changed ratio of these two intensities allowed Hambly *et al.* (2015b) to pinpoint a cross-

connection between potable and reticulation pipelines in a Victoria, Australia, neighbourhood.

Besides the peak-picking FEEMs data could be analysed using the Parallel Factor Analysis (PARAFAC), a mathematical multivariable model that allows to specify the components of the mixed spectrum of the three-dimensional matrices and correct the data in terms of inner filtering and fluorescence quenching effects of the water sample itself (Andersen & Bro, 2003; Bro, 1997; Murphy *et al.*, 2013; https://cran.r-project.org/web/packages/staRdom/vignettes/PARAFAC_analysis_of_EEM.html). This method is now used for evaluation of organic matter in natural and artificial aquatic systems (Murphy *et al.*, 2014). Recently it has been proposed as a surrogate for conventional water quality parameters to assess the water treatment process performance since the fluorescence intensities of peak T show correlations over 0.85 with BOD, COD, and TOC (Baker & Inverarity, 2004; Hudson *et al*., 2008).

2.4.4.2 Virus abundance estimations

The only attempt to estimate virus abundance in water using FEEM was reported by Pollard (2012a, 2012b), who used SYBR® Gold to stain the samples. This technique revealed a distinct fluorescent peak with the excitation/emission spectra typical of the SYBR®/DNA complex. The intensity of this peak correlated (r^2 = 0.84 for natural water and r^2 = 0.96 for the wastewater dilution series) with the virus numbers, determined by epifluorescence microscopy. However, the non-specific fluorescence of the water sample background can affect the results since in Pollard's experiments it comprised up to 70% of

the total signal. Also, samples rich in humic substances emitted auto-fluorescence in the same spectra that overlapped with the virus signal, which interfered with virus estimates.

Nevertheless, FEEM-based virus abundance estimation, with some further optimization and sensitivity improvement, has considerable potential to be adapted for online monitoring of virus removal during water treatment processes. Its combination with other online water quality parameters appraisal could function as early warning for some level of treatment process failure.

Chapter 3: Colloid chemistry pitfall for flow cytometric enumeration of viruses in water.*

3.1 Introduction

Viruses are the most numerous microbial group and impact on the dynamics of aquatic ecosystems (Kauffman et al., 2018; Steward et al., 2013). They influence biogeochemical cycles through gene regulation and configuring microbial communities, and by "killing the winning" prokaryotic or eukaryotic species (Weinbauer & Rassoulzadegan, 2004), they help maintain diversity and dynamic functioning of natural (Fauvel *et al*., 2017) and artificial (Withey et al., 2005) ecosystems. Key features include short-duration virus infection cycles, highly abundant viromes and rapid changes in virion abundance and diversity.

To investigate viruses in environmental waters, transmission electron microscopy (TEM) was one of the first methods, which demonstrated much higher abundances of viruses in marine waters compared to plaque forming unit enumeration (Bergh *et al*., 1989). With the development of sensitive fluorescent dyes, TEM was replaced by epifluorescent microscopy (EFM) (Noble & Fuhrman, 1998; Patel et al., 2007), which has demonstrated even higher counts, compared with TEM (Hermes & Suttle, 1995; Weinbauer & Suttle, 1997). Though sensitive, these methods are labor intensive and time consuming. Flow cytometry (FCM) enumerations of virus has neither of these shortcomings, and was first

* Dlusskaya, E. A., Atrazhev, A. M., & Ashbolt, N. J. (2019). Colloid chemistry pitfall for flow cytometric enumeration of viruses in water. Water Research X, 2, 100025. https://doi.org/10.1016/j.wroa.2019.100025

reported in 1979 (Hercher et al., 1979), but was not widely used in ecological studies until twenty years later with the availability of bright fluorescent DNA-binding dyes. Since then, flow cytometric virus enumeration has become a standard approach in water research (Marie et al., 1999).

The efficiency of virus-targeted FCM is usually estimated by its comparison with TEM or EFM virus counts in environmental samples. To our knowledge only Tomaru and Nagasaki (2007) attempted to compare FCM counts with most probable number estimates, based on a culture and extinction dilution method (Suttle, 1993) using single virus cultures. In general, SYBR® Green I is preferred for virus staining since this fluorescent dye is affordable and results in higher virus counts when compared to other dyes (Brussaard 2004).

The aims of this study were to illustrate likely artifacts and understand their mechanisms when staining wastewater bacteriophages with SYBR® Green I for FCM enumeration, and to estimate the sensitivity and accuracy of FCM for lambda (λ), P1, and T4 bacteriophage enumeration compared to PFU estimations.

3.2 Materials and methods

3.2.1 Bacteriophage sample preparation

Bacteriophages of three genome sizes: 48,502 bp dsDNA lambda (Sanger *et al*., 1982); 93,601 bp dsDNA P1 (Łobocka et al., 2004); and 168,903 bp dsDNA T4 (Miller et al., 2003) were propagated in *E. coli* hosts TG1 (Lucigen), MG1655 (ATCC 47076), and BL21DE3 (Sigma-Aldrich) respectively. The *E. coli* cultures were grown in LB broth (BD, REF# 241420) at 37^{o}C and 250 rpm to optical densities of 0.6-0.7, then either innoculated with an appropriate bacteriophage suspension or an equal volume of sterile PBS (host control). The incubation was continued overnight at 37 ^{o}C with no shaking. To disrupt the cells, the host control cultures (50 mL) were sonicated on wet ice for 1 min by using a horn-equipped ultrasonic apparatus (XL2020, 20 kHz) at 50% power setting. Then both bacteriophage-infected and sonicated control cultures were treated identically.

Overnight cultures were centrifuged at 4,000 *g* for 30 min to precipitate bacterial cell debris, supernatant was filtered through 0.22 µm syringe filter (Merck Millipore, REF # SLGS033SS) into a sterile Amicon Ultra 100K centrifugal filter device (Merck Millipore, REF # UFC910024), and centrifuged again at 4,000 *g* for 20 min to eliminate any influence of growth media on flow cytometry analysis. Bacteriophage remaining on the filter part of the device (in about 250 µL), was treated with DNAse I (Roche Diagnostics, REF # 10104159001) to remove residual host DNA by adding: 25 µL of 10x DNAse I buffer (100 mM Tris HCl pH 7.5, 25 mM $MgCl_2$, and 5 mM $CaCl_2$ in MQ water) and 1 µL of 2.5 mg/mL DNAse I, dissolved in storage buffer (20 % glycerol in 75 mM NaCl) to the bacteriophage

suspensions and incubated for 45 min at 37 ℃. All chemicals were purchased from Sigma, unless stated otherwise.

After the incubation, bacteriophage samples were rinsed with 10 mL of 1x HyClone PBS (HyClone Laboratories, REF #SH30256.02) that was filtered through 1 kDa Macrosep Advance Centrifugal device (PALL, REF # MAP001C36), resuspended in PBS to the initial volume and analysed.

3.2.2 Bacteriophage double agar overlay plaque enumeration assay

Solid and soft Trypticase Soy Agar was prepared from BBL Trypticase Soy Broth (BD, REF # 211768) with addition of 1.5 and 0.6% agar respectively. Triplicate decimal dilutions of bacteriophage (T4, λ or P1) samples were prepared in 900 µL of 1x HyClone PBS and the double-layer agar assay was carried out as described previously (Kropinski *et al.*, 2009). Standard deviations and P-values were calculated with Microsoft Excel™.

3.2.3 SYBR Green I auto-fluorescence

The molecular structure of SYBR® Green I (*National Center for Biotechnology Information. PubChem Compound Database; CID=10436340, https://pubchem.ncbi.nlm.nih.gov/compound/10436340 (accessed July 20, 2017)*) implies a hydrophobic compound, which is not fully soluble in aqueous solvents. Hence, to estimate fluorescence of colloidal SYBR® particles, we prepared stabilized emulsions of SYBR® with one of the following surfactants: Triton-X100, IgePal-630, Tween 20, NP 40, Brije 35, and Sodium Dodecyl Sulfate (SDS). SYBR® Green I (ThermoFisher, REF#S7563) was added to 1 % solution of a surfactant in 1 kDa – filtered Tris-EDTA (TE) buffer pH 8.0 to

final concentration of 50x. All samples and SYBR Green I stock in this study were diluted, stained, and stored in black microcentrifuge tubes (Agros Technologies, REF# T7100BK).

Duplicate dilutions of SYBR in TE were prepared at 0.5x, 1x, 5x, and 50x concentrations; one set was heated at 80 ℃ for 10 min, and the other was analysed unheated. All TE buffer was 1 kDa – filtered before use. Crimson fluorescent 0.2 µm FluoroSpheres® (ThermoFisher Scientific #F8806) were added to a final concentration of $3.4x10^7$ beads·mL^{-1} for quality control.

The working stock of SYBR® Green I should not be filtered due to interactions that remove this hydrophobic dye from solution (Figure 3.1). This effect is based on well understood selective wettability and capillary force mechanisms in colloid systems (Yu et al., 2016).

Fluorescence was observed with a conventional benchtop UV transilluminator (UVP, ThermoFisher Scientific) as well as an EVOS FL fluorescent cell imaging system (ThermoFisher Scientific). For the wet mount, 25 µL of fresh samples were placed on new pre-cleaned microscope slides (ThermoFisher Scientific #12-550-A3) and covered with glass coverslips (ThermoFisher Scientific #12-540B). The EVOS images were captured in TxRed (585/29 Ex 624/40 Em), GFP (470/22 Ex 510/42 Em), and TRANS channels and image overlays were created.

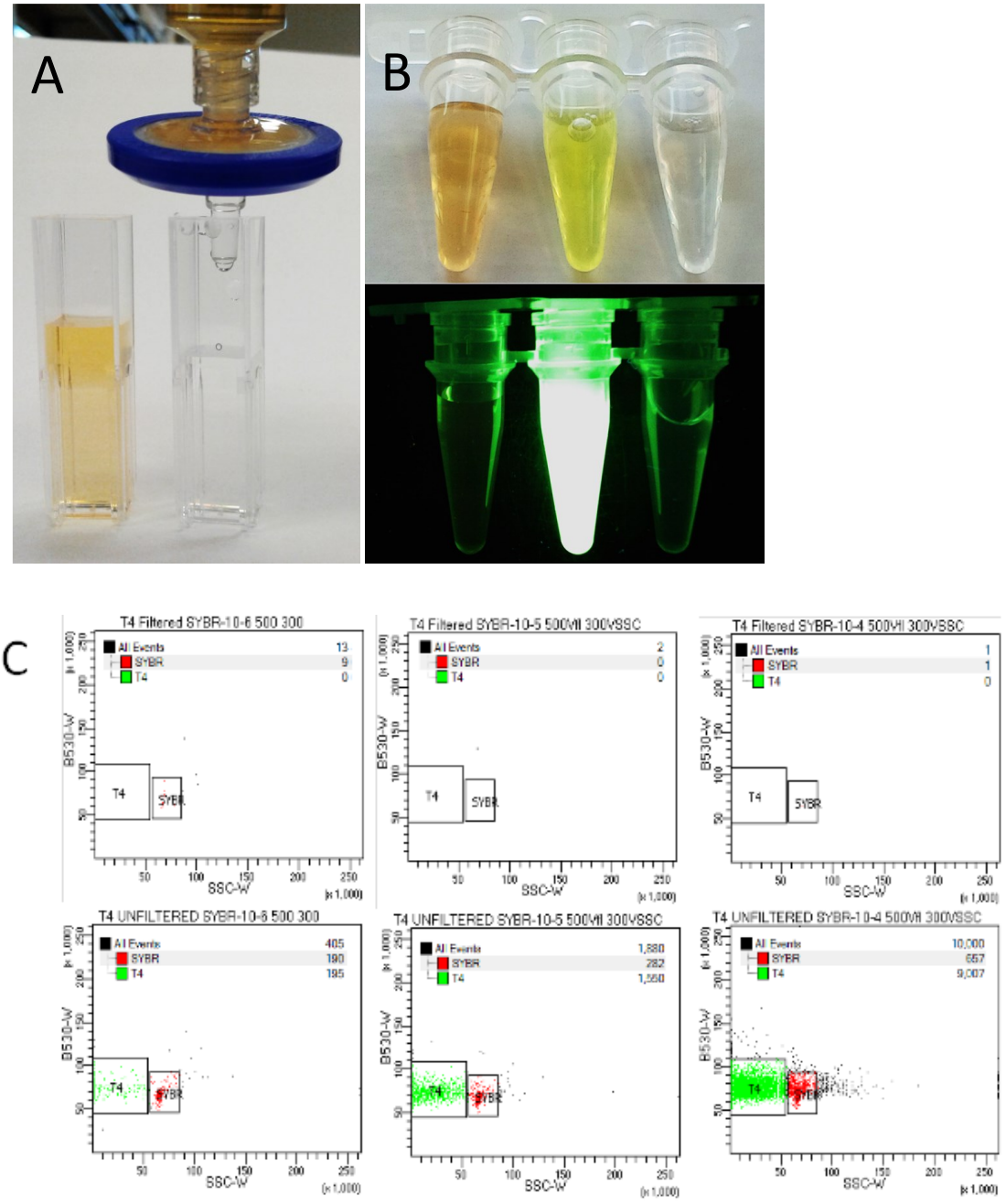

Figure 3.1. Removal of SYBR Green I from solution by coalescence on a membrane for aqueous solutions sterilization (EMD Millipore REF# SLGS033SS). Panel A - 50x SYBR Green I in 1-kDa filtered 1x TE before (left) and after (right) filtration through 0.22 µm syringe filter. Panel B - Left to right: 25x unfiltered SYBR Green I in TE buffer; 25x unfiltered SYBR

Green I and 150 ng·µL^{-1} double stranded DNA in TE; 25x filtered SYBR Green I and 150 ng·µL^{-1} double stranded DNA in TE. Visualised in natural light (top) and in UV (bottom). Panel C - Flow cytometric signal of bacteriophage T4 dilution series, stained with filtered (upper row) and unfiltered (lower row) SYBR Green I.

3.2.4 Flow cytometry

SYBR® Green I samples were diluted in TE buffer to final concentrations 0.1x, 0.2x, 0.5x, 1x, and 2x, with one set heat treated and the other not, as described above.

Bacteriophage decimal dilutions were prepared in triplicate in TE buffer and stained as described (Brussaard 2004) with 0.5x and 1x SYBR® Green I. TE buffer was also prepared with the SYBR® dye as negative control.

Flow rate was estimated with 1 µm latex bead FluoroSpheres® (ThermoFisher, REF# F8823). The beads were first briefly vortexed and then bath-sonicated for 1 min as recommended by the manufacturer; noting that vortexing only gave inconsistent results (data not shown). Triplicate 100-fold serial dilutions were prepared to 10^{-4}, and then decimally to 10^{-6}, immediately after the sonication step. It is important to pay attention that no droplet was left on the outer side of the pipette tip. Dilutions, used for analysis, were briefly vortexed and sonicated again right before being analysed. As each batch of beads has a Certificate of Analysis with the number of beads per mL indicated, it was possible to calculate the number of beads per mL of the working dilution. To calculate the flow rate, the number of events in the bead population was divided by bead concentration in the working dilution. Flow rate was calculated each time samples were analysed.

Flow cytometric analysis was carried out with BD LSRFortessa™ X-20 cell analyzer (BD Biosciences, USA) equipped with 488 nm excitation laser with standard filter setup. The trigger was set to 200 on green fluorescence (FITC channel). Data was collected using FITC-W / SSC-W dot plots. FITC channel was set at 500V and SSC channel was set at 300V.

Events were gated based on SYBR in TE samples with no virus and T4 SYBR-stained decimal dilutions.

Also, an older model of flow cytometer, Gallios™ (Beckman Coulter), also equipped with 488 nm excitation laser, was used to compare sensitivity of the two instruments. Data were collected as FL1 (525/40 nm) INT / FL2 (575/25 nm) INT and/or FL1 TOF / SSC TOF plot, with the same no virus and T4 SYBR-treated samples used on the BD LSRFortessa™.

3.3 Results

3.3.1 SYBR® auto-fluorescence interference

Microscopic examination of SYBR® Green I partly dissolved in TE buffer revealed the presence of fluorescent particles in all dilutions, in both heated and unheated samples. Critical to the presence of possible artifacts analysed by FCM, this dye produces small crystals or amorphous mass, which may also lead to uneven distribution of the SYBR fluorophore among the aliquots used for sample staining (Figure 3.2). Centrifugation of SYBR stock is still not recommended as another well understood (Becher & Fishman, 1965) mechanical method for breaking an emulsion in addition to filtration. Addition of surfactants to 1% final concentration to aid colloid dispersion (relevant to maximum levels expected in wastewater (Adak *et al*., 2005)) resulted in intense fluorescence of SYBR® Green I (Figure 3.3) even with no DNA present. Similar results were obtained with SYBR Gold (ThermoFisher, REF#S11494) at 50x final concentration, Hoechst 33342 Ready Flow Reagent (ThermoFisher, REF#R17753) at 10% of commercial stock concentration, and

some other fluorescent dyes (Figure 3.4). Numerous fluorescing SYBR® Green I particles were observed by microscopy (Figure 3.5), and FCM signal was also more intense when compared to controls with no surfactant added.Flow cytometric analysis of various concentrations of SYBR® Green I in TE demonstrated a distinct population of fluorescent particles. Event counts in some random sample tubes were much higher than in other replicate tubes with supposedly the same concentration of SYBR® (data not shown). Most likely, this variability was the effect of non-uniform dispersion of SYBR® Green I in the stock solution. Moreover, the event counts noticeably increased after bath-sonication, pipetting, or just hand shaking of the samples and decreased in the samples subsequently kept undisturbed, as illustrated in Figure 3.6 when using the Gallios™ instrument and on the BD LSR Fortessa™ X-20 (see Figure 3.7).

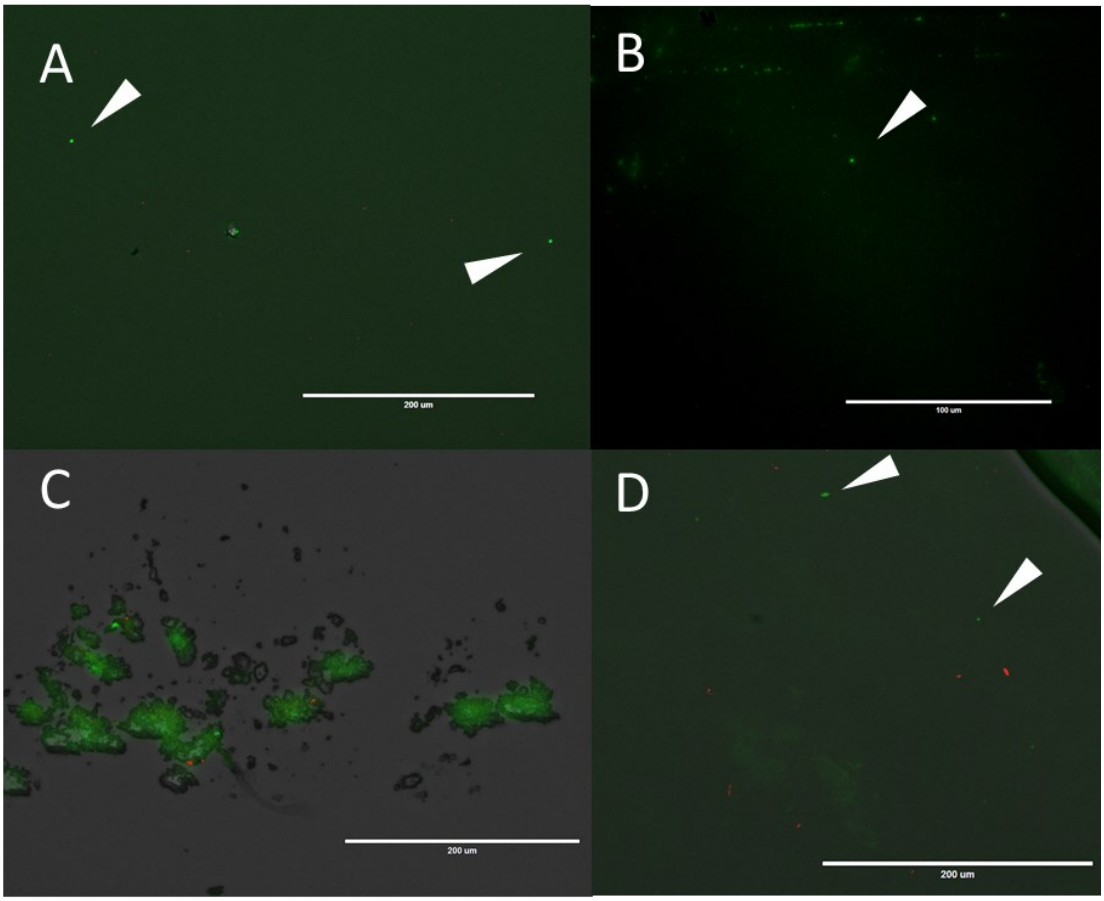

Figure 3.2. SYBR® Green I by fluorescent microscopy in TE buffer: A) 0.5x; B) 1x; C) 2x; D) 5x. Red dots – 0.2 µm crimson FluoroSpheres®. Arrows indicate SYBR®-colloid particles.

3.3.2 Bacteriophage detection and enumeration

Double agar overlay plaque assay showed 9.98 ± 0.09 log PFU.mL^{-1} of T4, 10.36 ± 0.25 log PFU.mL^{-1} of P1, and 9.3 ± 0.15 log PFU.mL^{-1} of λ bacteriophages. However, both Fortessa™ X-20 and Gallios™ instruments failed to detect Lambda (data not shown) and P1 (Figure 3.8) bacteriophages. On the other hand, bacteriophage T4 was resolved as a distinct population of events when analysed on the Fortessa™ X-20 (Figure 3.9, A-C), but not with the Gallios instrument (Figure 3.6). Two distinct populations were identified (P1 & P2), with only the number of events in P2 changing according to dilutions of the T4 bacteriophage, thus confirming P2 largely contained the target population. Bacteriophage host controls (Figure 3.10) also prove that the signal in the population T4 (which is same as P2, the name was changed after one of the instruments software updates) belongs to the virus and not to debris or bacterial cell structures (like vesicles). Bacteriophage-free host *E. coli* cells were disrupted by sonication during the sample preparation as plating of 10µL of lysate indicated no survivors. T4 bacteriophage FCM counts of the same bacterial lysate that was shown on Figure 3.9 revealed no significant difference (by two-tailed unpaired T-test) between either 0.5x or 1x SYBR stained samples at either 10^{-5} or 10^{-6} dilutions, as well as when compared with the plaque assay counts (Figure 3.11). However, significant disturbing of the samples led to decreased FCM virus events (Figure 3.9, D-F), and estimated numbers did not correspond to the plaque assay data. Therefore, care in sample handling is also important when quantifying (T4) bacteriophages by FCM.

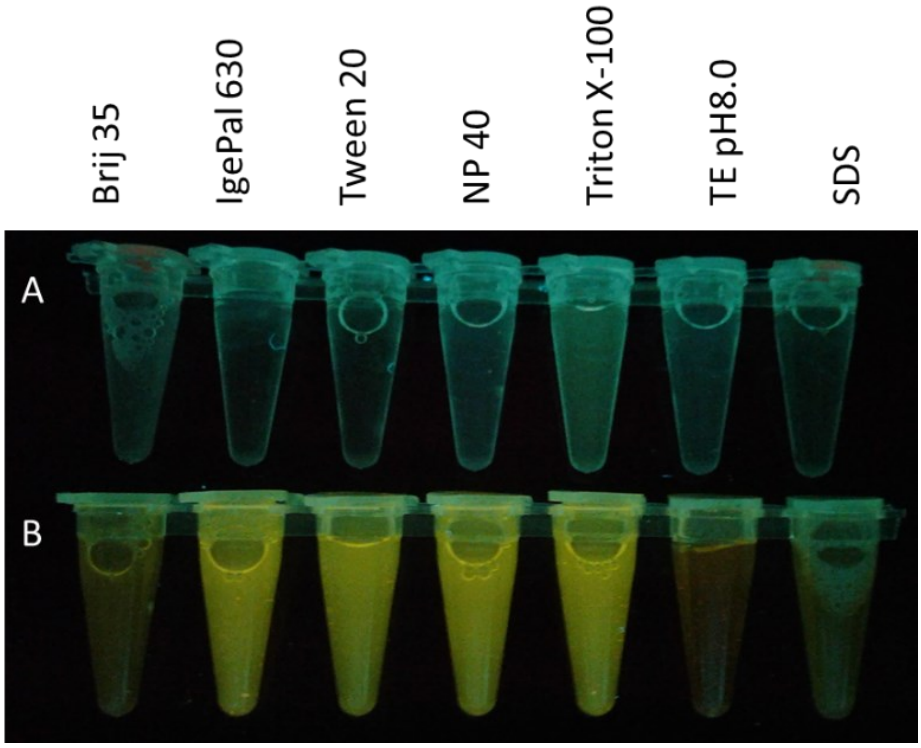

Figure 3.3. Fluorescence of SYBR® Green I emulsified with various surfactants.

A) 1 % surfactants in 1 kDa-filtered TE pH 8.0; B) with 50x SYBR® Green I added

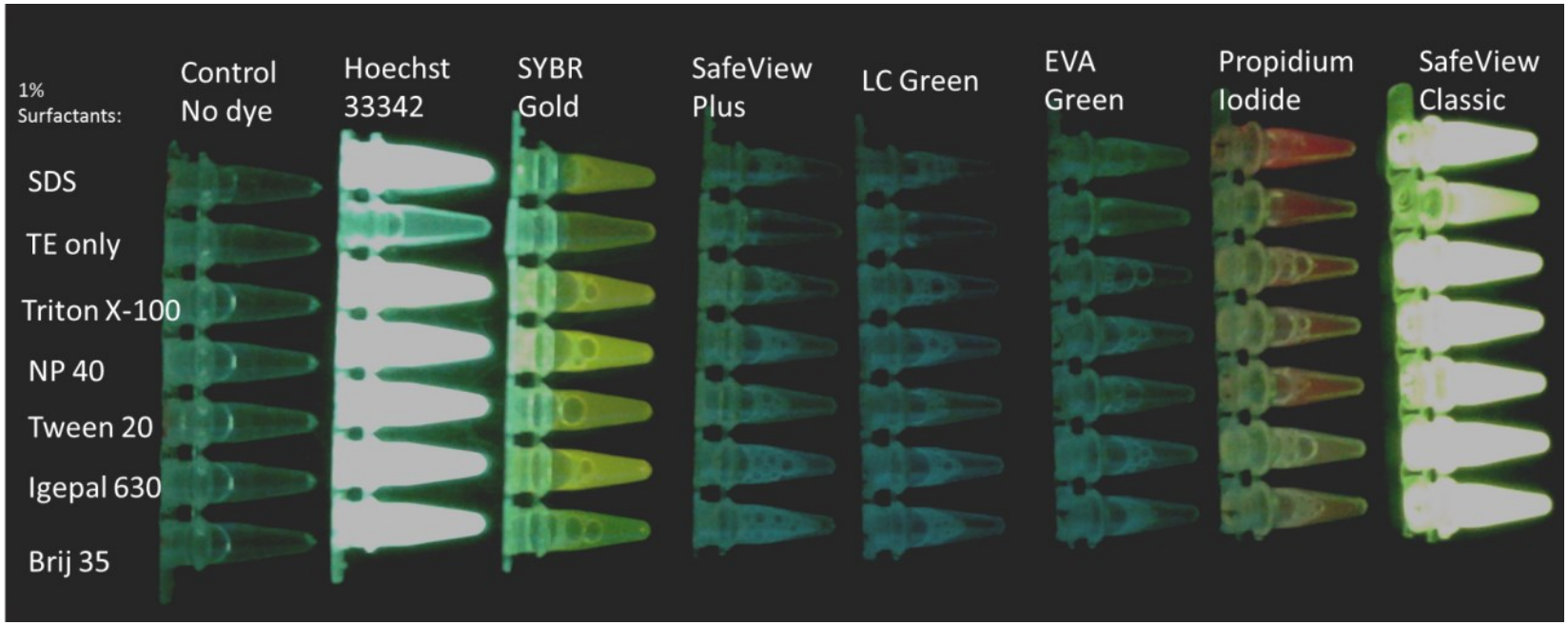

Figure 3.4. Fluorescence of DNA-binding dyes emulsified with various surfactants. Fluorescence of SafeView Plus, LC Green, and EVA Green is less intense in this experiment due to lower concentrations these dyes are sold at; microscopic examination still revealed auto-fluorescing particles present.

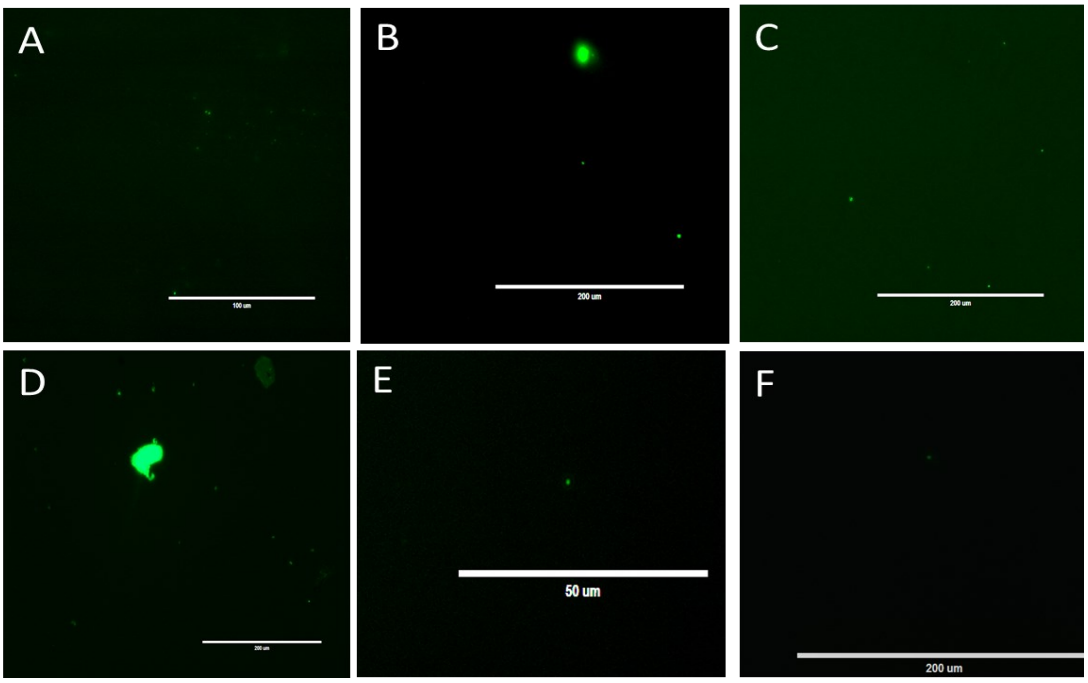

Figure 3.5. SYBR® Green I emulsions by fluorescent microscopy. Prepared with:

A) BRIJ 35; B) Tween 20; C) NP 40; D) Triton-X100; E) EGEPAL CA-630; F) SDS.

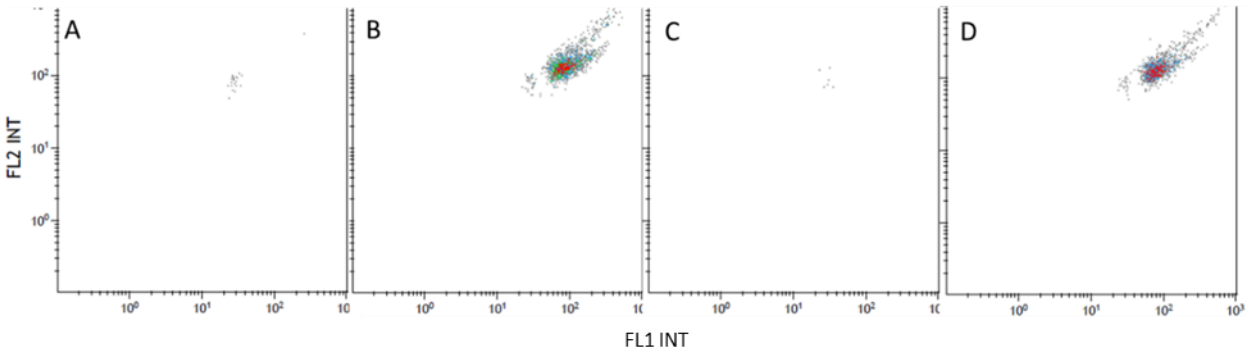

Figure 3.6. Fluorescent signal artifact of SYBR® Green I-stained T4 bacteriophage sample (~10^6 PFU.mL^{-1}), obtained by GALLIOS™ flow cytometer in FL1 (525/40) intensity vs FL2 (575/25) intensity parameters. Measurements were performed in the same tube: sample gently transferred into FCM tube after staining (A), immediately after vigorous hand-shaking (B), 20 min after hand-shaking (C), after shaking second time (D). Enhanced FCM signal was only observed after shaking.

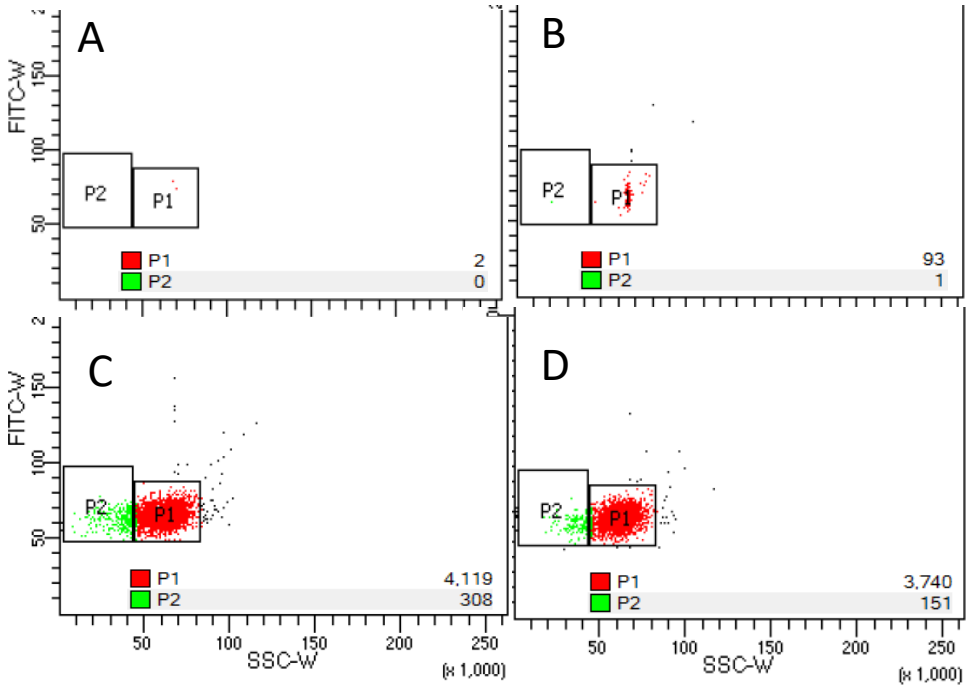

Figure 3.7. FCM fluorescent signal of 0.5x SYBR® Green I in TE buffer, presented as a plot of fluorescence (490 ex/530 em) duration (FITC-W) vs Side Scatter duration (SSC-W). No SYBR TE control (A), gently handled sample (B), and the same sample immediately and 10 minutes after vigorous pipetting (C and D). P1 – gated SYBR® particle population, P2 – gate based on T4 virus signal. Immediately after pipetting SYBR® population overlapped with the P2 gate.

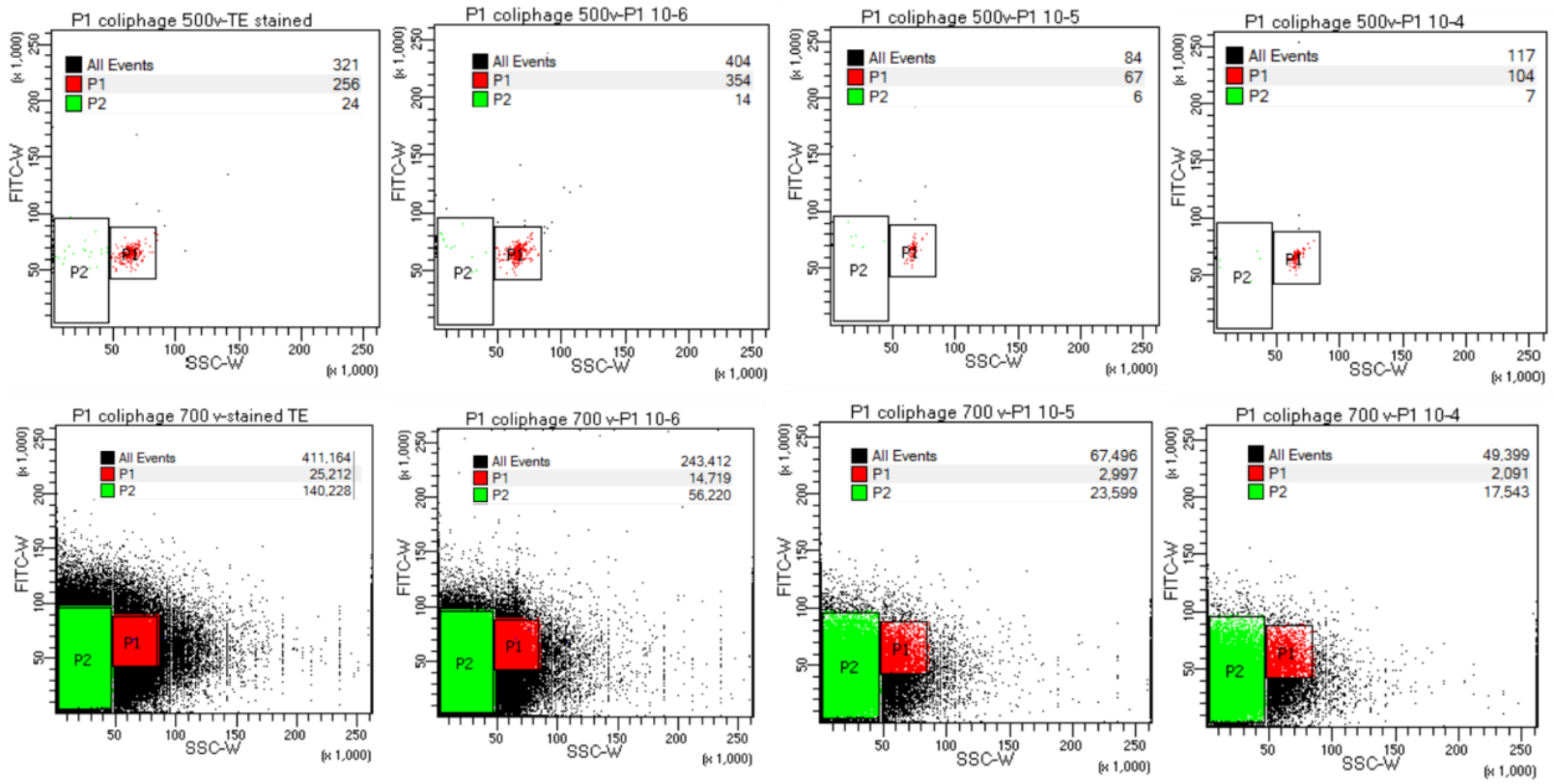

Figure 3.8. Unsuccessful attempt of bacteriophage P1 (100 kbp DNA genome) FCM detection. P1 – SYBR Green I signal population; P2 – expected virus. In the upper row, stained TE buffer control and decimal dilutions of the phage were analyzed at the 500V FITC voltage settings, and in the lower row the same samples were analyzed at the 700V for higher sensitivity of the instrument. In lower row the number of FCM signal events decreased from the virus-free control toward samples with higher concentrations of the virus, hence proving that the appeared signal is not related to the bacteriophage P1.

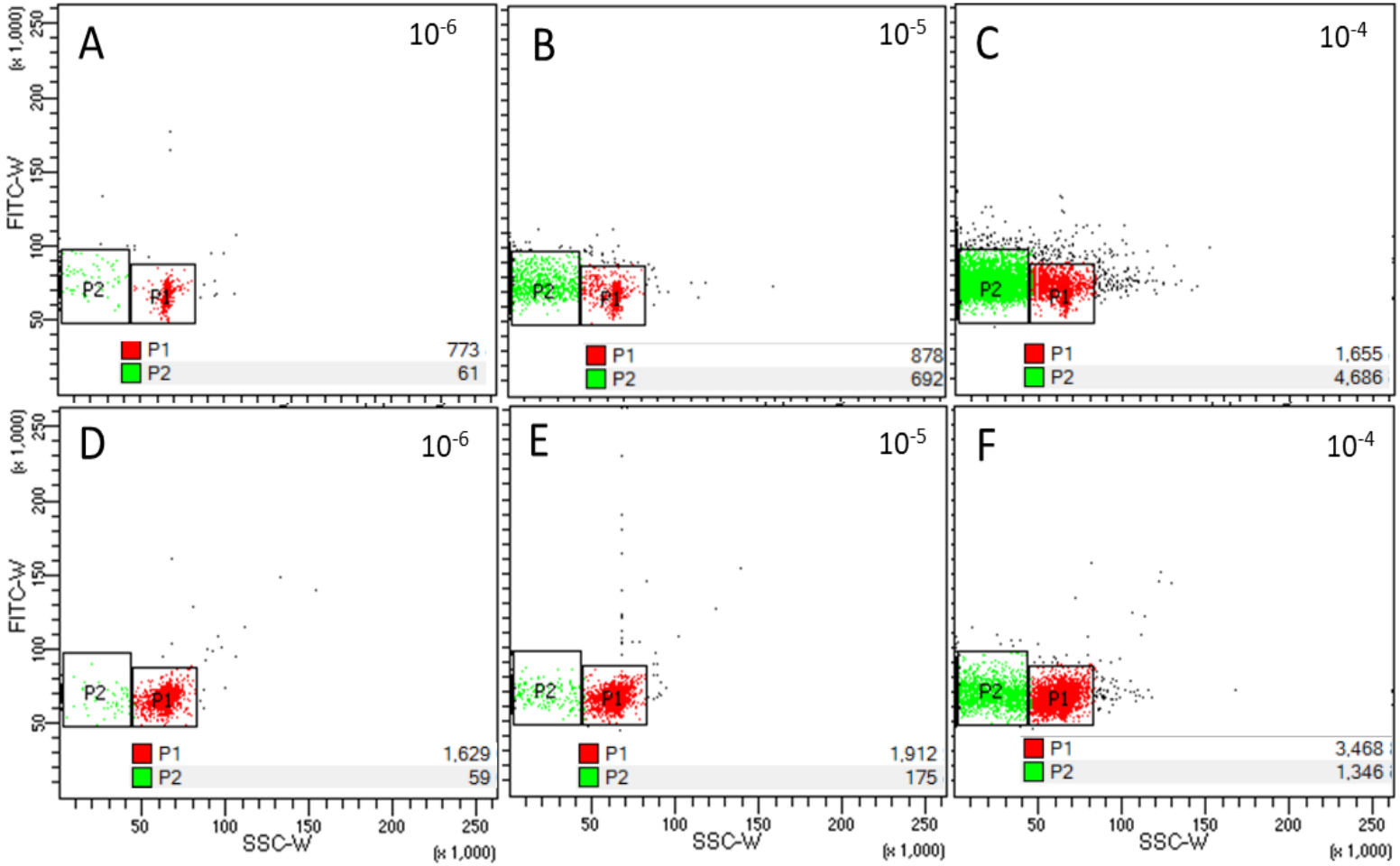

Figure 3.9. Flow cytometric analysis of SYBR® Green I-labeled bacteriophage T4 at indicated decimal dilutions (population P2) (A-C). D-F: the same samples after vigorous pipetting.

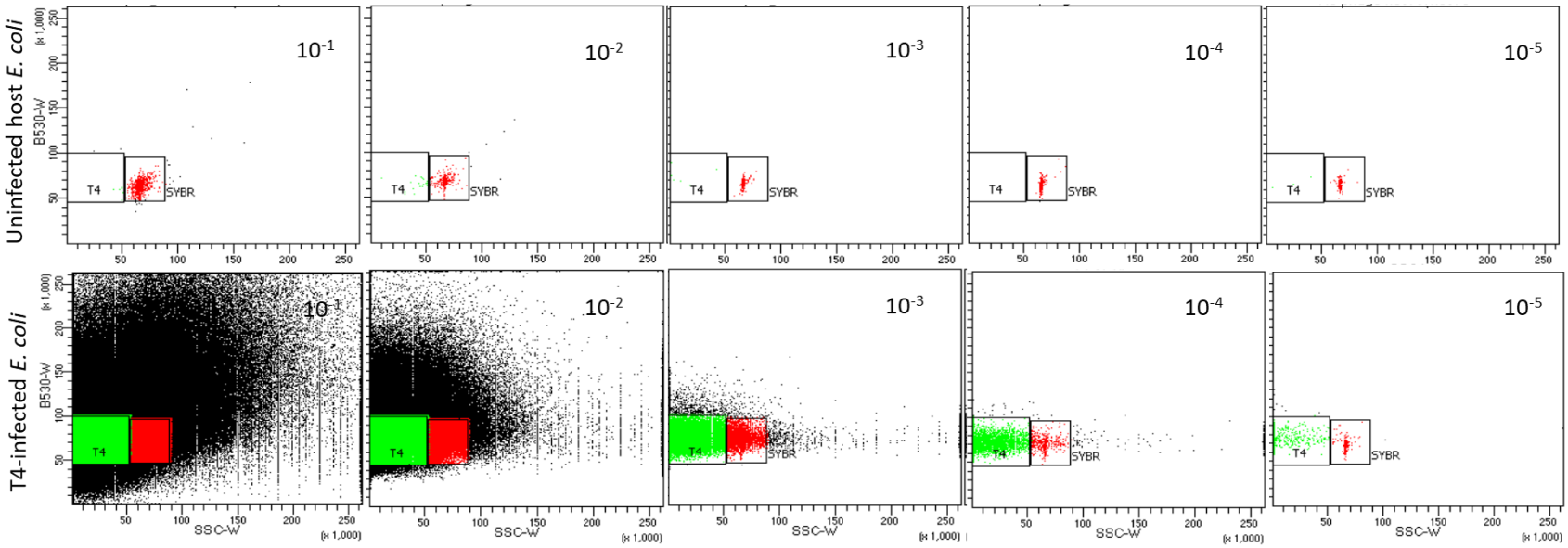

Figure 3.10. Uninfected host *E. coli* control demonstrated no FCM signal in the population gated as virus (T4). The relative fluorescence duration (B530-W) is plotted as a function of the side scatter signal duration (SSC-W). Upper row: decimal dilutions of uninfected host *E. coli* BL21DE3 culture preparation. Lower row: the same decimal dilutions of T4-infected *E. coli* culture preparation. The uninfected host culture was sonicated to disrupt the cells and then both cultures were treated identically according to Bacteriophage sample preparation protocol.

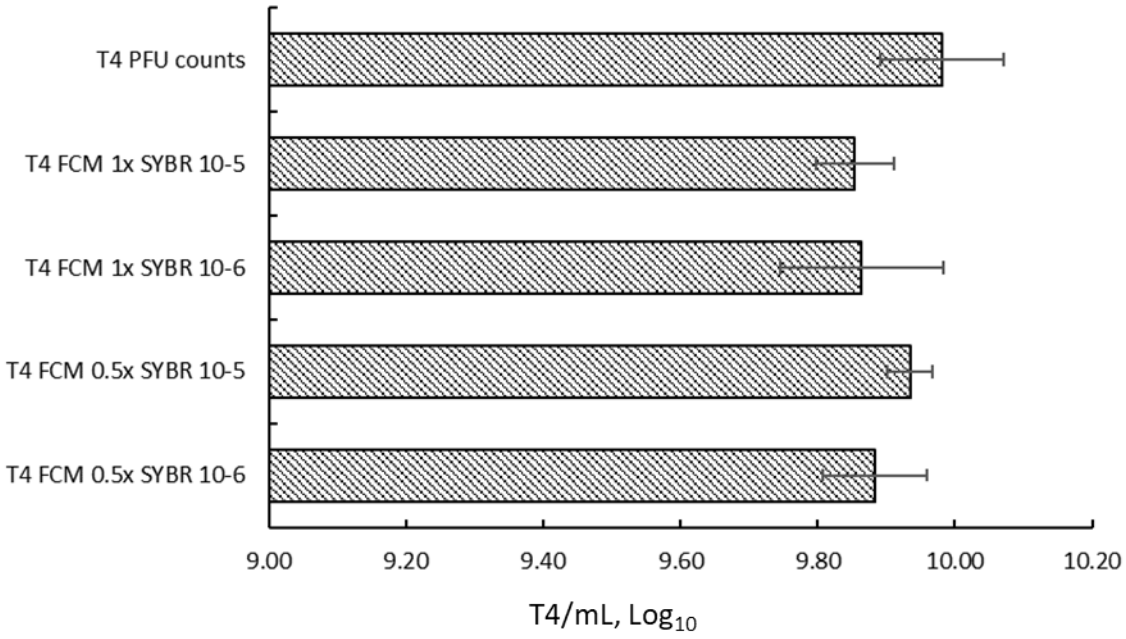

Figure 3.11. T4 virus enumeration by double agar overlay plaque assay (PFU/mL) and FCM using SYBR® Green I labelling (n=6, error bar is 1 SD)

3.4 Discussion

3.4.1 FCM artifacts due to colloidal fluorophore particulates:

As SYBR® Green I is a hydrophobic chemical with low solubility in aqueous solvents (*National Center for Biotechnology Information. PubChem Compound Database; CID=10436340, https://pubchem.ncbi.nlm.nih.gov/compound/10436340 (accessed July 20, 2017)*) there are inherent problems in using such fluorophores when targeting small particles like viruses by FCM. Though not widely discussed in the microbiological literature, therefore, SYBR® Green I forms a disperse colloid rather than a homogenous molecular solution. Disperse systems can be formed via two main routes: mechanical dispersion or condensation from oversaturated solutions (Shchukin *et al.*, 2001). For example, heating samples to 80 °C during staining procedure enhances oversaturation of the solution, and colloid particles start forming as the temperature decreases. The fact that fluorescent particles appear in both heated and unheated samples demonstrates that either route or both routes together, might contribute to SYBR® Green I dispersion.

A further indication of this dispersion was seen by the SYBR-FCM signal increase by shaking, sonication or pipetting, presumably due to the increase in auto-fluorescing dye colloidal particles. To the best of our knowledge, this is the first report of such chemical aspect of fluorescent dyes and its associated interference with small-particle enumeration by FCM. Consequently, keeping samples undisturbed for certain amounts of time reduces these apparent ‘virus’ event counts. This is a typical behaviour of lyophobic disperse systems (Shchukin *et al.*, 2001). In such systems, mechanically dispersed colloid particles tend to coagulate, and if interaction energy between the particles allows, they will

coalesce into larger particles. When the interaction energy is insufficient, due to low concentration and small diameter of remaining particles, coalescence becomes impossible and the disperse system self-stabilizes (Shchukin *et al.*, 2001). Our findings demonstrate that SYBR® Green I, as it is used in flow cytometry for virus enumeration, looks like a good example of a self-stabilized or pseudolyophilic system.

Such behaviour is not unique to SYBR® Green I, as the fluorescent dye SafeView Plus™ (Applied Biological Materials Inc., REF #G468) was also shown to self-stabilize in solution in the same manner, which we confirmed by flow cytometry. Furthermore, the addition of surfactants to a panel of SYBR® Green I solutions generated and stabilized artifact particles into emulsions (Figure 3.3), which could be misidentified as virus populations by FCM. Hence, when high gain levels are used to enumerate small-particle virions by FCM, hydrophobic fluorophores may generate various levels of false positive 'virus' signals. The same phenomenon was observed earlier by Pollard (2012), who compared the excitation and emission spectra of organic matter in water, in parallel with intact virus particles, and confirmed that about 70 % of the fluorescent signal was associated with the water matrix itself independently of the presence or absence of virus. Although Pollard did not use flow cytometry, his findings contribute to our observations that fluorescent colloid dye particles, present in dye-stained virus suspensions, can comprise a significant portion of the FCM signal.

Hence, the use of fluorescent dyes for virus enumeration by flow cytometry may produce false-positive signals and lead to overestimation of total virus counts by misreporting colloid particles as virions, depending on instrument sensitivity. Further

research is needed to optimize reporting procedures involving small-particle count in pseudolyophilic colloid systems, to address stained-virus and no-virus but stain-present controls as discussed below.

3.4.2 Precautions for identification of target virions populations by FCM:

To reduce misidentification of virions in environmental matrices, the instrument and assay sensitivity could be estimated using a panel of bacteriophages of various genome sizes. As such, the target population(s) could be identified by gating it/them from the total stained suspension signal. As illustrated in the current work, serial dilutions of the sample need to be correlated with the decline in target signal, which should be independent of dye concentration and should appear as a defined target population (*e.g.* Figure 3.9). Once the population is identified and gated, FCM signal counts should correlate to bacteriophage enumeration by a second established method, such as culture-based plaque assay (*e.g.,* Figure 3.11). Stained no-virus aqueous phase control should always be applied during target identification, to minimize false-positive signals.

In addition, staining of virus particles with nucleic acid stains may require heating of the samples to 80^{o}C, in order to expose viral nucleic acid (Brussaard, 2009). Successful enumeration of nucleic acid targets relies on gentle handling of such heated samples. We speculate that in order for the number of fluorescent signals to correlate to the number of target nucleic acid molecules associated with virions, the freshly heated and released viral DNA needs to remain compact. Rough handling of the sample could untangle the DNA molecule, creating distant contact points with the dye, and therefore decreasing the intensity of dye signal associated with a single DNA molecule.

3.5 Conclusions

Commonly used fluorescent dyes create pseudolyophilic colloid systems, which auto-fluoresce as stained virus-like particles even in the absence of DNA. The presence of surfactants further enhances non-specific fluorescence of such dye colloids and, therefore, use of surfactants for sample preparation should be avoided. Altogether, these interfere with small-particle enumeration by fluorescence-based assays, such as flow cytometry.

Successful enumeration relies on correct identification of the target population by the careful use of negative virus control samples. The instrument sensitivity should be assessed by comparison with established culture-based methods.

Given the pseudolyophilic colloidal nature of fluorophores used in FCM, sample handling can additionally affect the accuracy of virus enumeration. Overall, further research is needed to optimize the use of fluorescent dyes for virus quantification from environmental matrices by sensitive assays, such as flow cytometry.

Chapter 4: Outer limits of flow cytometry to quantify viruses in water[†]

4.1 Introduction

Source water scarcity is a global concern (WHO, 2009), exacerbated by changing precipitation patterns as well as urban growth. Municipal wastewater is now seen as one of the largest untapped water resources available to secure water services for future urban generations (UNESCO, 2017). Yet, concerns with chemical and microbial hazards in reclaimed wastewater have limited its wider use as a potable resource (Po *et al.,* 2005; Stenekes *et al*., 2006; Turner *et al.*, 2016).

Of the microbial hazards present in municipal wastewater, human enteric viruses cause infections at the lowest doses (Fong & Lipp, 2005; Peter *et al*., 2008; Yates, 2013). Also, infected humans shed waterborne viruses in the highest numbers of any pathogen group (Bosch *et al*., 2008; Caballero *et al*., 2003; Costafreda *et al*., 2006). Pathogenic viruses can exceed 10^6 virions per L of untreated sewage (Eftim *et al*., 2017). Enteric viruses are also highly resistant to water treatment processes and persist in the environment (Fong *et al*., 2010; Grabow, 2007). Hence, human enteric viruses are a critical pathogen group to control in discharged wastewaters and for water reuse.

Wastewater industry regulations are moving towards virus testing of sewage-impacted waters (US EPA, 2015a). To ensure public health and safety, the efficiency of pathogen

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removal during wastewater treatment requires a set of robust, well-validated methods. Standard methods exist for the assay of human enteric virus surrogates, such as somatic and F-specific coliphages (USEPA Methods 1601 & 1602), and the phage numbers are used to validate the removal performance of treatment processes (Lee *et al.*, 2019). However, there are few suitable methods for the **real-time** wastewater treatment process assessment (Rajnovic *et al.*, 2019). These methods are especially needed for wastewater intended for reuse in higher risk scenarios, such as potable reuse (Antony *et al*., 2012; Tchobanoglous *et al*., 2015).

Counting viruses using Flow cytometry (Flow virometry) has become an increasingly popular tool since the development of sensitive fluorescent dyes (Zamora & Aguilar, 2018). It is used for detecting viruses in wide range of situations from vaccines *(*Vlasak *et al*., 2016) to wastewater (Ma *et al.*, 2013) to marine environments (Marie *et al*., 1999). Despite the relatively high instrument cost and need for skilled personnel, it pushed back against the “old school” methods of plaque assay, transmission electron microscopy and epifluorescent microscopy due to their time and labor costs.

Here we examine flow cytometry for virus enumeration (flow virometry) to be used as a convenient monitoring tool for near real-time assessment of wastewater treatment efficiency. We focused on wastewater-borne bacteriophages as human enteric virus surrogates for log-removal estimates (Zimmerman *et al*., 2016).

While flow virometry was first applied for marine virus enumeration some twenty years ago (Marie *et al*., 1999) there are still gaps in our understanding of its application to

natural and engineered aquatic environments. How sensitive and accurate is flow virometry in these complex and relatively unknown environments. The nature of colloids that form during sample preparation and analysis is also poorly understood *(*Dlusskaya *et al*., 2019). Here we assess these interferences in flow virometry that result from the wastewater sample matrix as well as the sensitivity and accuracy of FCM to detect viruses in water. We look at methods to improve flow virometry sensitivity and define the current limits of its application to aquatic environments.

4.2 Materials and Methods

4.2.1 SYBR® Green I at various concentrations of TE buffer

Tris-EDTA (TE) buffer stock (Sigma Aldrich, Ref# T 9285) was filtered through 10 kDa molecular sieve (Millipore Sigma, Ref# UFC 9010) and diluted in triplicate in molecular grade water (GE Healthcare Life Sci., Ref# SH30538.01) to final concentrations of 0, 5,10, 20, 30, 40, 50, 60, 80 and 100 mMol (0 to 10x). SYBR® Green I (Life Technologies Corp.) was added to a 0.5 x final concentration. Samples were heated at 80℃ for 10 min to aid dissolution and handled gently to avoid additional emulsification of the SYBR® fluorophore (Dlusskaya et al., 2019).

4.2.2 Wastewater samples

Wastewater was sampled in July and August 2018 at the EPCOR Gold Bar treatment plant, located in Edmonton, Alberta, Canada. https://www.epcor.com/about/who-we-are/where-we-operate/edmonton/Documents/GoldbarTreatmentProcess.pdf. One-liter

samples of post-grit, primary effluent, secondary (final) effluent, and further tertiary treated – membrane filtered (0.45 µm pore size) water were transported on ice to the laboratory and processed immediately.

4.2.3 Virus-free wastewater

Wastewater sample aliquots were filtered through a sterile Amicon Ultra 100K centrifugal filter device (Merck Millipore, Ref # UFC910024) by centrifugation at 4,000 *g* for 20 min to remove the native microbial and virus populations (Pollard, 2012b). The pore size of 100 kDa filters is around 6 nm (Guo & Santschi, 2007), some three times smaller than the smallest viruses. This virus-free matrix water was used for bacteriophage T4 resuspensions as well as the diluent for the preparation of original wastewater (Fig. 4.3) or serial dilutions of T4-spiked samples (Fig. 4.4B) before flow virometry. Aliquots of the virus-free wastewater were also stained with 5 mMol (0.5x) SYBR® Green I following the same procedure that was used for TE buffer staining.

4.2.4 Preparation of bacteriophages and phage-spiked wastewater samples

The stock culture of coliphage T4 (ATCC® 11303B4™) was propagated in host *E. coli* BL21 DE3 (Sigma-Aldrich). The *E. coli* stock culture was incubated overnight in flasks containing Luria-Bertani (LB) broth (BD DifcoTM Ref # B244620) at 37℃ and 250 rpm. To produce an exponentially growing *E. coli* culture, fresh LB was inoculated with 10% v/v of the overnight *E. coli* stock and incubated at 37℃ and 250 rpm for 2 h. Then the host culture

was infected with 2% v/v of ~10^{10} PFU/mL bacteriophage stock and incubated an additional 6 h.

Aliquots of the culture lysate were centrifuged at 4,000 *g* for 30 min to remove bacterial cell debris. The supernatant was filtered through a sterile 0.22 µm syringe filter into Amicon Ultra 100K centrifugal filter devices and centrifuged at 4,000 *g* for 20 min to remove the culture medium. The retentate that remained in the filter part of the device (~250 µL) was treated with DNAse I (Roche Diagnostics) to remove the residual host DNA. DNAse I buffer was added to a final concentration of 1x (10 mM Tris HCl pH 7.5, 2.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$). DNAse I, dissolved in storage buffer (20% glycerol in 75 mM NaCl), was added to a final concentration of 10 ng/µL. All chemicals were purchased from Sigma unless stated otherwise. The retentates were incubated for 45 min at 37℃. After the incubation, they were rinsed at 4,000 *g* for 10 min with 5 mL of Phosphate Buffered Saline (PBS) buffer (GE Healthcare Life Sci., Ref # SH30256) to minimize stress and damage to the virions and prevent viability loss.

Rinsed bacteriophage T4 concentrates were re-suspended in the virus-free matrix wastewater (from which all naturally occurring viruses were removed by ultrafiltration as described above) from either Post-Grit, Primary effluent or Secondary effluent samples to their original volume.

For flow virometry of bacteriophage T4-spiked wastewater, decimal dilutions were prepared in triplicate in TE buffer (diluent control), and in the same virus-free matrix wastewater in which bacteriophage T4 was re-suspended. Samples were stained with 0.5x

SYBR® Green I as described by Brussaard (2004). Virus-free matrix wastewater (100K Amicon filter flow-through) samples were stained with 0.5x SYBR® Green I as negative-virus controls.

4.2.5 Sample preparation for FCM enumeration of viruses in wastewater

Wastewater samples were centrifuged at 4,000 *g* for 20 min. The supernatants were treated as follows: for samples with "the original background water" 10 mL aliquots of wastewater were only filtered through a 0.22 µm syringe filter. The samples in which the original background water was replaced with TE buffer were either DNAse treated or not (No-DNAse). Triplicate 10 mL aliquots of wastewater samples were filtered through 0.22 µm syringe filters into Amicon Ultra 100K centrifugal filter devices and centrifuged again at 4,000 *g* for 20 min to remove the background water. The sample retentates intended for "No-DNAse" were rinsed with 5 mL of TE at 4,000 *g* for 10 min and resuspended in TE to the original 10 mL volume. For DNAse treated samples DNAse buffer and DNAse were added to the retentates in the Amicon device filter part (250 µL) to achieve final concentrations of 1x and 10 ng/µL respectively. Samples were incubated at 37°C for 30 min, then the retentates were rinsed and resuspended in TE as described above.

4.2.6 Bacteriophage recovery from Amicon Ultra 100K centrifugal filter device

Aliquots of a bacteriophage T4 bacterial culture lysate were processed in a similar way to wastewater virus samples, except PBS buffer replaced TE. This was to minimize

physiological stress on the virus. Culture lysate controls were only filtered through 0.22 µm syringe filters, then plated as described previously (Kropinski et al., 2009). Triplicate No-DNAse treated and DNAse treated samples were split to enable parallel enumeration by plaque counts and by flow cytometry (virometry).

4.2.7. Flow Cytometry

The flow cytometer used was a BD LSRFortessa™ X-20 cell analyzer (BD Biosciences, USA) equipped with a 488 nm excitation laser and a standard filter setup. The trigger was set to green fluorescence (FITC[B530] channel). Data was collected using FITC (B530)-W / SSC-W (signal duration) and FITC(B530)-H / SSC-H (signal fluorescence intensity) dot plots. FITC channel was renamed into B530 during our experimental phase after one of the instrument's software updates. There were no changes in the channel optical setup. The virus was gated on FITC (B530)-W / SSC-W plot and signal events were recorded for 1 min. The virus gating was based on SYBR®-stained TE control samples with no virus added as well as SYBR®-stained bacteriophage T4 decimal dilutions. All decimal dilutions for flow virometry were prepared in TE buffer.

The instrument performance at the settings used for virus detection was estimated with FluoroSpheres® 0.02 µm latex beads (ThermoFisher, Ref # F8787) (Figure 4.1). The flow rate was estimated with FluoroSpheres® 1 µm latex beads (ThermoFisher, Ref# 8823) as described previously (Dlusskaya et al., 2019). The beads were first briefly vortexed and then bath-sonicated for 2 min as recommended by the manufacturer. Bead decimal dilutions were briefly vortexed and sonicated again right before analysis.

Photographs of Amnis®Cell Stream® flow cytometer (Luminex) capillary (Figure 4.2) were taken with the built-in quality control camera.

Virus-Like Particle (VLP) numbers in DNAse-untreated wastewater samples with TE buffer background were calculated as follows: VLP=(N·1mL)/(F·t·D). Where N was the number of events within the population gated as a virus, F was the flow rate (mL/min), t was the time of the data recording (min), D (dilution) was the amount of the original wastewater sample (mL) in 1 mL of the sample assayed by flow virometry.

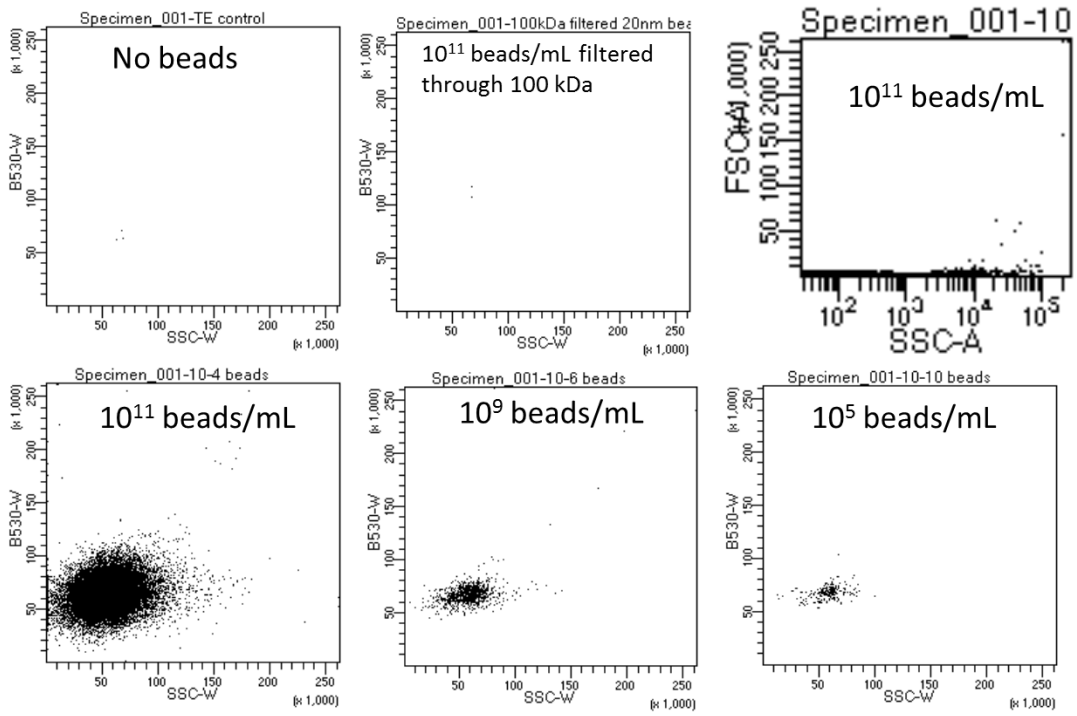


Figure 4.1 Fortessa X-20 flow cytometer was able to detect green-fluorescent beads 20 nm-diameter (Thermofisher, Ref# F8787) by fluorescence only and not by forward scatter at the voltage instrument settings we used for virus detection.

4.3 Results

4.3.1 SYBR® Green I stain alone produces a colloidal VLP signal

The SYBR® Green emulsion in TE buffer (both viral free) observed in the flow cytometer capillary (Cell Stream, Millipore Sigma) is shown in Figure 4.2. As a control, we used unstained viral free TE buffer (Figure 4.2A). Notice this capillary was empty. In contrast, SYBR® Green in TE buffer showed fluorescent VPLs (Figure 4.2B). These fluorescent VLPs were not viruses. The VLPs in Figure 4.2B were due to the SYBR® Green stain forming colloids that appear as VLPs to the flow cytometer.

Figure 4.3 shows viral free SYBR® Green stain (fixed concentration of 5 mM) mixed with increasing concentrations of viral free TE buffer. In this figure SYBR® Green fluorescent VPLs are plotted as a function of the TE buffer concentration. Increasing concentrations of TE buffer did not change the intensity of non-specific SYBR® Green VPL signal. Therefore, TE buffer concentration had no impact on the flow cytometer signal in the presence of the SYBR® Green stain (Figure 4.2B).

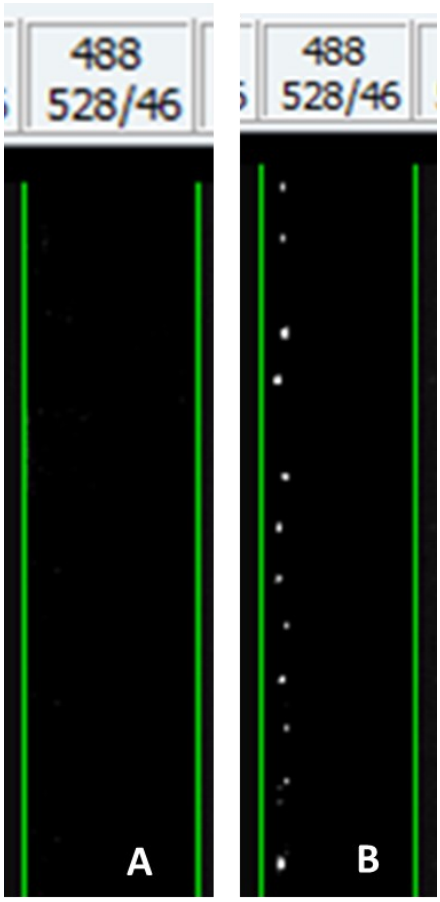


Figure 4.2 Snapshot of flow cytometer capillary (Amnis® Cell Stream®, Luminex). Viral free TE buffer is running through both capillaries. Viral free SYBR® Green stain was only added to capillary B. There are no particles showing in capillary A. However, fluorescent VPLs appeared in capillary B. Therefore, the VLPs in capillary B are not viruses, but rather they are artifacts related to the SYBR® Green stain.

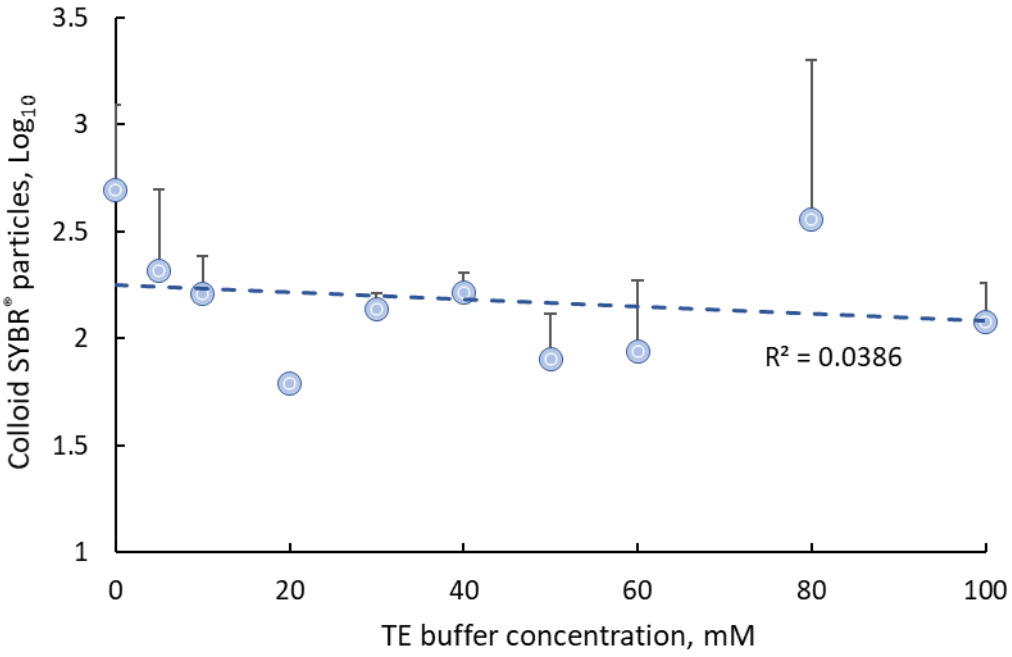


Figure 4.3 Colloidal SYBR® Green FCM signal, obtained using Fortessa X20, is plotted as a function of the increasing TE buffer concentration. There was no correlation between the TE buffer concentration and the FCM signal (R^2=0.0386): increasing the concentration of TE buffer did not increase the FCM signal. Therefore, TE buffer was not responsible for the formation of the non-specific colloidal SYBR® Green fluorescent FCM signal (all samples were viral free).

4.3.2 Wastewater samples with viral population removed

The non-specific flow virometry signal of SYBR®-stained virus-free wastewater decreased from Post-Grit to Secondary effluent samples (Figure 4.4). These results were consistent with organic substances removal from Post-Grit to Secondary effluent during the wastewater treatment process (https://www.epcor.com/products-services/water/water-quality/wqreportsedmonton/wwq-edmonton-2018.pdf).

In the T4-spiked wastewater samples, the concentration of bacteriophage T4 was the same in both Figure 4.5 plots since the dilutions were prepared from the same T4-spiked stock. The total organic carbon (TOC) concentration in TE-diluted (Figure 4.5A) and virus-free WW-diluted (Figure 4.5B) samples differed by two orders of magnitude due to the dilution with the TE buffer. Figure 4.5B illustrates how the matrix wastewater background obscured the T4 virus signal, making it increasingly difficult to resolve possible virus events against non-specific SYBR® signal. In the sample with a higher TOC, the bacteriophage signal receded and/or fused with this non-specific signal.

Similarly, the SYBR® and bacteriophage T4 signals merged when samples contained high numbers of a virus. When the high-TOC background culture medium was replaced with TE or PBS buffers, still there was a gradual increase in the SYBR® and bacteriophage T4 signals merging as the bacteriophage concentration increased (Figure 4.6).

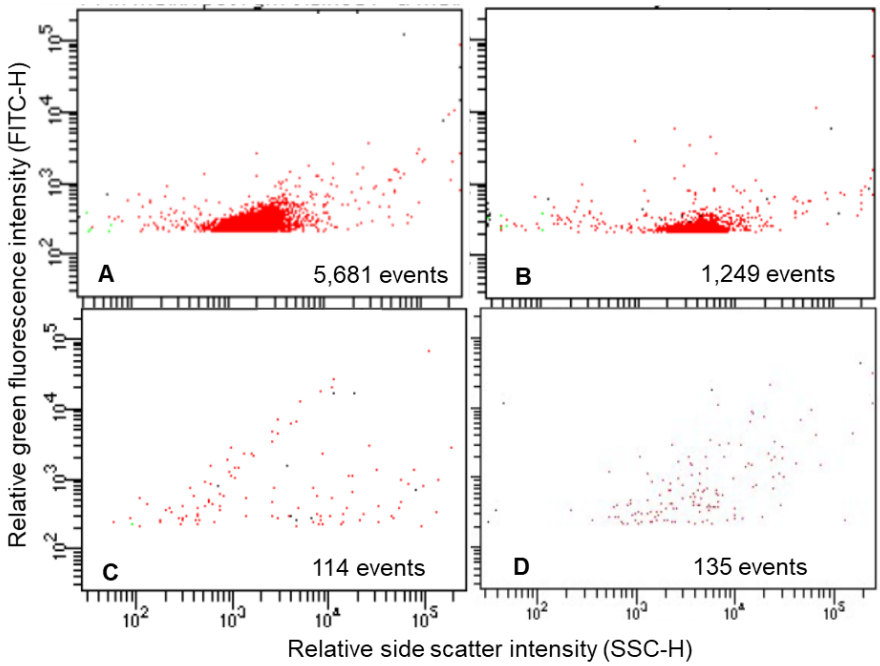


Figure 4.4 Three (A to C) background SYBR® stained wastewater (WW) samples from which all viruses had been removed by 100 kDa ultrafiltration. The WW samples were taken from three stages of treatment (A - Post-Grit; B - primary effluent; C - Final (secondary) effluent. Plot D shows viral free SYBR® stained TE buffer control. The plots show green fluorescence intensity (FITC-H) vs Side Scatter intensity (SSC-H). As the WW treatment progressed (A to C) more organic carbon material was removed from samples. There was a corresponding rapid decline in the VPLs as the treatment process removed organic matter. The analysed sample volume was standardised for 60 seconds. The organic matter in the background WW appeared responsible for the VLP signal. These VLPs were not viruses.

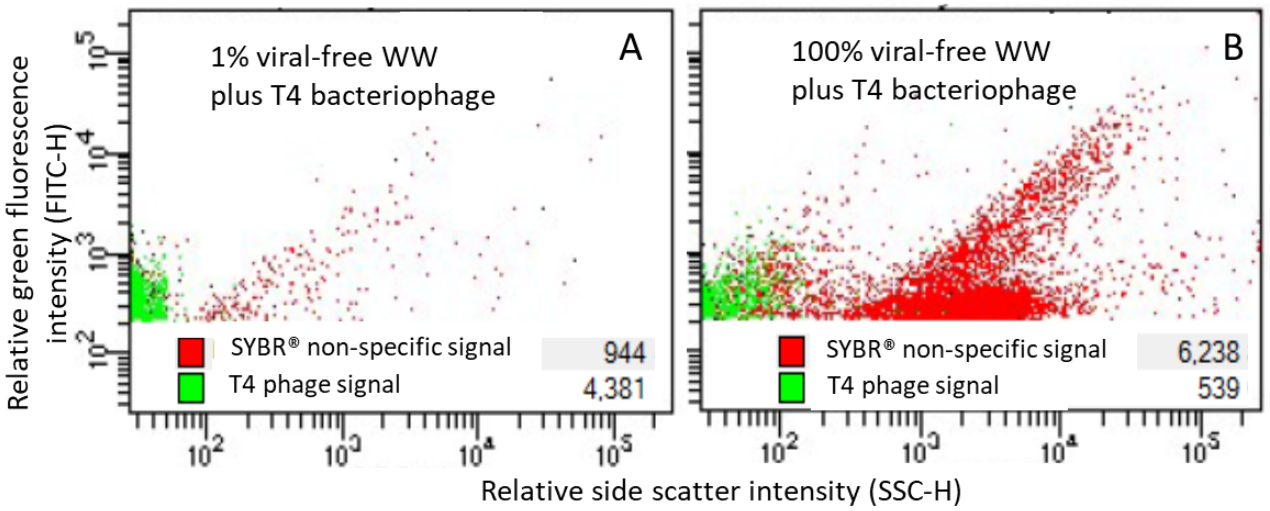


Figure 4.5 WW background matrix enhances the artificial SYBR® Green signal that is unrelated to virus and obscures the FCM signal generated by the virus. The FCM signal is shown as fluorescence *vs* side scatter intensity (H) plot. Virus-free (100 kDa-filtered) WW was spiked with purified bacteriophage T4 suspension of a known concentration. The sample was diluted with either TE buffer (1% of WW matrix remained) (A) or with virus-free WW (100% of WW matrix remained) (B). The 100% WW (B) lead to a significant underestimate of T4 phage compared to 1%WW sample: 539 VPLs *cf* 4,381 VLPs. At the same time non-specific SYBR® Green VPL numbers increased. Only in the 1% WW TE-diluted sample were the virus counts consistent with the plaque (PFU) enumeration.

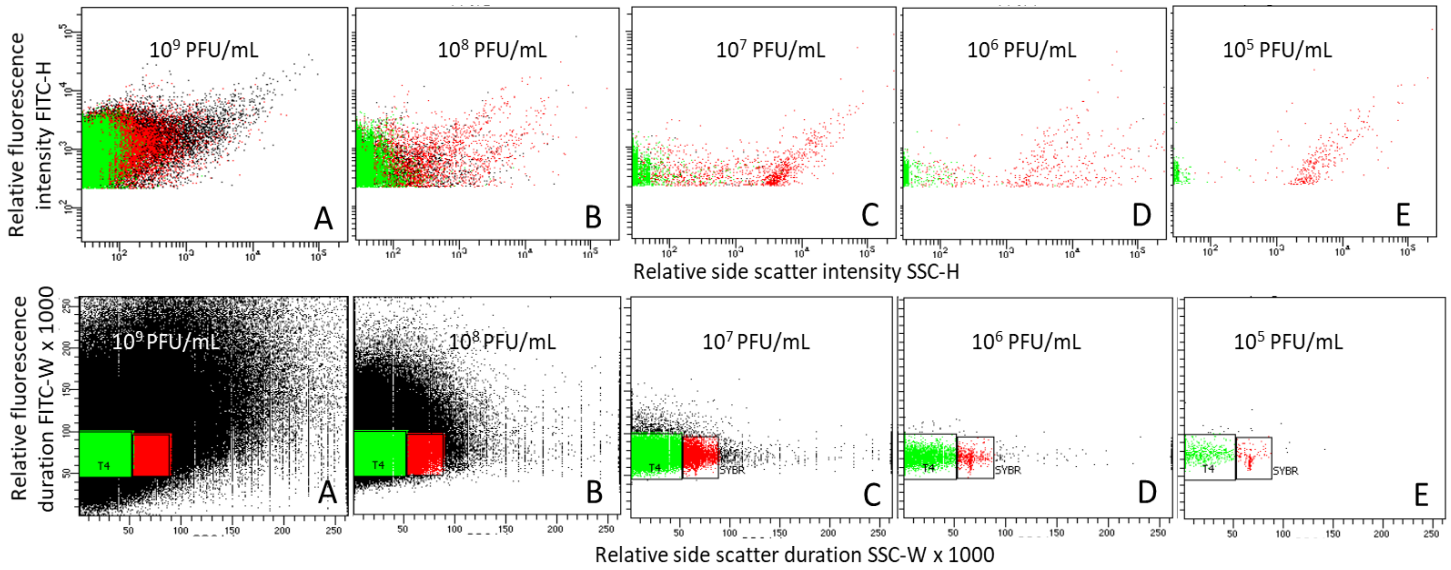


Figure 4.6. Changes in the flow cytometric signal as concentrations of T4 phage in TE buffer (A to E green dots) decrease across the panels (A-E); PFU/mL determined for each dilution is shown on each plot. The red dots are VLP colloidal artifacts generated by the SYBR® stain. The same T4 phage dilution series was used for both H- (top) and W-plots (bottom). The relative fluorescence intensity is plotted as a function of the side scatter signal intensity (top row) and of the signal duration (bottom row). The W-plot is a good quality control. When the FCM signal leaks from the gated area and overwhelms the plot (black dots) – then the results are unusable (bottom A, B, C) and that the more diluted sample should be used for viral enumeration. In an H-plot a high-virus sample produces a compact population of dots that might be mistakenly gated as single virus while it includes both virus and SYBR® signals (top A and B).

4.3.3 Virus recovery using Amicon Ultra 100K centrifugal filter device

Nearly all bacteriophage T4 was recovered once the background culture medium was removed using an Amicon Ultra 100K centrifugal filter device. Culture-based plaque-forming unit (PFU) counts showed 92-93% recovery for both No-DNAse treated and DNAse treated samples. Flow virometry counts were: 93% recovery for No-DNAse treated versus 87% for DNAse treated samples ($p=0.003$, $n=6$). Thus, the additional DNAse digestion step was omitted for wastewater samples.

4.3.4 Use of Flow Cytometry to determine viral numbers in wastewater

Our attempts to enumerate viruses using flow virometry in the samples with the original wastewater background were unsuccessful. We found that the calculated VLP numbers were inconsistent between the decimal dilutions of the same sample, and it was impossible to decide which dilution produced the correct result (Fig. 4.7). Only when the background WW matrix was replaced with TE buffer (see Methods) did the dilutions measure statistically the same VLP/mL counts (by two-tailed unpaired T-test, $p>0.05$), and only those were taken into consideration.

Flow virometry total virus estimates for various stages of the wastewater treatment process are presented in Figure 4.8. VLP counts were calculated as described above, were between 5.5 and 6.5 Log_{10}/mL for post-grit, primary, and secondary effluent samples. No significant decrease in VLP counts was observed. The 0.45 µm-filtered samples (tertiary treated wastewater) produced zero VLP counts in four samplings out of five. Only the last

sampling, which was taken within two hours after backwashing the full-scale membrane filtration system at the sewage treatment plant, yielded 2.5 Log_{10} VLP/mL, inferring some 3 to 6 log-reduction.

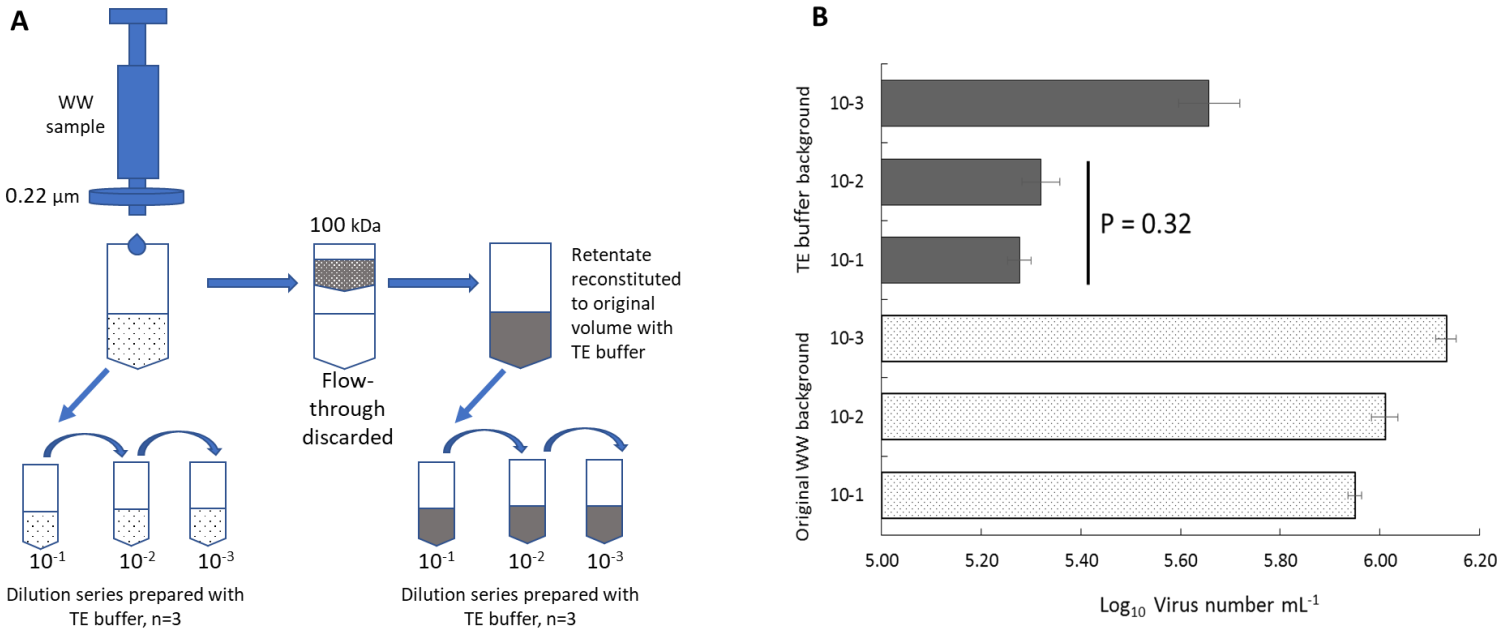


Figure 4.7 Improvement of the accuracy in relative VLP quantifications in WW due to removal of the original background matrix water. Removing the original background matrix in the wastewater virus sample by filtration on the 100 kDa sieve and reconstitution of the virus in the TE buffer (A) led to significantly consistent virus counts between decimal dilutions of the sample (unpaired two-tailed T-test, n=3) (B). Virus numbers (VLP mL^{-1}) were calculated for each dilution of a sample.

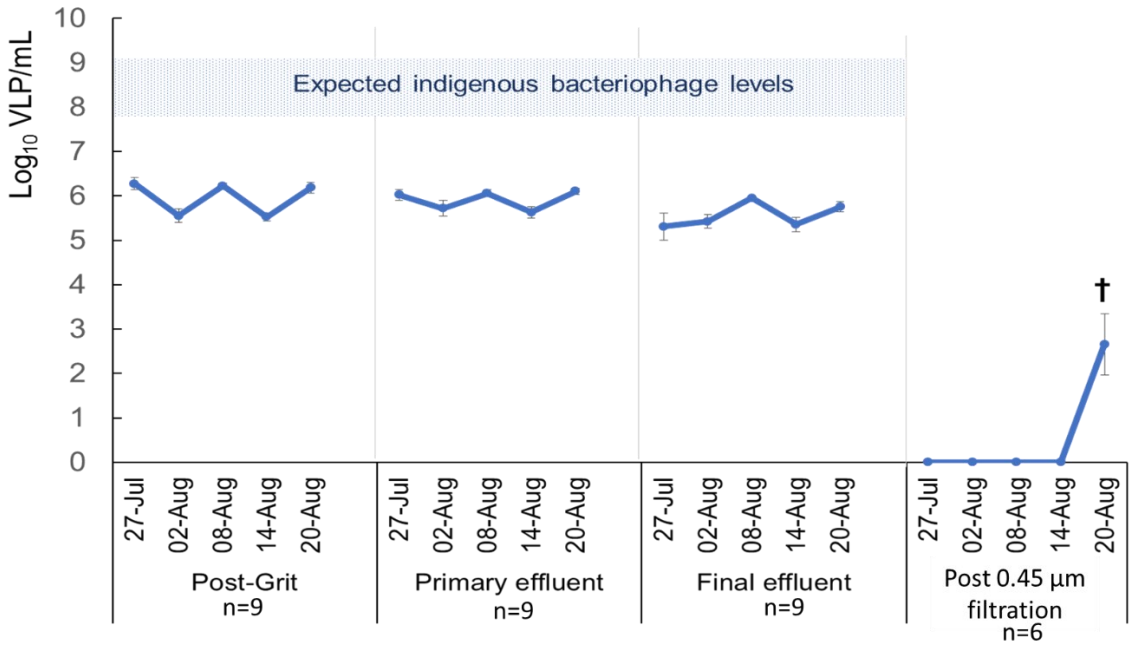


Figure 4.8 FCM counts of wastewater virus (VLP/mL) were consistent at all stages of the treatment process except for post 0.45 µm filtration. In four out of five post-filtration samples no FCM-detectable virus was found. Samples for the virus enumeration were prepared with the TE buffer as the background. The detection limit of Fortessa X-20 is ~150 kbp virus genome size, hence viruses with smaller genomes are not detected.

† - sample was taken within two hours after the filtration system backwash

4.4 Discussion

4.4.1 Preparation of wastewater samples for flow virometry

The decrease in instrument signal of virus-free wastewater samples from post-grit to final effluent (Figure 4.4) inferred that the non-specific SYBR® fluorescence signal in virus-free wastewater was an artifact, possibly enhanced by synthetic surfactants from shampoo or laundry formulations present in raw wastewater (Dlusskaya *et al*., 2019; Palmer & Hatley, 2018). Other organics likely contribute, as we also observed a high non-specific SYBR® fluorescence signal in virus-free samples from a freshwater lake with noticeable eutrophication (data not shown). Microorganisms are known to produce a variety of amphiphilic biological compounds (natural surfactants), like glycolipids, lipopeptides, lipoproteins, phospholipids, etc. (De *et al*., 2015; Holmberg, 2001; Rosenberg & Ron, 1999). These microbial total organic carbon (TOC) constituents of the samples' colloid systems could play a role that produces a VLP signal that is not due to viruses. The TE buffer is not a source of the false VLP signal since we found no emulsifying properties of the TE buffer that would intensify the non-specific SYBR® signal (Figure 4.3).

The interference of high-TOC sample background and the resulting merging of viral and non-specific SYBR® signals in such samples (Figure 4.5) could lead to either overestimation or underestimation of the bacteriophage counts. depending on the interpretation of the resulting flow cytometer plots by the analyzer.

The sample preparation protocol described by Marie *et al*. (1999) is widely used by marine researchers for flow virometry. This requires heating the sample to 80°C for 10 min. This step causes the denaturation of virus capsid proteins. Denatured and partially denatured proteins can potentially act as surfactants (Kato *et al*., 1981; Wilde *et al.*, 2004) in the colloid system of a sample. So, at higher concentrations, these may also increase the SYBR® non-specific VLP signal. At the same time, denatured proteins tend to aggregate (Amin *et al*., 2014; Fink, 1998) causing virus particles to aggregate in a sample. To our knowledge, there is no published high-resolution microscopic image of a virus stained according to the Marie *et al*. (1999) protocol.

4.4.2 Both W-plots and H-plots should be employed for results interpretation

In Figure 4.6 the analysed samples consisted of TE buffer, purified bacteriophage T4, and 5 mM SYBR®. It shows a disproportionate increase in the VLP signal as the T4 virus numbers in the sample increased. We gated the virus-associated VLP signal in signal duration parameter plot (W) when the dilutions of the T4 bacteriophage produced a distinct population of signal events that could be easily resolved from the SYBR® self-fluorescence signal (Dlusskaya et al., 2019). The SYBR® self-fluorescent population was very compact in the W-plot due to the equal volumetric mass density of SYBR® colloid particles and, therefore, relatively equal time of a particle passing the laser beam. Hence, it was easy to gate in the virus-free SYBR® -stained controls. In contrast, in the signal intensity parameter plot (H) the same SYBR® signal was usually quite scattered and

difficult to gate. We suspect that fluorescence intensity of SYBR® colloid particle artifacts depends on their diameter, noting that larger droplets self-quench.

It is easy to see (Fig. 4.6) that as the concentration of the virus in the sample increases, the virus-associated VLP and the SYBR® autofluorescence populations fuse at first, and then the total signal overwhelms the W-plot. This is a good quality control. When the signal leaks the gated area and overwhelms the plot – it indicates that the results are unusable (Figure 4.6 bottom A, B, C) and that more diluted sample should be used for the enumerations. However, the same total signal appears as a tidy and very compact population of events when depicted by the signal intensity (H) plot. Yet it is totally unclear what proportion of this signal belongs to single viruses and what proportion belongs to virus aggregates or other VLP artifacts (Figure 4.5 top A, B). If the virus signal has not been gated based upon control bacteriophage serial dilutions prior to the virus enumeration in environmental samples, all these signal events in the H-plot compact population will be misinterpreted and gated as a virus.

We find that W and H-based plots are useful as quality controls for each other. The increased signal duration without much increase in fluorescence intensity is an indicator of virus aggregates. If the populations are gated correctly on the W plot and appropriate dilution is used, they will be clearly separated on the H plot as well.

An increased photomultiplier tube (PMT) voltage gain, as sometimes recommended for higher instrument sensitivity, may also produce an electron noise artifact (Snow, 2004). This electron noise artifact signal may fuse with part of the virus signal if no appropriate

control is applied during optimization of the instrument voltage. Modern flow cytometry instruments are extremely sensitive and very precise with signals of any nature, including the instrument electronic noise. Therefore, the reproducibility of samples does not equal accuracy, nor is reproducibility proof of viral identity.

4.4.3 Importance of correct dilution and background control

In our previous experiments with flow virometry of purified bacteriophage stock dilutions (Dlusskaya et al., 2019) we noticed the same quantification problem as seen when plaquing on solid media: higher dilutions tend to overestimate counts, whereas lower dilutions tend to underestimate them. When we recalculated VLP counts across two or three dilutions in a row, usually the mid-dilution range produced VLP/mL counts that were statistically the same (by unpaired two-tailed T-test). Also, these counts were consistent with bacteriophage PFU/mL counts. In the wastewater virus enumeration experiments we observed the same effect in the samples in which background matrix wastewater was removed and replaced with TE buffer (Figure 4.7). We consider VLP counts from these dilutions to be the most reflective of actual viruses of detectable genome size (detection limit varies among instruments and needs to be established for the model used).

Samples with original matrix wastewater did not reproduce the same VLP/mL counts between dilutions (Fig. 4.7B). We attribute this to interference from TOC present in wastewater. As a point of difference with seawater, TOC concentrations in wastewater are 2-4 orders of magnitude higher (Huang *et al.*, 2010; Ozturk & Yilmaz, 2019; Thermo

scientific, 2007) and their composition varies substantially. For marine water, Brussaard *et al*. (2010) recommended using filtered autoclaved sample at the same dilution factor as the sample to serve as a control for each dilution. Though these recommendations are technically correct, we found that serial dilutions of wastewater samples with TE buffer instead of original matrix wastewater background generated more consistent results that were easier to interpret and required only one control – stained TE buffer.

4.4.4 Flow virometry is missing most viruses in wastewater

Given wastewater contains some 107-108 bacterial cells/mL depending on the treatment process stage (Ewert & Paynter, 1980; Foladori et al., 2010; Ma et al., 2013) and, if assumed average burst size to be 50 bacteriophages per bacterial cell and around 30% of bacterial population is infected (Bergh et al., 1989), expected indigenous bacteriophage counts should be at least one order of magnitude higher than those of host bacteria – 10^8-10^9 bacteriophages per mL (Maranger & Bird, 1995; Wommack & Colwell, 2000). Yet the VLP counts we estimated for post-grit and effluent samples were 10^5-10^6 VLP/mL, at least two orders of magnitude lower than estimated indigenous bacteriophage numbers (Figure 4.8). VLP counts were also 1 to 2 orders of magnitude lower than the counts previously reported for sewage and mixed liquor by transmission electron microscopy (Ewert et al., 1980), and for wastewater by epifluorescent microscopy (Pollard, 2012b). Hence, most wastewater viruses appear undetectable by current flow virometry. Similarly, Steward et al. (2013) reported the same underestimates of total viruses for marine systems.

4.4.5 Virus size matters

In four post-filtration (0.45 µm) full-scale wastewater samples out of five, no virus was detected, and only the sample that was taken right after the microfiltration membrane backwash contained detectable virus (Fig. 4.8). At first glance, the flow virometry results for microfiltration virus removal look striking. Antony *et al*. (2012) reported a microfiltration membrane process additionally improved virus removal because of a caking layer on the membrane surface. However, Qui *et al*. (2015) unambiguously demonstrated the presence of human enteric viruses in post-filtration samples from the same facility using cultural and molecular techniques. They reported that 2-4 Log_{10} of the tested viruses naturally present in wastewater were removed by ultrafiltration, but up to 3 Log_{10} gene copies per liter of norovirus, reovirus, and astrovirus were remaining. Only a minority (<1 Log_{10}) of relatively large-sized virus particles is expected to be removed by 0.45 µm pore-sized filtration. Therefore, apparent zero FCM virus counts in post-filtration wastewater samples might be dangerously misleading, especially in water intended for reuse, if the system's performance is not thoroughly understood.

The detection limit of the Fortessa X-20 instrument used in our experiments was ~150 kbp of virus genome size (Dlusskaya et al., 2019). Hence flow virometry is grossly underestimating viral numbers in wastewater by missing most bacteriophages that have small genome sizes (see below), and it will not detect human enteric viruses, which have similarly smaller genomes (<150 kbp). This non-detection occurs even though the Fortessa's sensitivity for a fluorochrome is nearly 10 times higher than that of previously

used analog instruments (e.g. BD-FACS): detection limits being 80 molecules of equivalent soluble fluorescein on the Fortessa X-20[3] versus 750 for analog instruments[4] .

Hatfull (2008) analyzed 500 sequenced bacteriophage genomes to show that most genomes were smaller than 50 kbp. We updated Hatfull's histogram using 2550 complete bacteriophage genomes, available in the NCBI virus database in 2019. The frequency (number) of complete bacteriophage genome sequences and their cumulative % of occurrence are plotted as a function of their genome size distribution (Fig. 4.9). The viral genome size distribution remained the same. Half of all genomes were 50 kbp or smaller. Hence Hatfull's histogram appears to reflect bacteriophage genome size distributions in nature.

The viral genomes range from 3.4 kbp to 497.5 kbp. In terms of FCM analysis, more than 85% of those genomes fell below the Fortessa X-20 detection limits of 150 kbp. Most (70 to 87%) of viruses in marine environments are not FCM detectable either (López-Pérez *et al*., 2017). To answer the question Steward *et al*. asked in 2013: "Are we missing half of the viruses in the ocean?"; FCM is missing most natural viruses in the case of bacteriophage-dominated wastewater. However, it is orders of magnitude lower, not half.

[3] https://www.bdbiosciences.com/documents/bd_lsr_fortessax20_techspecs.pdf
[4] https://www.bdbiosciences.com/documents/FACSCalibur_FlowCytometry_TechSpec.pdf

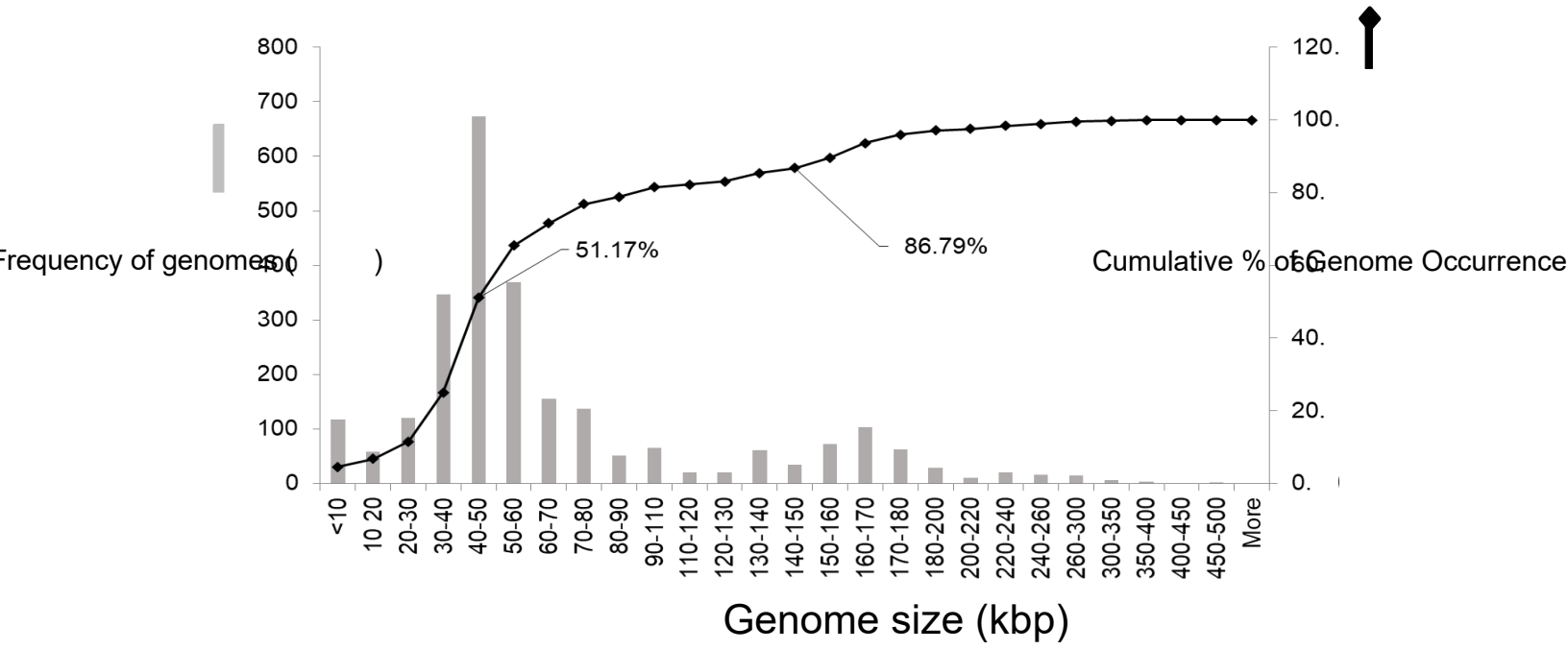


Figure 4.9. The frequency (number) of complete bacteriophage genome sequences and their cumulative % of occurrence are plotted as a function of their genome sizes (kbp). As of July 2019, the NCBI viral database contains 2,550 complete viral genomes that range from 3.4 to 497.5 kbp. Of these genomes 87% fall below the FCM detection limit of 150 kbp. Most, 70% to 87%, of those in marine environments are not detectable (also see Lopez-Perez et al., 2017) using FCM. We have not considered the very small human pathogenic viruses causing respiratory diseases, such as MERs and SARS (COVID-19) as these RNA viruses all have small (~ 30kb) genomes. These would be impossible to detect using flow virometry with its current limits of sensitivity

4.4.6 Need for improved QC for flow virometry

Some authors report high sensitivity of flow virometry (Brussaard, 2004; Huang *et al*., 2015b) and even reported single virus particle separation using a flow cytometric cell sorting (Allen et al., 2011). However, these and earlier publications may lack important controls that prove the flow virometry signal reported as virus is in fact due to viral presence.

Fluorometers, including flow cytometry instruments do not measure the absolute number of photons emitted by fluorescently labeled samples. Instead, they are equipped with photomultiplier tubes which produce an electrical signal that is proportionate to the number of photons hitting the detection window. This electrical signal (analog voltage) is measured and converted into a numerical value (Snow, 2004). Hence these numerical values are relative to the number of emitted photons and reported as relative fluorescence units (RTU). This makes flow cytometry a semi-quantitative method. As any semi-quantitative method, it calls for a robust set of controls to which sample fluorescence can be compared. Stained virus-free buffer controls are important in defining and gating the fluorophore-generated artifacts. They will subsequently help to separate the virus signal from the artifact. It is also important to remove the background matrix of unknown chemical composition, which has been shown to affect these artifact levels.

Ma *et al*. (2013) and Allen *et al*. (2011) report virus enumerations or sorting using unstained virus samples as a control. Although important, the unstained sample is the control for the dye only, but does not address the potential for the dye to interact with all

sample constituents, not just virus. Therefore, unstained virus-containing sample cannot be used as the only control for virus population identification. Likewise, Brussaard (2004) and Allen *et al*. (2011) omit descriptions of bacteriophage sample preparations in terms of presence/absence of TOC-rich host culture media, dilution effects, and the kind of diluent used, representing factors potentially confounding the flow virometry results. To stain viruses, Brown *et al*. (Brown *et al*., 2019; Brown *et al*., 2015) used filtered SYBR® solutions from which the hydrophobic dye emulsion was largely removed by filtration (Dlusskaya *et al*., 2019).

We have shown that these controls, are important to reliably demonstrate virus detection and quantification in future virometry studies in waters. This is especially critical when these results are intended for Quantitative Microbial Risk Assessment (QMRA) and/or Log-reduction target verification (Antony *et al*., 2012). As stated by Snow (Snow, 2004) in using flow cytometry: “As the operation of these instruments becomes more user-friendly and increasingly automated, in the absence of rigorous controls and calibrators, it becomes easier to generate bad data and not know it”. When public health is at stake, bad data are unacceptable.

Overall, I would recommend the following steps for sample preparation and virometry analysis. Samples should be processed immediately or flash frozen. Use of chemical preservatives or surfactants is not encouraged to prevent additional damage to the viruses or unforeseen chemical reactions with sample constituents. Sample needs to be filtered through 0.22µm pore-size filter to remove large debris and bacterial cells. Then

the background matrix water - with largely unknown chemical composition - should be replaced with an ultrafiltered standard chemically defined buffer (*e.g.,* saline or TE). It is critical that confounding sample constituents be removed, yielding a simpler and more predictable colloid system formed after the sample fluorescent staining. At staining, the fluorophore interacts with all the chemicals present in the sample, not just viruses. Using the same buffer as the background and the diluent during sample preparation ensures that only one parameter in the sample changes, which is virus concentration. Therefore, serial dilutions of the sample and virus-free buffer can be compared. Stained virus-free buffer should be used as negative control to gate non-specific fluorophore signal that is unrelated to the virus. The viral identity of the signal of interest must only be proven by using sample dilution series. If the event counts in the target population do not change according to the dilutions, adjustment of the instrument settings might help but only in case if the virus genome size is above the instruments' detection limit.

4.4.7 The future of flow virometry for sensitive viral enumeration

Flow virometry's most limiting factor is the lack of sensitivity. Most viruses are too small to be detected in natural environments. However, nanotechnology offers a way to overcome the sensitivity issue. Zhu *et al*. (2014) built a laboratory scale instrument to separate and detect particles to a transmission electron microscopy scale with a viral counting efficiency of 10,000 particles per minute. They use an advanced high sensitivity flow cytometry (HSFCM) that achieves real-time light-scattering detection of single nanoparticles as small as 7 nm in diameter. This sensitivity could be applied to high

resolution counting of fluorescence-stained viral particles to capture smallest viruses that have so far remained elusive to flow virometry in natural and engineered aquatic environments.

4.5 Conclusions

- To date, inadequate controls are used to show that FCM signals are indeed viruses. Future research needs to address the controls that can be standardized.
- The accuracy and sensitivity of FCM for virus enumeration needs validating in natural and engineered environments.
- High background organic content in the various water samples, regardless of its origin, interferes with virus enumeration so much so that viral like particles are not necessarily due to viruses.
- More than 85% of sequenced bacteriophage genomes are less than 150 kbp and, therefore, below the current FCM detection limit.
- Flow instruments must be markedly more sensitive and specifically designed for virus applications to detect most viruses in nature that have genome sizes <150 kbp, which includes known human enteric viruses.
- FCM (flow virometry) is currently neither sensitive nor accurate enough for Quantitative Microbial Risk Assessment applications or wastewater treatment performance assessment (targeting at least 4 log-reductions).

Chapter 5: Optimizing fluorescence-based assay for virus monitoring of wastewater[5]

5.1 Introduction

Reclaimed municipal wastewater is becoming a necessary alternative source water, especially in growing urban areas (Salgot *et al*., 2018). Globally, wastewaters are typically discharged to surface waters where residual infectious human pathogens impact on recreational activities (Benjamin-Chung et al., 2017), food production/safety (Adegoke *et al*., 2018) and unintended reuse via drinking waters (US EPA, 2017). In all these applications, the hazard group requiring the largest reductions are human enteric viruses (Gerba *et al*., 2017; Gonzales-Gustavson *et al*., 2019; Schoen *et al*., 2017).

Currently, employed treatment processes remove bacterial or parasitic pathogens more efficiently than pathogenic viruses (Abbaszadegan *et al*., 1997; Amarasiri *et al*., 2017; Rose et al., 1996). Viruses pose the greatest risk of all groups of pathogens due to a combination of their high numbers and diversity in wastewater and low infectious dose (Gerba et al., 2018). The concern about viral pathogens monitoring using currently available methods is that their detection limits are usually higher than the concentrations considered to be safe (WHO, 2017b). Traditional virus enumeration techniques like electron (TEM) or epifluorescent microscopy (EFM) and culture-based methods are labour and time-consuming which makes them unsuitable for real-time monitoring of the

[5] Dlusskaya E.A., Pollard P. C., & Ashbolt N. J. Optimizing fluorescence-based assay for virus monitoring of wastewater. Ready for submission.

treatment process. PCR-based methods, though relatively fast and sensitive, detect only the target pathogen(s) and have poor ability to identify infectious virions (Young *et al*., 2020), which limit their use for overall process performance assessment. However, as COVID-19 pandemic has shown, in case of an active outbreak, PCR-based methods of surveillance of the known causative agent in water can provide substantial epidemiological information (Arora *et al.*, 2020; Medema *et al*., 2020; "Novel Coronavirus Found in Sewage," 2020). Flow virometry requires costly equipment, highly skilled personnel, and currently its accuracy and sensitivity with wastewaters are questionable (Dlusskaya *et al*., submitted for publication).

To ensure public safety water reuse systems need an accurate, inexpensive and rapid method of virus concentration assessment during the treatment. Pollard (2012a, 2012b) has reported a fluorescence scanning method to quantify viruses in various waters. Unlike flow virometry, this method measures total DNA/RNA fluorescent signal rather than single particle fluorescens and, therefore, is less prone to biases caused by virus particle aggregation or weak individual particle signal. This method has the potential for automation and on-line monitoring as sample preparation for analysis is not overly complicated. However, signal formation mechanisms and sensitivity improvement is required for practical applications, such as to assess virus removal performance during wastewater treatment, something the U.S. Environmental Protection Agency (US EPA, 2015b) is looking into.

Hence, the aim of this study was to optimize the fluorescence scanning protocol for sensitive and accurate virus estimations by fluorescence scanning with the use of DNA assays, bacteriophage standard cultures and wastewater samples.

5.2 Materials and Methods

5.2.1 Bacteriophage stock culture preparations

The stock culture of coliphage T4 (ATCC® 11303B4™) was propagated in host *E. coli* BL21 DE3 (Sigma-Aldrich). The *E. coli* stock culture was incubated overnight in Luria-Bertani (LB) liquid medium (BD DifcoTM Ref # B244620) at 37˚C and 250 rpm. To produce an exponentially growing *E. coli* culture, 40 mL of fresh LB was inoculated with 10% v/v of the overnight *E. coli* culture and incubated for 2 h under the same conditions. This 2-h host culture was infected with T4 coliphage (2% v/v) and incubated an additional 6 h.

Aliquots of the culture lysate were centrifuged at 4,000 *g* for 30 min to remove host cell debris, the supernatant was filtered through sterile 0.22 µm syringe filter into Amicon Ultra 100K centrifugal filter devices (Merck Millipore, Ref # UFC910024) and centrifuged at 4,000 g for 20 min to remove the culture medium. The retentate that remained in the filter part of the device (~250 µL) was treated with DNAse I (Roche Diagnostics) to remove the residual host DNA. DNAse I buffer was added to a final concentration of 1x (10 mM Tris HCl pH 7.5, 2.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$); and DNAse I, dissolved in storage buffer (20% glycerol in 75 mM NaCl), was added to a final concentration of 10 ng/µL. All

chemicals were purchased from Sigma unless stated otherwise. The retentates were incubated for 45 min at 37˚C. After the incubation, they were rinsed at 4,000 *g* for 10 min with 10 mL of Phosphate Buffered Saline (PBS) buffer (GE Healthcare Life Sci., Ref # SH30256). Rinsed bacteriophage T4 concentrates were re-suspended in the PBS to the original aliquot volume. The plaque-forming units were enumerated as described by Kropinski (23).

5.2.2 DNA extraction

E. coli ATCC 25922 was grown overnight at 37˚C and 250 rpm. Cells from 5 mL of the culture were harvested at 4,000 *g* for 7 min and the supernatant was discarded. The cell pellet was resuspended in 2 mL of lysis buffer (0.1M EDTA and 1% Sodium Dodecyl Sulphate) and 1 mg of proteinase K powder (Thermo Fisher, Ref # AM2542) was also dissolved in this mixture. The bacterial lysate was incubated at 56˚C for 4 h and then heated to 75˚C for 10 min to inactivate the proteinase. After the heating step, the sample was transferred into 50 kDa pore size dialysis tubing (Spectrum™ labs, Ref # 08-700-129) and dialyzed against $T_{10}E_1$ buffer in a cold room. After 24 h the old buffer was replaced with a fresh portion and dialysis continued for an additional 48 h. After the dialysis, the DNA concentration was 248.6 ng/µL and the 260/280 ratio was 2.03 as measured with the NanoDrop™ spectrophotometer (Thermo Scientific™).

5.2.3 Generating Fluorescence Excitation Emission Matrices (FEEMs) and total fluorescence measurement.

Fluorescence Emission Excitation Matrices (FEEM) were created using a Cary Eclipse Scanning Fluorescence Spectrophotometer (Varian, since taken over by Agilent) that was set at a speed of 9600 nm/s and 800 V. Samples were scanned at 200-700 nm excitation and 220-850 nm emission wavelengths. Each fluorescence intensity data set was adjusted using the sterile viral free MQ water blank treated as per a sample. Total fluorescence values (presented in relative (arbitrary) units, a.u., by the instrument software) for SYBR® Green I /DNA peaks were calculated for excitations between 475 and 505 nm and emissions between 520 and 550 nm.

5.2.4 Sample preparation for fluorescence analysis

Autofluorescence of bile salts, detergents, and humic acid. Triton X-100 (CAS 9002-93-1), Brij 56 (CAS 9004-95-9), Tween 20 (CAS 9005-64-5), Sodium Dodecyl Sulphate (CAS 151-21-3), NP-40 (CAS 9016-45-9), Igepal-630 (CAS 9016-45-9) were purchased from Sigma. Bile salts (Oxoid™, Ref # LP0055J) were purchased from Thermo Fisher. Humic Acid (Ref # AC 120860010) was purchased from Acros Organics.

To generate FEEMs, these reagents were diluted in sterile MQ water. The same MQ water was used as a blank control to adjust the fluorescence.

Humic acid interference with the DNA fluorescence. The original DNA extract and Humic acid (HA) were diluted in MQ water.

To measure DNA fluorescence in the HA gradient, binary dilutions of HA from 500 ppm to 0.98 ppm were prepared in triplicate in MQ water and in 0.1 ng/µL DNA solution. Total fluorescence of the samples within $\lambda_{ex/em}$ = 475 to 505 nm/520 to 550 nm range was measured before and after SYBR® Green I staining to 1x final concentration of the dye.

To measure DNA gradient fluorescence at a constant HA concentration, 5 ng/µL DNA working stock was diluted in triplicate to final concentrations of 0.01, 0.02, 0.05, 0.1, and 0.2 ng/µL and stained with 1x SYBR® Green I. Total fluorescence was measured before and after the addition of the 1000 ppm HA stock solution to final concentration of 15 ppm (M. Huang et al., 2010).

The samples were blanked with MQ water.

Bacteriophage. The bacteriophage stock serial dilutions were prepared in MQ water. The samples were stained with 1x SYBR® Green I for 10 min either at room temperature or at 80˚C. Unstained MQ water was used as a blank.

Wastewater samples. Wastewater was sampled at the EPCOR Gold Bar treatment plant, located in Edmonton, Alberta, Canada[6]. One-liter samples of post-grit, primary effluent, and tertiary treated – membrane filtered (0.45 µm pore size) water were transported on ice to the laboratory and processed immediately. Wastewater sample aliquots were filtered through a sterile 0.22 µm syringe filter to remove debris and all

[6] https://www.epcor.com/about/who-we-are/where-we-operate/edmonton/Documents/GoldbarTreatmentProcess.pdf

microorganisms except viruses. Virus-free wastewater was prepared by centrifugation through Amicon Ultra 100K centrifugal filter device (Merck Millipore, Ref # UFC910024) at 4,000 *g* for 20 min to remove the native virus populations (Pollard, 2012b).

Samples were stained with 1x SYBR® Green I for 10 min at 80˚C. Unstained virus-free wastewater was used as a blank.

5.3 Results

The Varian Cary Eclipse Spectrophotometer resolves the T4 DNA fluorescence signal from the background starting at 10^5 PFU/mL. Then the total fluorescence increased logarithmically with bacteriophage concentration until signal saturation at 10^8 PFU/mL (Figure 5.1).

Heating the sample to 80˚C during the SYBR® staining stage enhanced the sample total fluorescence, thus making the method substantially more sensitive. Figure 5.2 shows the fluorescence excitation-emission matrices (FEEMs) of bacteriophage T4 samples, stained at room temperature and at 80˚C.

Removal of background water from samples and replacing it with MQ water leads to better resolution of the bacteriophage T4 signal. The total fluorescence of 10^6 PFU/mL of the bacteriophage T4 was not significantly different from the virus-free controls in the samples with post-grit and post-membrane water backgrounds. The same concentration bacteriophage suspensions in MQ water produced significantly higher total fluorescence signal ($p < 0.001$) when compared to virus-free controls (Figure 5.3).

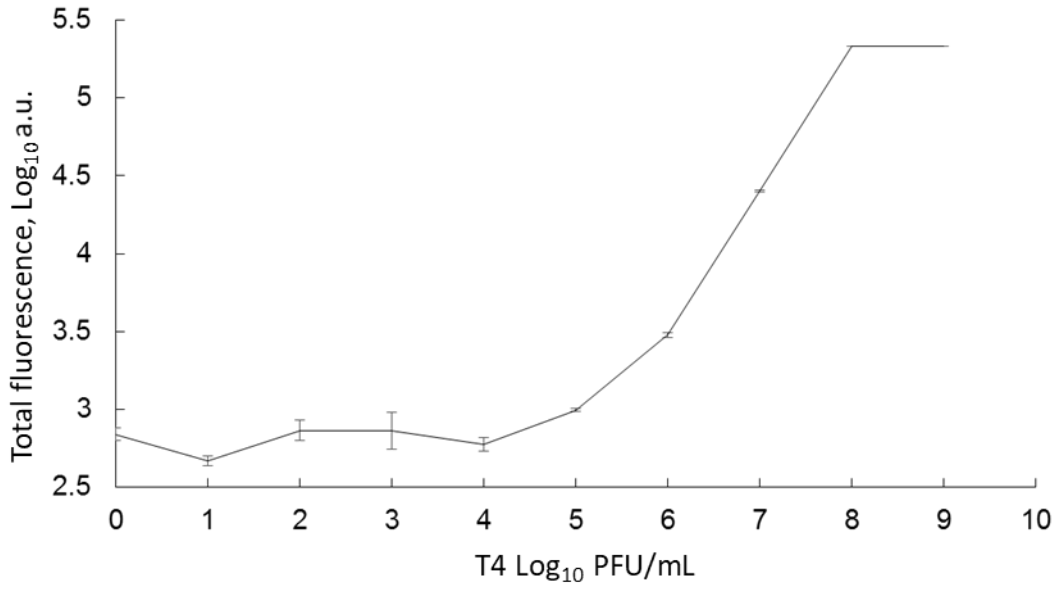


Figure 5.1. Total $\lambda_{ex/em}$ = 475 to 505 nm/520 to 550 fluorescence of the SYBR-stained bacteriophage T4 in MQ water. The Cary Eclipse Spectrophotometer starts resolving theT4 fluorescence signal from the background between 10^5 and 10^6 PFU/mL.

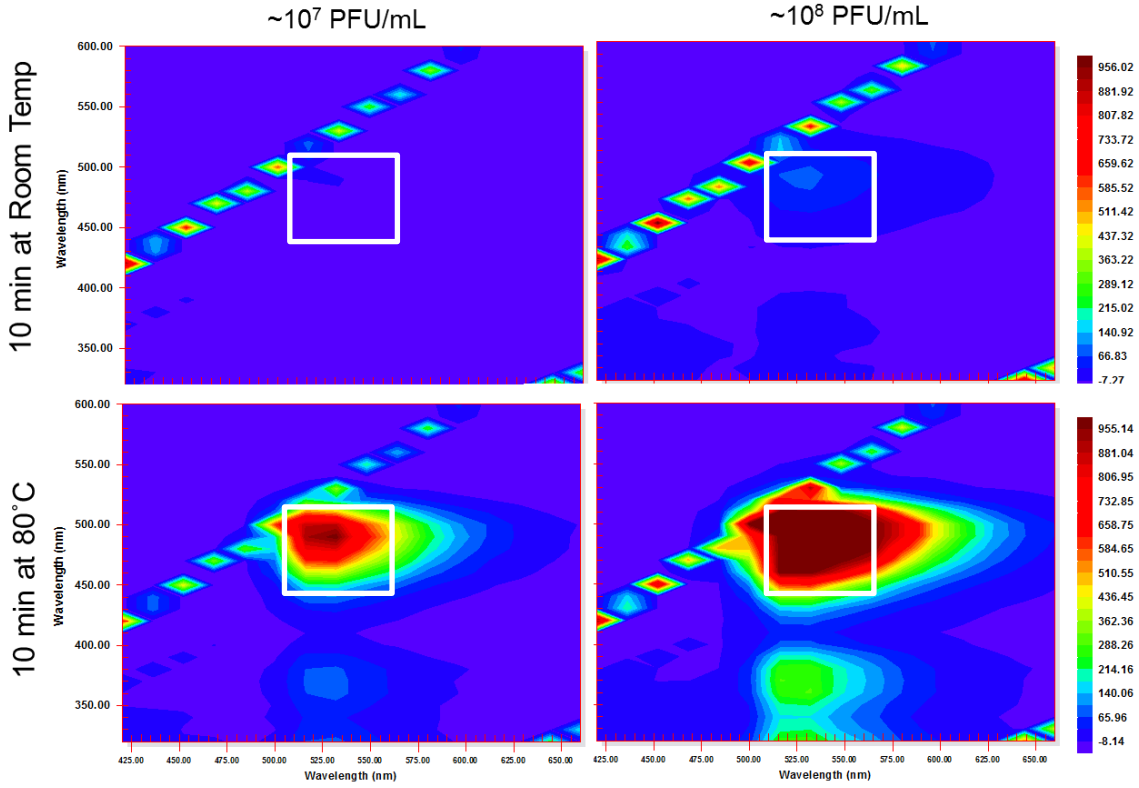


Figure 5.2. Heating the bacteriophage T4 sample to 80˚C during SYBR staining process enhances total fluorescence signal, thus increasing sensitivity. The box highlights the DNA/SYBR® target region.

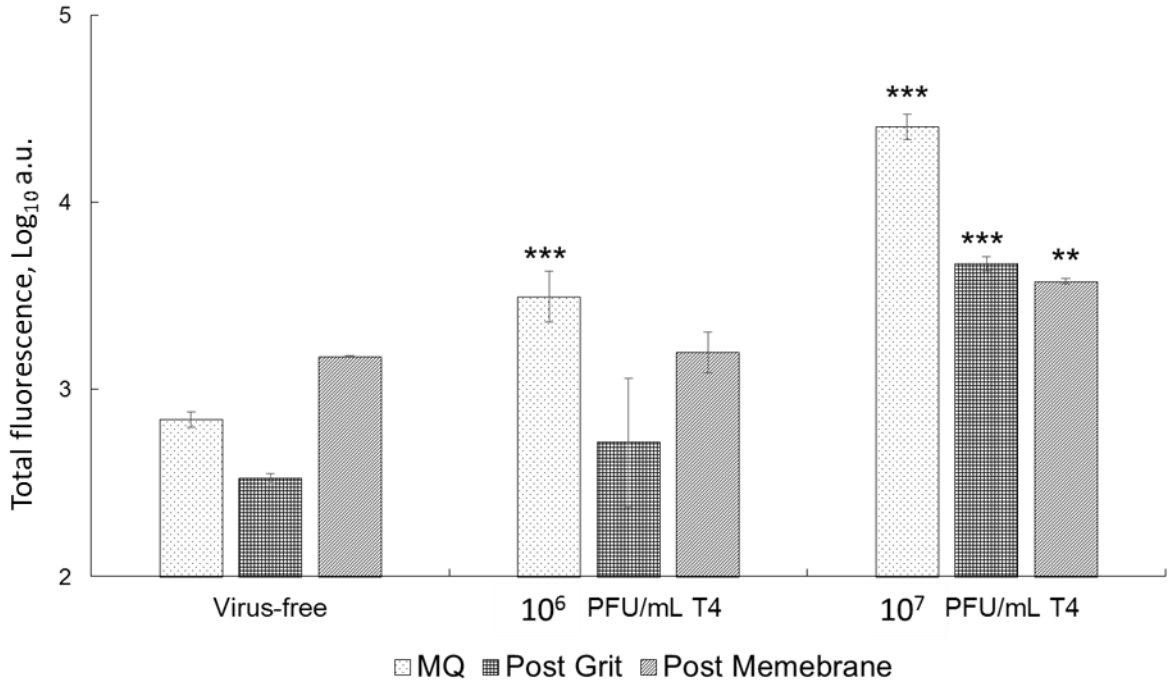


Figure 5.3. Total $\lambda_{ex/em}$ = 475 to 505 nm/520 to 550 fluorescence of the bacteriophage T4 in MQ water, Post-Grit, and Post-UF Membrane water background. The Cary Eclipse Spectrophotometer resolved the bacteriophage signal in the MQ water suspensions more efficiently than in wastewater suspensions. This is likely due to the absence of organic colloids that are present in wastewater that interfere with SYBR® Green fluorescence. A two-tailed Student t test yielded P values of <0.05 (**) and <0.001 (***).

Concentration-dependent, humic acid auto-fluoresces in the same $\lambda_{ex/em}$ (475 to 505 nm/520 to 550 nm) range the DNA/SYBR® complex does. This auto-fluorescence intensity increases with an increase in humic acid (HA) concentration, reaching a peak at around 125 ppm, and then diminishing as concentration of HA increases (Figure 5.4 and 5.5C). HA significantly increases the total fluorescence signal of SYBR®-stained *E. coli* dsDNA dissolved in MQ water**.** Figures 5.5 A and B show FEEMs and total fluorescence plotted as a function of different concentrations of dsDNA at a constant 15 ppm concentration of HA. The total fluorescence increases with the increase of the DNA concentration. Figure 5.5C shows a significantly higher total fluorescence of 0.1 ng/µL SYBR-stained dsDNA when HA concentrations are between 2 and 15 ppm. At the HA concentration of 125 ppm, the total fluorescence of SYBR®-stained dsDNA becomes indistinguishable from the HA autofluorescence. It remained indistinguishable as the HA concentration further increased. At the same time, 15 ppm HA added to wastewater samples with the original background matrix, decreased the total fluorescence within the DNA/SYBR® complex $\lambda_{ex/em}$ range (Figure 5.6). Neither bile salts nor surfactants produced an auto-fluorescence signal in the same $\lambda_{ex/em}$ (475 to 505 nm/520 to 550 nm) range the DNA/SYBR® complex does (data not shown).

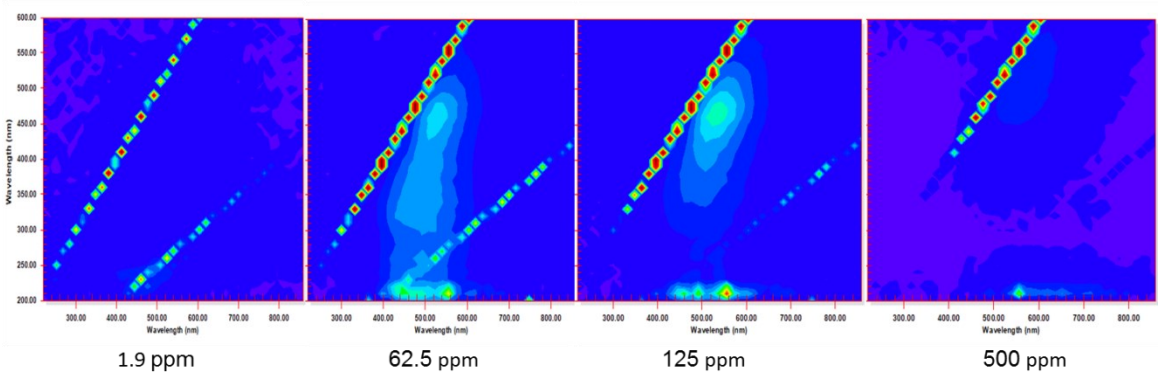


Figure 5.4. At concentrations between ~50 to 200 ppm, Humic Acid emits auto-fluorescence at the same excitation/emission range ($\lambda_{ex/em}$ = 475 to 505 nm/520 to 550 nm) as DNA/SYBR® complex, which might potentially affect the DNA concentration estimates.

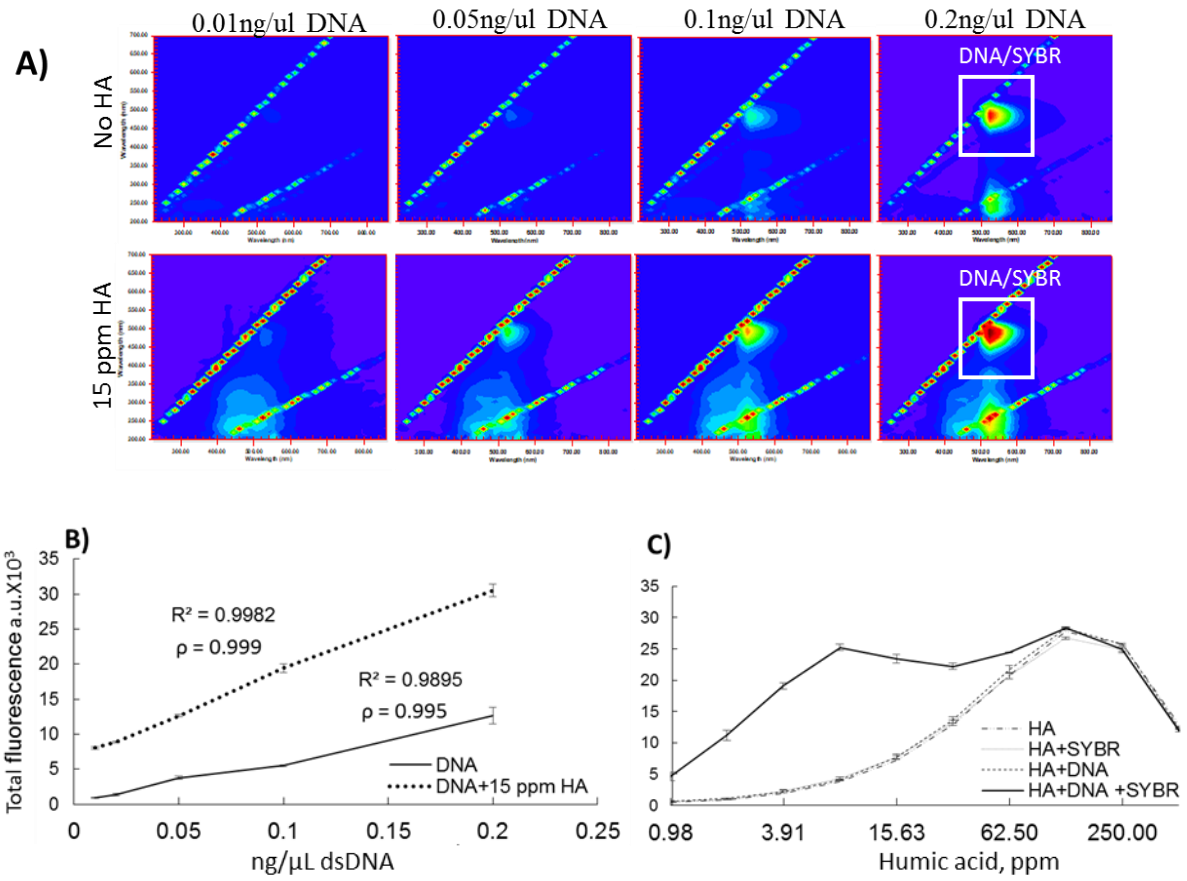


Figure 5.5. Humic Acid enhances fluorescence of dsDNA in MQ water.

A) Addition of 15 ppm of Humic Acid (bottom) led to more intense fluorescence of the DNA/SYBR® complex as visualized by FEEMs (the white box highlights the DNA/SYBR® target region of $\lambda_{ex/em}$ = 475 to 505 nm/520 to 550 nm). It allowed to detect 0.01 ng/µL DNA, which was not possible without Humic Acid addition.

B) The total fluorescence of SYBR®-stained DNA is some five times higher when Humic Acid is added, meaning better sensitivity.

C) Total fluorescence of 0.1 ng/uL DNA in Humic Acid gradient graph allows to determine the concentration of Humic Acid at which the difference between its self-fluorescence and the DNA/SYBR® complex fluorescence is the highest.

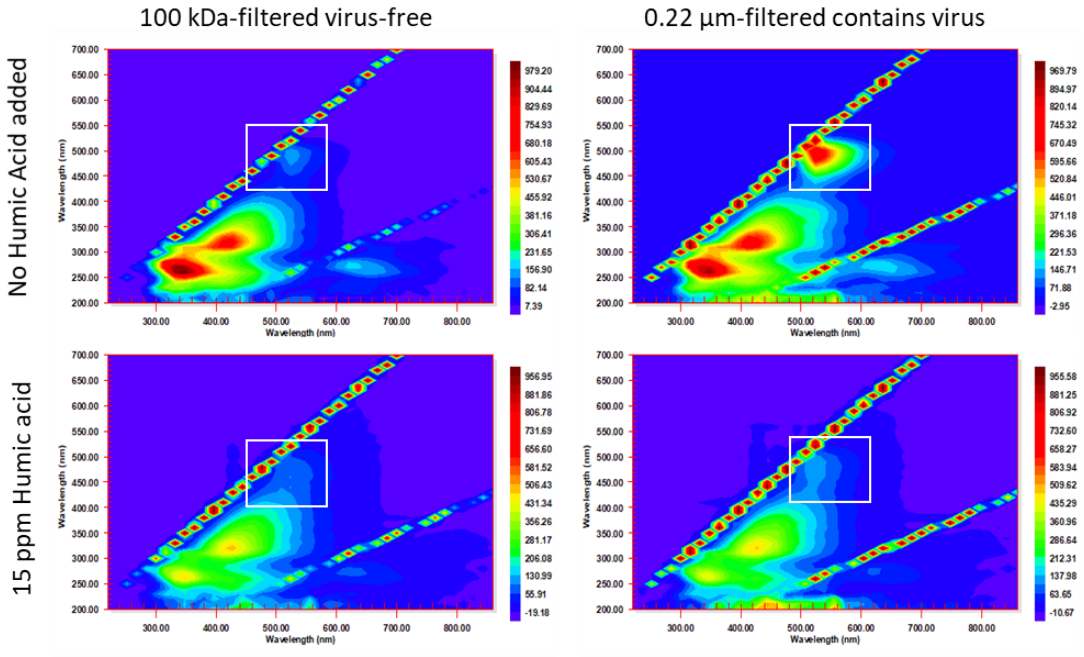


Figure 5.6 In wastewater samples addition of Humic Acid might decrease the total fluorescence within the DNA/SYBR® target region of $\lambda_{ex/em}$ = 475 to 505 nm/520 to 550 nm due to complex and largely unpredictable colloidal interactions with wastewater background matrix constituents other than DNA.

The same Post-Grit sample was filtered through 0.22 µm pore size membrane to remove larger debris and bacterial cells and through 100 kDa molecular sieve to remove viruses. Upper row depicts FEEMs of SYBR®-stained sample and virus-free control with no Numic Acid added and bottom row shows the same samples after addition of 15 ppm of Humic Acid. The white box highlights the DNA/SYBR® target region.

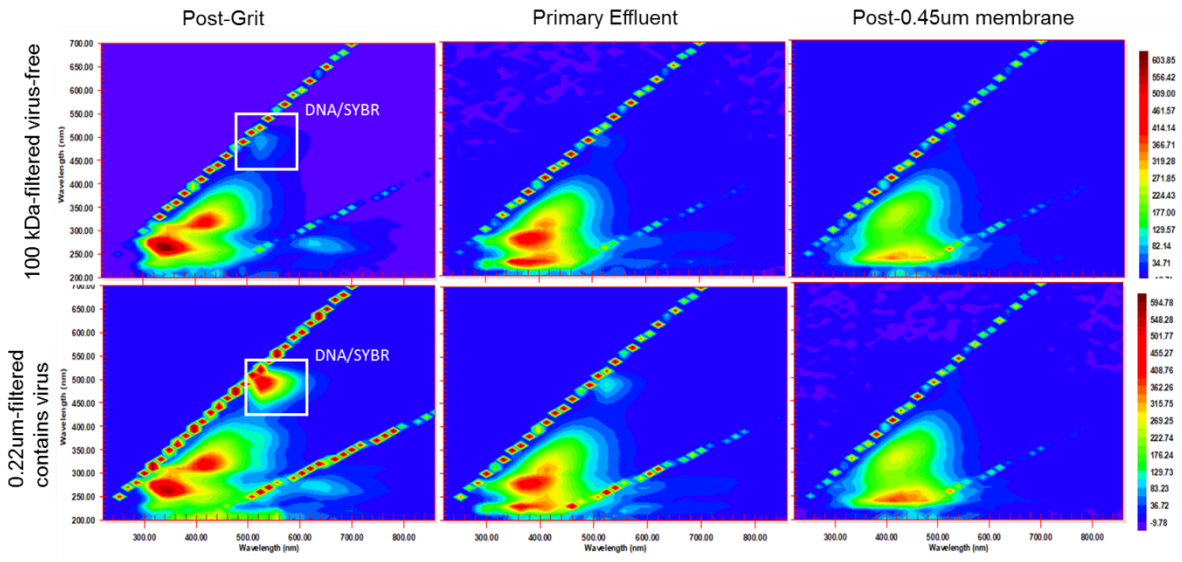


Figure 5.7. Fluorescence excitation emission matrices of municipal wastewater at different stages of the treatment process. Upper row: all viruses from samples were removed by ultrafiltration through 100 kDa molecular sieve. Bottom row: Samples were filtered through 0.22 µm pore size membrane to remove larger debris and bacterial cells. Virus DNA total fluorescence can be calculated by subtracting the total fluorescence of the virus-free control from the total fluorescence of the virus-containing sample. Also noticeable is the decrease in DNA/SYBR® complex fluorescence intensity as the wastewater treatment progresses from Post-Grit to Post-membrane.

Virus DNA total fluorescence can be calculated by subtracting total fluorescence generated by the virus-free control from the virus-containing sample total fluorescence. FEEMs, shown in Figure 5.7, demonstrate the difference between virus-containing wastewater samples and the controls, from which all the viruses were removed by ultrafiltration. The post-grit sample produced the highest fluorescence within the 475 to 505 nm/520 to 550 nm $\lambda_{ex/em}$ range for both virus-containing samples and controls. The fluorescence decreased in the wastewater samples from more advanced stages of the treatment process. These results are in good agreement with the removal of organic substances during the treatment process (https://www.epcor.com/products-services/water/water-quality/wqreportsedmonton/wwq-edmonton-2018.pdf). Raw sewage contains the highest levels of dissolved organic carbon, part of which are viruses and free DNA.

5.4 Discussion

In determining the feasibility of using a total fluorescence measurement method for rapid estimations of the virus numbers in wastewater we identified organic carbon and free DNA factors that may affect the accuracy of virus estimations. However, a solution to these issues was identified, along with modifications to enhance the total fluorescence measurement sensitivity, even using an older generation (Varian Cary Eclipse) Spectrophotometer.

5.4.1 Fluorescent excitation-emission matrix (FEEM)

Fluorescence spectroscopy has long been proposed as a tool for monitoring water quality (Baker *et al*., 2015; Hambly *et al*., 2010; Henderson *et al*., 2009). The development of rapid assessment methods for wastewater process performance has been an area of key interest in terms of recycled water use (Henderson et al., 2009).

The FEEMs we obtained for wastewater samples show the same Tryptophan-like fluorescence peaks described by other authors and typical for wastewater with protein-derived fluorescence due to high bacterial activity (Baker & Spencer, 2004; Nowicki et al., 2019; James P.R. Sorensen et al., 2018). These peaks were absent in the samples with the MQ water background. A distinct fluorescence peak in the $\lambda_{ex/em}$= 475 to 505 /520 to 550 nm region appeared when wastewater samples were stained with SYBR® Green I. A similar peak in water samples was reported by Pollard (Pollard, 2012b) with the use of SYBR® Gold.

However, organic matter (OM), as represented by HA in the current study, also produced a peak within the same $\lambda_{ex/em}$= 475 to 505/520 to 550 nm region as the SYBR®/DNA complex (Figure 5.4). The maximum self-fluorescence was observed at around 125 ppm HA and at higher concentrations it self-quenched likely due steric interference HA is known to be prone to (Ohno, 2002). The peak has longer ex/em wavelength than humic-like peaks reported by Hambly *et al.* (2010) but is in good agreement with the HA spectra reported by Mobed *et al*. (1996) and Pollard (2012b). This wavelength difference

might be due to the sample pH. Spencer *at al*. (2007) reported a distinct redshift of the humic-like peak spectra with a pH increase.

Wastewater virus-free controls, generated by ultra-filtration, still showed some fluorescence in the SYBR®/DNA complex peak range, presumable due in part to free DNA present in raw sewage (*e.g*. Zhang *et al*., 2018). However, as the intensity of the fluorescence peaks decreased through stages of sewage treatment (Figure 5.6), organics removal generally (including microorganisms, OM and free DNA) may all contribute to FEEM signal reduction. Henderson *et al.* (2009) emphasized the matrix water effects that might alter the fluorescence readings: inner filtering/steric effects, fluorescence quenching, *etc*. The chemical composition of wastewater fluctuates seasonally, daily, and hourly (Cheremisinoff, 2019; Heukelekian & Balmat, 1959), and it changes dramatically during the treatment process[7]. All these changes undoubtedly affect the fluorescence, thus making the interpretation of the quantitative results more challenging (Henderson et al., 2009). Temporal i*n-situ* quantification of the virus in the wastewater by total fluorescence measurement was beyond the scope of this study.

A simple solution to FEEM interferences in wastewater was to filter and compare to the non-UF-filtered sub-sample. As shown on Figures 5.1 and 5.5, in the experiments with free DNA and bacteriophage T4 serial dilutions in MQ water, the total fluorescence signal was quite precise and highly correlated with the increase of the DNA ($\rho = 0.995$) or phage

[7] https://www.epcor.com/products-services/water/water-quality/wqreportsedmonton/wwq-edmonton-2018.pdf

concentration (within the detection limits of 10^5 to 10^8 PFU/mL, ρ = 0.99). Pollard (2012b) also demonstrated a strong correlation between virus counts by epifluorescent microscopy and total fluorescence in natural water samples and wastewater within 10^7-10^8 Virus/mL range. Noting that for wastewater samples, bacterial-size and larger particles that would interfere with the virus assay were removed by pre-treatment through 0.45 µm then 0.22 µm filters before SYBR® staining.

5.4.2 Impact of Humic Acid on the SYBR Green I/ DNA complex fluorescence in wastewater and control samples.

Hambly *et al.* (2010) showed the presence of humic compounds in wastewater and Huang *et al.* (2010) reported about 12 ppm of HA in a residential wastewater sample, taken in Shanghai, China.

In our experiments HA significantly increased total fluorescence of DNA in the samples with MQ water background at concentrations between 2 and 15 ppm (Figure 5.5), reaching the maximum of fluorescence at ~8 ppm. With the further HA concentration increase, the total fluorescence produced by the SYBR®/DNA complex became less distinguishable from the HA auto-fluorescence (Figures 5.4 and 5.5C). But when added to SYBR®-stained wastewater samples in 15 ppm final concentration, HA quenched the fluorescent signal in all SYBR®/DNA (ex/em = 490/530 nm) and the Tryptophan–like (ex/em = 250-300/350-400 nm) peaks (Additional material). We attribute this effect to complex molecular and colloid interactions between HA and other constituents of wastewater matrix. These constituents likely include surfactants and/or bile acids as a

part of the sample colloid system, despite no self-fluorescence of surfactants or bile acids in spectra close to the SYBR®/DNA complex.

By contrast to our findings, Zipper *et al*. (2003) reported the quenching effect of 10 ppm HA on the SYBR®/DNA complex in distilled water. Humic acid is a heterogeneous macromolecule and its composition might depend on the source it was extracted from and the method of extraction. Unlike the German team, we used HA in the sodium salt form, which is more water-soluble, remains a true solution and starts forming colloid particles at higher concentrations than the protonated acid form. Binding of two fluorophores with similar ex/em spectra gives us a base to suggest that fluorescent resonance energy transfer (FRET) to be the mechanism behind the enhancement of the SYBR®/DNA complex fluorescence. Though in their experiments Zipper *et al.* showed no HA binding to the SYBR®/DNA complex, we assume that the deprotonated form of HA might behave differently and bind to it. In all our experiments HA stock solution was added **after** staining the DNA with SYBR®. The total fluorescence of each sample was measured before and after the HA addition.

5.4.3 Measuring viral abundance by FEEM.

The proposed protocol provides improved sensitivity and accuracy of virus quantification in water samples regardless of their source. Though it is possible to calculate the virus fluorescence in a sample by subtracting total fluorescence of the virus-free control (as it was shown on Figure 5.6), this approach is more applicable to monitoring water sources of relatively stable chemical composition, for example, post-

membrane treated wastewater. In cases of untreated wastewater or environmental waters with fluctuating chemical composition the “matrix effects” might introduce significant confounders and require exhaustive controls to compensate for inner filtering or quenching effects (Lakowicz, 2006). While the matrix wastewater is the analyte for other fluorescence-based water monitoring applications, in the case of virus quantification it can be removed and the virus concentrated by 3-4 orders of magnitude by filtration on 100-300 kDa molecular sieves or other devices like hollow-fibre filters (Smith & Hill, 2009). Resuspension of viruses in a standardized buffer will alleviate the “matrix effect”. Staining the sample at 80°C in the presence of HA sodium salt as a fluorescence enhancer will improve the sensitivity. All the sample preparation steps described here in combination with modern sensitive fluorescence detection instruments can bring virus detection limits 3-5 orders of magnitude down. They can also be automated, making this protocol adaptable to on-line monitoring of wastewater treatment performance. The TEM or EFM-based standard curves (*e.g*. Pollard, 2012b) for virus quantification need to be prepared and regularly updated for individual water sources as dominating virus genomes size distributions might vary and the fluorescence intensity of SYBR®/DNA complex is proportional to the DNA concentration.

Further validation of this protocol, optimization of the optical instruments, and overall method standardization are needed prior to its consideration for on-line virus monitoring by the water industry.

Chapter 6: Final discussion and conclusions

With continued climate change, population growth, and urbanization - there is increasing demand for fresh water. However, as natural fresh water sources continue to diminish and water becomes the most important at-risk natural resource (Rose, 2007), there will be a growing need to reclaim water for various domestic, agricultural, and industrial applications. There is growing recognition that municipal wastewater can become a reliable, convenient, as well as environmentally and economically advantageous alternative source of fresh water (UNESCO, 2017; Wu *et al.*, 2013). Yet water reuse might be Janus-faced: in that inadequately treated municipal wastewater poses a serious risk for both the environment and public health (Chen *et al*., 2013; Prüss-Ustün *et al*., 2014).

The WHO (2001), European Union (Marecos do Monte, 2007; Luigi Rizzo *et al*., 2018), US EPA (2017), and other national water quality strategies, as in Australia (National Water Quality Management Strategy, 2006) have their focus on the prevention of waterborne illnesses, which in turn is based on a proactive water safety framework. Water quality monitoring is a major part of this proactive approach along with the risk assessment (WHO, 2001b)

Municipal wastewater that mostly consists of human waste is a source of human pathogens by the mere nature of it. These pathogens must be efficiently removed and/or inactivated before the treated wastewater is fit-for-use (Chhipi-Shrestha, *et al*., 2017a, 2017b). The municipal wastewater treatment process is a continuous operation and the treated wastewater is discharged constantly. This is what makes all culture-based and many molecular-based water quality monitoring methods limited or inadequate to the

task. These methods are time consuming and reflect the quality of the water that had been discharged few hours or days before. For high risk scenarios these methods are not amenable to timely detection of hazardous events, such as pathogen break-through of treatment barrier(s), and it is these sporadic hazardous events that pose much higher risk for health and safety (Bartram *et al*., 2018; Beaudequin *et al*., 2015; Hrudey *et al.*, 2006; Medema & Ashbolt, 2006) compared to normal conditions.

The online monitoring of the wastewater treatment process and recycled water quality is of paramount significance to ensure public and environmental health. Turbidity, electrical conductivity, pH and disinfectant residual are monitored routinely as standard water quality parameters and indirect indicators of pathogen (including virus) removal (Vanrolleghem & Lee, 2003; Von Sperling, 2015). Fluorescent spectroscopy has been used to monitor fluorescent organic matter in wastewater treatment process (Carstea *et al*., 2016). However, of all pathogen groups, viral reduction need is highest (CSWRCB, 2018; Schoen *et al.*, 2017) and there is no method for direct *ex-tempore* monitoring of virus removal during the wastewater treatment process.

As an endeavor to find a solid and reliable method of virus monitoring during wastewater treatment, my research proceeded from a basic validation of flow cytometry as a method for virus enumeration in general to a more specific assessment of this method for its applicability at wastewater treatment facilities. To assess the sensitivity of flow cytometry in virus enumeration, five pure cultures of coliphages (MS2, phiX174, Lambda, P1, and T4) with different genome sizes (3.6, 5.4, 48.5, 93, and 169 Mbp respectively) were analyzed using four different commercially available flow cytometers

(BD FACScanto, BD Fortessa X-20, Gallios by Beckman Coulter, and Cellstream by Millipore Sigma). During this initial stage it became apparent that flow cytometry as a method for virus enumeration was not as sensitive and straightforward as previously reported (Brussaard, 2004) and it does rely heavily "on the skills of the operator". A possible explanation why marine virologists initially found FCM virus enumeration simple and uncomplicated is the fact that in open water environments there are relatively high numbers of algal viruses, up to 10^5-10^6 VLP/mL^{-1} (Hara *et al*., 1996; Sandaa, 2009; Weynberg, 2018), and genome sizes of algal viruses are large [160-600 kbp] (Mirza *et al.*, 2015; Sandaa *et al.*, 2020; Tawaha *et al*., 2020; Van Etten *et al*., 2002) compared to genomes of bacteriophages that dominate WW environments. Some protozoan viruses can reach even 400 nm in diameter with genome sizes above 1000 kbp (Colson *et al*., 2017; Desjardins, 2012). Hence, FCM analysis of environmental waters may be more amenable for these larger viruses given the published conditions in the literature.

My findings demonstrated that the virus sample, prepared for the analysis, is a colloid system to which all the laws of colloid chemistry apply (Michen & Graule, 2010; Pal *et al*., 1998; Wilkinson & Lead, 2007). However, what may be less recognized by microbiologists is the colloid nature and self-fluorescence of the fluorescent dyes that are used to stain viruses before analysis, which can seriously complicate the analysis itself as well as the interpretation of the results by flow cytometry. The data presented in Chapter 3 provided the fundamental understanding of the physics and chemistry behind flow cytometric virus enumeration (flow virometry).

When undertaking flow virometry with wastewater, special attention must be paid to the chemical composition of the background water matrix. I clearly demonstrated interference with soluble organic substances in wastewater sample on virus enumeration. High organic substance background enhances the emulsifying of the fluorescent dye and increases the false signal of virus-like particles (VLPs) in the sample. The enhanced VLPs at the same time obscured the true virus signal. The results, presented in Chapter 4, provide critical insight to inform decisions regarding the workability of flow virometry in water reuse scenarios, such as the core need to replace the wastewater matrix with standardized buffer and provide stained virus-free controls. In the future my detailed explanation of the basic mechanisms behind VLP artifacts in wastewater samples will help to ameliorate this method for the real-time analysis of viruses in water.

While flow virometry needs enhanced instrument sensitivities and further development the method protocol before it can be used for wastewater monitoring, Chapter 5 provides preliminary data on an alternative fluorescence-based method of virus estimation: fluorescent spectroscopy. Fluorescent spectroscopy was previously reported as an effective method for detection of water contamination with sewage (Carstea *et al*., 2016b). Moreover, tryptophan-like fluorescence (measured at excitation-emission wavelengths of 280 nm and 360 nm) can be monitored in real-time to detect faecal contamination of drinking water (Sorensen et al., 2018). In the current work, I described how fluorescent spectroscopy has potential for real-time detection of viral nucleic acids stained with SYBR® Green fluorescent dye to monitor the wastewater treatment process. The fluorescence intensity of samples highly correlated with nucleic acid content and was

significantly increased with the addition of a humic acid sodium salt (Chapter 5). The enhancement of fluorescence intensity of SYBR®/DNA complexes with humic acid appeared to be due to a FRET mechanism. Potentially this effect could be further developed to improve the sensitivity of both flow cytometric and spectroscopic methods for the assessment of virus abundance in water.

Real time virus monitoring is needed for timely detection of sporadic hazardous events (Chen *et al*., 2013). Automated flow virometry or viral NA fluorescence intensity scanning of raw sewage (Figure 6.1 A) might play a key role in early detection of a hazardous event and initiating the enhanced treatment protocol specifically designed for such events. Tolerable virus concentration in reclaimed water is based upon tolerable disease burden and can be calculated (Kamizoulis, 2008; WHO, 2017b; Guidelines for the Safe Use of Wastewater, Excreta and Greywater, 2006). The difference between virus concentration in raw sewage and tolerable concentration is the virus LRV requirement and it can also be calculated for individual WWTPs (Gerba & Betancourt, 2019; Ito *et al*., 2017). *Ex tempore* virus monitoring in final effluent (Figure 6.1 B) will become a critical control point (CCP) that ensures that the virus removal by the system is operating as designed. In case of insufficient LRVs after primary and secondary treatments the operator will be able to make instant adjustments to subsequent tertiary treatment processes like chlorination, AOP, or UV disinfection prior to discharging the treated water into the receiving waterbody. In water reuse scenarios the final effluent passes through additional pathogen barriers like chemical coagulation, membrane-based processes (MF, UF, RO) and AOPs. These processes have been shown to have highest LRVs (Zhang *et al*., 2016) as the result

of physical removal or chemical disintegration of viral particles. The *ex-tempore* virus monitoring in the post-tertiary effluent (Figure 6.1 C) should be the final CCP for the fit-for-purpose treated water, which in the case of the above the threshold virus signal will be diverted from the distribution line for further/emergency treatment to prevent adverse public health outcome. These three virus' monitoring points will add to other online-monitored parameters to "tailor" the treatment process to suit momentary wastewater virus load.

The virus load differs greatly between raw sewage and final or post-tertiary effluents. Therefore, further investigations and validation of virus concentration methods are needed. Dead end ultrafiltration-based methods have been previously demonstrated as easy to use and efficient (Smith & Hill, 2009;Wommack *et al*., 1995). However, due to the relatively low viral load post-treatment, effluents and especially post-membrane effluents require higher levels of concentration, two to three orders of magnitude versus 5 to 10 times as validated previously. General chemistry findings of this research provide a fundamental basis for development and validation of optimal virus enumeration protocols that are suitable for automated virus monitoring by fluorescence spectroscopy or flow virometry. The methods for establishing the thresholds for virus-generated fluorescent signal as well as the guidelines for including these thresholds into facility operation procedures also need to be addressed in the future. The findings I present in this dissertation lay the foundation for fundamental understanding of the nature of fluorescence-based virus enumeration methods and the virus-wastewater system as a whole. They also serve as a starting point for much needed industrial applications of

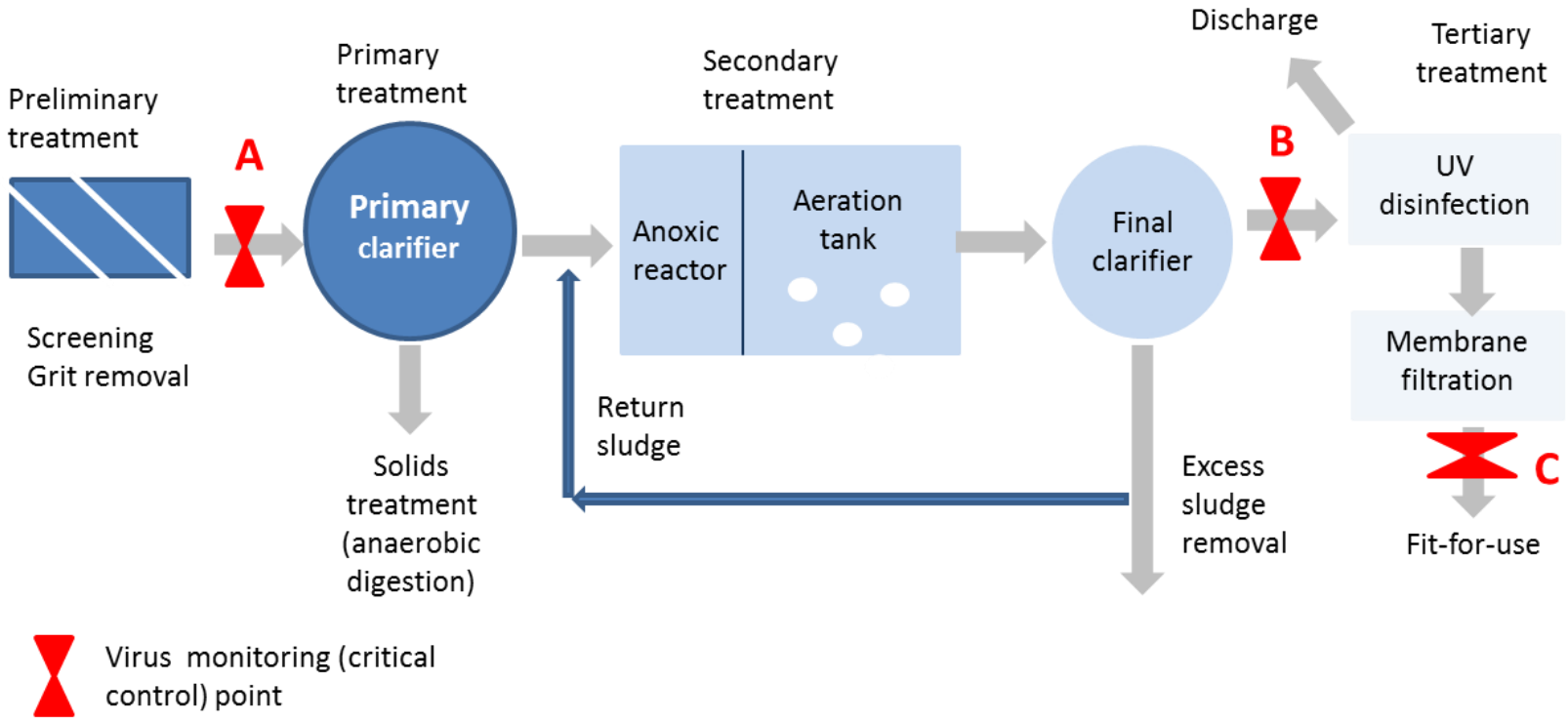


Figure 6.1 Potential virus monitoring points along the wastewater treatment train by the example of Gold Bar WWTP process.

fluorescence-based *ex tempore* virus monitoring to protect environmental and public health and safety.

Overall, this thesis research provides the following conclusions:

1. Flow virometry with complex water matrices is currently neither sensitive nor accurate enough to be used in water reuse scenarios;

2. Fluorescent spectroscopy has potential to provide useful, real-time monitoring of fluorescently labeled viral nucleic acids during wastewater treatment process; and

3. Understanding the fundamental colloid mechanisms in sample matrices along with the development of more sensitive instruments for analysis could turn flow virometry into a useful tool for water quality monitoring.

Appendix 1: Staining of nucleic acids with fluorescently labeled recombinant DNA and RNA-binding proteins.

Expression and purification of DNA-binding protein Sso7d and DNA labeling.

Having failed to detect small viruses (with the genome below 150 kbp) with flow cytometry directly, I attempted another strategy: to create a fluorescently labeled DNA-binding protein with the rationale that the protein molecules are much larger than SYBR and, therefore, can add some "bulk" to virus DNA and increase the side scatter signal of the latter. I chose the 7 kDa, thermostable, basic (PI=9.7), and hydrophilic (GRAVY = -0.998) DNA-binding protein Sso7d from an archaeon *Sulfolobus solfataricus*. It has been studied extensively since its first description in 1988 and shown to bind DNA very efficiently with the binding site of about 4 bp of DNA per one protein molecule (Edmondson & Shriver, 2001) and create tight supercoiled structures by kinking the DNA strands (Choli *et al*., 1988).

Two versions of the Sso7d-encoding gene were assembled from a set of oligonucleotides: Wild type and E35L mutant. The E35L mutant (Shehi *et al*., 2001) was chosen in hopes to prevent viral RNA degradation in the sample because the Sso7d protein was also isolated as an unknown RNAse in a DNA-unrelated experiment (Fusil et al., 1993). In the assembly, I added a Cysteine label on N-terminus for subsequent fluorescent labeling of the recombinant protein (Figure A.1).

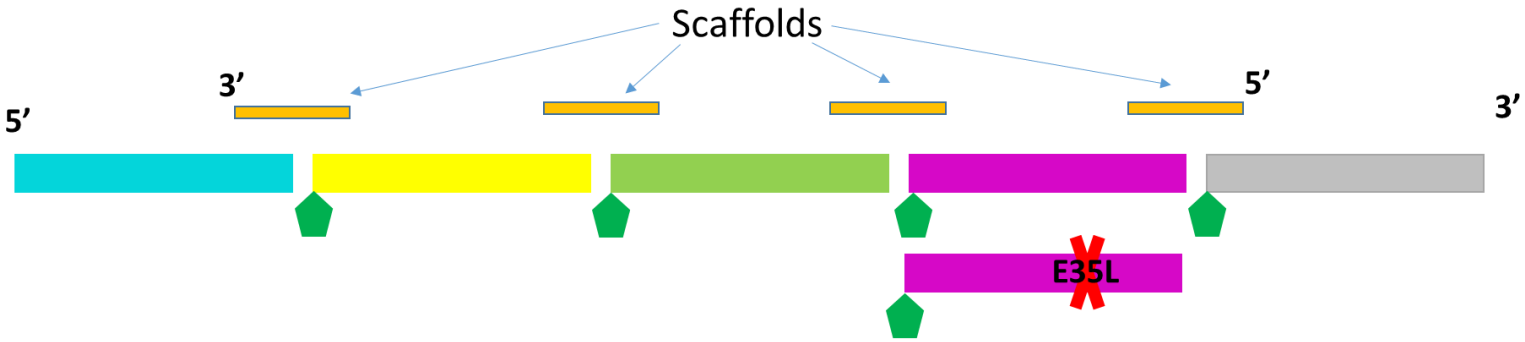
First, an oligonucleotide at 20 pmol concentration was phosphorylated for 60 min at 37 ℃ by T4 Polynucleotide Kinase in ligase buffer. Then the set of oligonucleotides and

scaffolds was assembled, annealed at 94 ℃ for 2 min, cooled down, T4 ligase was added and the ligation was carried out overnight at room temperature.

The final ligation product was visualized in 2% agarose gel with TAE buffer. Electrophoresis was carried out at 90v for 45 min. The 50 mL gel was stained with 0.5 µg/ml final concentration of ethidium bromide before casting. The correct sized bands were excised and used as a template for PCR reaction with LIC51 primers for pLATE51 vector, used in aLICator ligation independent cloning system (ThermoFisher). PCR product was visualized in 1 % agarose gel. After visualization, wells were excised right under the product bands, the gel was returned into the electrophoresis unit, wells were filled with fresh 1x TAE buffer, and the electrophoresis was run for 2.5 min. The liquid from the wells was collected and the product presence was confirmed by the UV imaging (Figure 5). The purified product was stored at -20℃. The assembled Sso7d gene was cloned in *E. coli* DH5α in the pLATE51 vector according to the manufacturer's instructions. Competent DH5α cells were transformed by standard heat shock (90 s at 42 ℃). After those plasmids were isolated, inserts were sequenced for quality, and correctly assembled vectors were cloned in expression *E. coli* strains BL21DE3 and BL21pLyzS. When induced with 0.4 mM IPTG, the clones did not produce any recombinant protein.

Attempts to clone the Sso7d gene in pET21b and express the protein in *E. coli* BL21DE3, BL21-SI, and BL21pLyzS have also failed.

TCT CTC ATA TGC ACC ACC ACC ACC ACC ACG GTG GCT GTT GCC CGG GTT GTT GCG GTG GC GCT ACC GTT AAA TTC AAA TAC AAA GGT
GAA GAA AAA GAG GTC GAC ATC TCC AAA ATC AAA AAA GTT TGG CGT GTT GGT AAA ATG ATA TCT TTT ACC TAC GAC GAA
GGT GGT GGT AAG ACC GGT CGT GGC GCC GTT TCC GAA AAA GAC GCT CCG AAG GAA CTG CTG CAG ATG CTG GAG AAA
CAG AAA AAA TAA



W- wild type Ssoo7d
E35L mutant – with Glutamic acid (GAA) replaced by Leucine (CTG)
- phosphorylation

Figure A.1. Sso7d gene assembly.

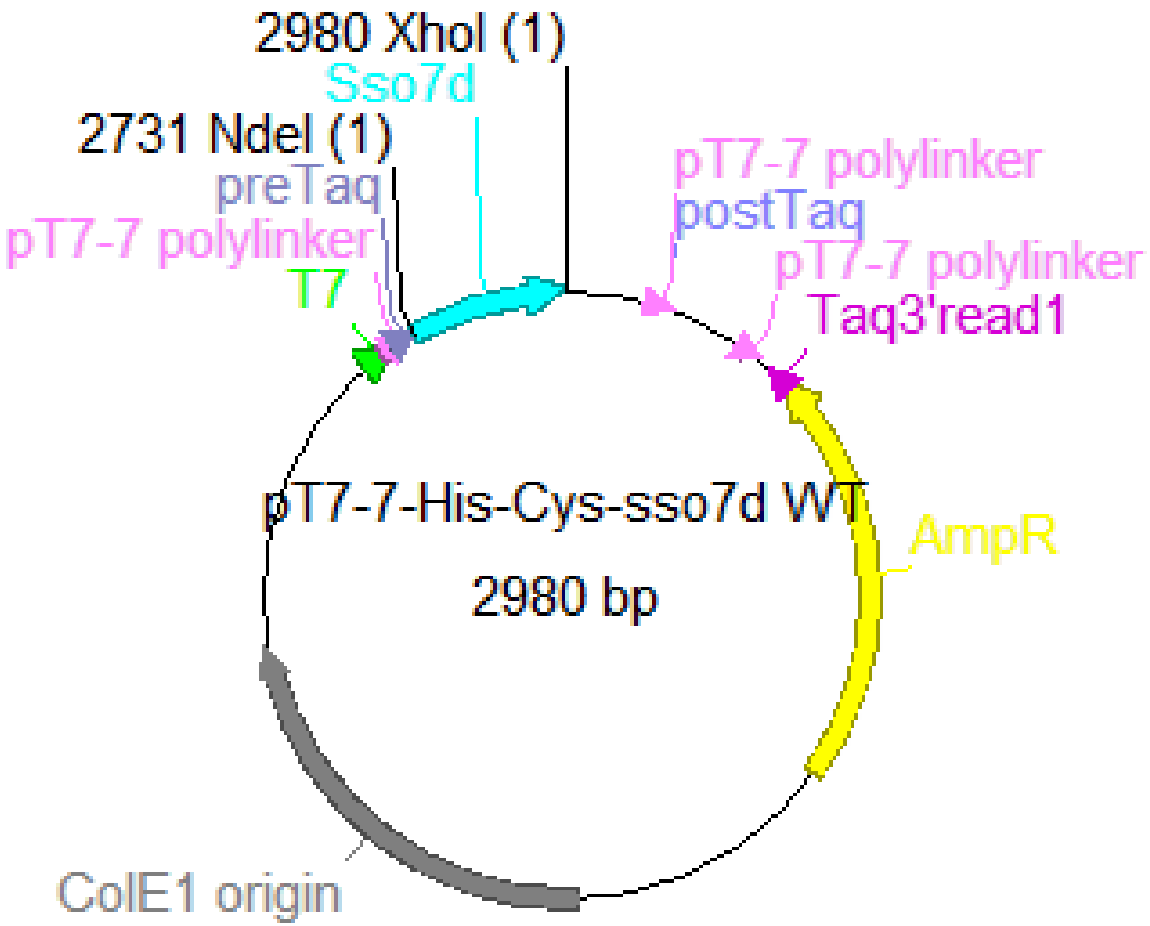


Figure A.2. Vector assembly of Sso7d in pT7-7 plasmid.

So, the Sso7d gene assembly was repeated with His and Cys tags incorporated on the N-terminus of the protein. In this case, the ligation was carried out in two steps: first, the oligonucleotides for the gene itself and appropriate scaffolds were annealed (95℃ / 2 min - 72℃ / 1 min - 60℃ / 1 min - 37℃ / 1 min – 20℃ / 1 min) and ligated for 40 min at 15 ℃, then the excessive amount of Cys/His-tag oligonucleotide and its scaffold were added to the mixture and the ligation was repeated. This was done due to suspected heterodimer formation between Sso7d 3 (Sso7d E35L) and Cys/His tag oligonucleotides. The ligation product was PCR amplified with the primers that contained NdeI and XhoI restriction sites, purified from gel as described above, and ligated into pT7 expression vector (Figure A.2). After sanger sequencing of the clones (propagated in DH5α cells), the Wild type Sso7dW, and mutant Sso7dE35L clones with correct open reading frame (ORF) were cloned in *E. coli* Rosetta-Gami B(DE3). Stock cultures of Sso7dW-RG and Sso7dE35L-RG were kept frozen at -80℃.

The protein expression and extraction were carried out as follows:

Stock cultures of Sso7dW-RG and Sso7dE35L-RG in Rosetta-Gami were inoculated into 50 mL LB + 100 µg/mL Ampicillin + 13 µg/mL Chloramphenicol and incubated at 37℃ and 250 rpm for 7 hours. Then stock cultures were inoculated into 2 L of LB with antibiotics (in 4 L flasks) and incubated at 37℃ and 250 rpm. When the OD_{600} reached ~0.3, the cultures were induced with 1 mM IPTG and incubated overnight at room temperature and 230 rpm.

To harvest the cells, the overnight cultures were centrifuged at 4,000 RCF for 15 min. Pellets were resuspended in 10 mM Tris-HCl pH 9.2 buffer, and after all the cells were

resuspended well, Tween 20 was added to the final concentration 0.1%. The suspensions in 50 mL aliquots were kept frozen at -20℃ until needed.

Frozen cell suspensions were thawed in 35℃ water bath and 0.1 M PMSF stock in isopropanol (a serine protease inhibitor) was added before sonication to a final concentration of 0.1 mM. Then the cell suspensions were sonicated on ice (using Misonix XL2020) for 1 min at 50% power output.

DNAse I (Bovine, Sigma Aldrich) was added to a final concentration of 10 µg/mL along with 5 mM $MgCl_2$ and 0.5 mM $Ca\ Cl_2$. Samples were incubated on ice for 1 hour and then NaCl was added to a final concentration of 0.3 M. Lysates were centrifuged at 10,000 RCF for 20 min. The supernatant extracts were stored at -20℃ until needed.

The his-tagged protein was purified with affinity chromatography (His-trap) as follows:

To a 45 mL aliquot of the cell extract, 2.7 mL of 5 M NaCl was added to bring salt concentration to 0.6 M (as 0.3 M was already there). Then 2 M Imidazole stock was added to a final concentration of 10 mM and 100 µL of 1 M DTT (a redox agent) was added as well.

The prepared extract was loaded onto the equilibrated Ni-NTA column and let go through at 4℃. Then the column was washed with 25 volumes of 25 mM Imidazole and a second time with 50 mM Imidazole in PBS. The protein was eluted with 250 mM

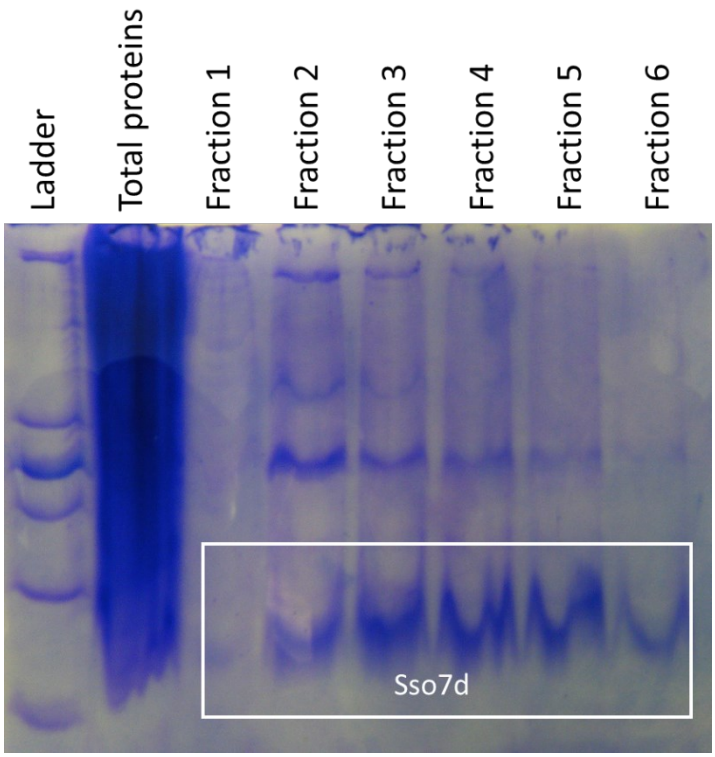


Figure A.3. Fractions of Ni-NTA purified his-tagged protein Sso7d.

Imidazole in PBS into 1 mL fractions. Each fraction was tested for protein presence (200 µL of Bradford solution + 10 µL of eluate).

Protein-positive samples were visualized in 16% Tris-Tricine gel (Figure A.3):

Resolving gel: 30% acrylamide – 5.4 mL; 3x gel buffer (3M Tris-HCl pH 8.45 + 0.3% SDS) – 3.3 mL; H_2O – 1.3 mL; 10% APS – 50 µL; and 10% TEMED in H_2O – 50 µL.

4% stacking gel (Laemli): 30% acrylamide – 750 µL, 1M Tris-HCl pH 6.8 – 625 µL, 10% SDS – 50 µL, H_2O – 3.575 µL. 10% APS – 25 µL; and 10% TEMED in H_2O – 25 µL.

Anode buffer: 0.2 M Tris pH 8.9

Cathode buffer: 0.1 Tris pH 8.25; 0.1 M Tricine; 0.1% SDS.

For gel loading 8 µL of the sample was mixed with 2 µL of the DTT loading buffer (1 M DTT; 2% SDS; 40% Glycerol; 0.5% Bromphenol Blue) and heated at 94℃ for 4 min. The electrophoresis was carried out at 60 mAmp for 120 min.

After the electrophoresis, the gels were rinsed in gel rinse buffer (5% acetic acid, 10% EtOH in H_2O) to remove SDS and stained overnight in the Coumassie: 0.3% Coumassie R250; 50% methanol; 10% acetic acid; 40% MQ water.

The resulting gels showed more than one band. Therefore, the samples required additional fractionation, as well as removal of excessive NaCl.

Gel purification in Sephadex G-50 resin.

Columns, made of 10 mL serological pipets, were filled with 12 mL of 50% Sephadex G-50 resin suspended in 10 mM sodium phosphate + 150 mM NaCl, pH 7.4. The Ni-NTA elution fractions with the purified protein were pooled together and let evaporate from ~4 mL to ~1 mL. Then the sample was loaded onto the column and washed/eluted with

the above buffer. First 4 mL of the flow-through were discarded, then 0.5 mL fractions collected and tested with Bradford reagent. Positive fractions were analyzed in Tris Tricine gel (20 µL sample + 4 µL of DTT loading buffer) (Figure A.4).

The electrophoretic mobility shift assay.

Nucleic acid and DNA-binding protein complex has decreased electrophoretic mobility compared to free NA. The fractions of pure Sso7d protein were mixed with dsDNA and analyzed with agarose gel electrophoresis, which confirmed high DNA-binding activity of both variants of the protein (Figure A.5).

Originally, Sso7d was gel-purified with PBS at pH7.4 and attempted fluorescent Cys-tag labeling with Atto 647 maleimide did not work. But in PBS pH 6.4 the protein was labeled successfully without any loss in its activity (Figure A.6).

Unfortunately, the Sso7dE35L mutant still demonstrated RNAse activity when incubated for 30 min at 44℃ with RNA, even in the buffer with no Ca^{2+} or Mg^{2+} and with EDTA added. On the other hand, Sso7dE35L-RG produces roughly 5 times more recombinant protein than the wild type.

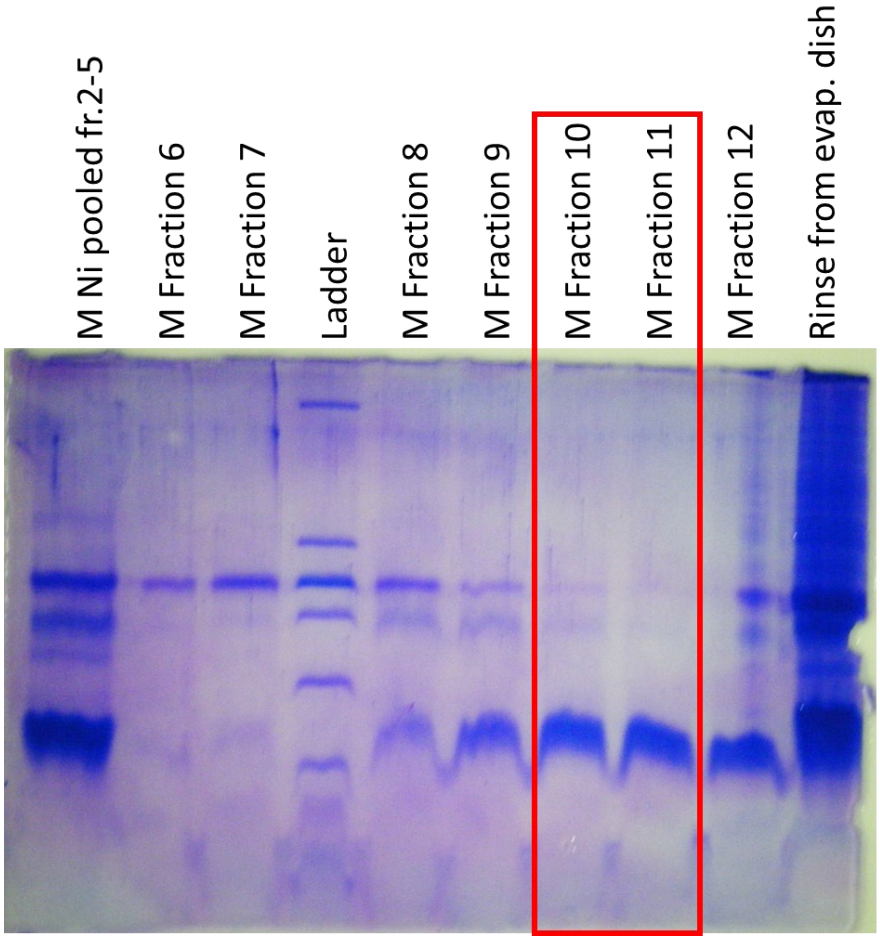


Figure A.4. Fractions of Sephadex G50-purified recombinant protein Sso7d.

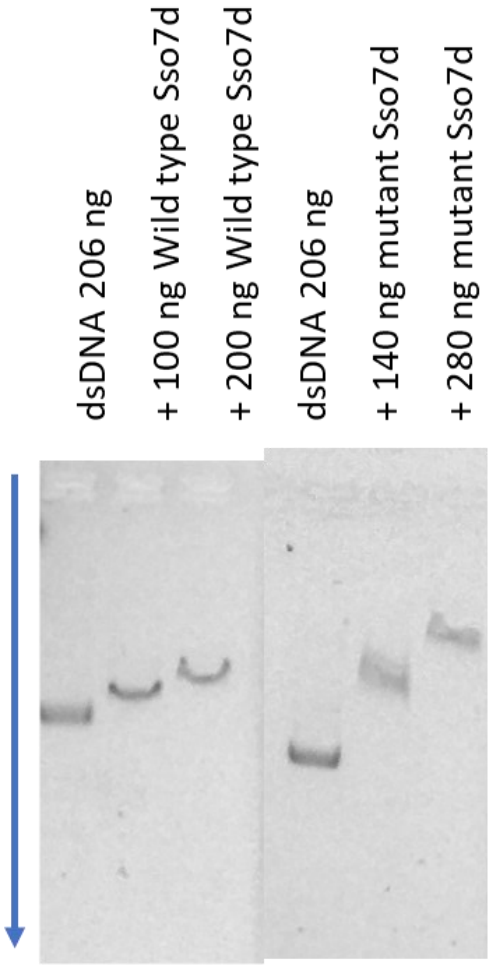


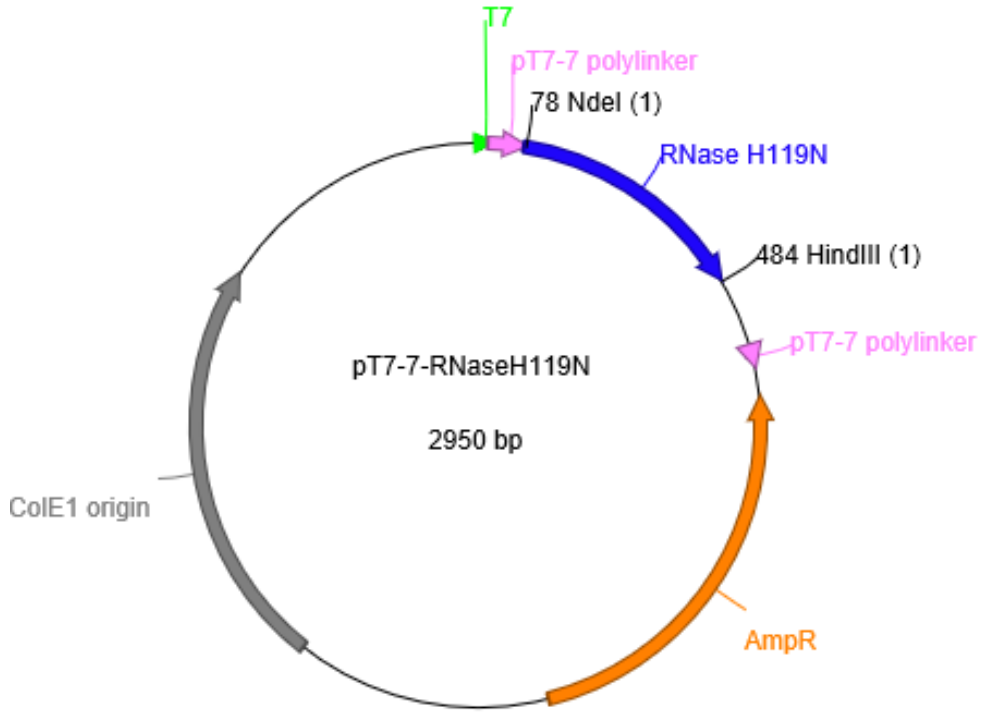
Figure A.5. Electrophoregram of pUC19 dsDNA. Added Sso7d DNA protein binds to the DNA and retards its movement.

Expression and purification of RNA-binding protein RNAse A H119N mutant and RNA labeling.

For RNA labeling I chose an RNAse A H119N mutant protein which has no RNAse activity but still keeps high substrate affinity (Panov *et al*., 1996). The custom-synthesized gene with two-point mutations, one - to replace Histidine with Asparagine, and the other one – to eliminate a NdeI restriction site inside the ORF, was ordered from IDT and cloned into *E. coli* Rosetta-Gami in pT7-7 vector through NdeI and XhoI restriction sites using the same protocol as for Sso7d (Figure A.7). The incubation, expression, and purification protocols for RNAse A H119N were like those for Ss07d as well, except for DNAse I treatment step that was omitted. The electrophoretic mobility shift assay demonstrated the affinity of the RNAse A H119N to both DNA and RNA and a lack of RNA cleavage. (Figure A.8)

Figure A.6. Sso7d protein, labeled with Atto 647, keeps its DNA-binding activity.

dsDNA (pUC19) – 200 ng per well; Sso7d – 270 ng/µL.



ctctctctcatATGCATCATCACCATCACCACGGCGGTAAAGAAACTGCGGCCGCAAAATTTGAACGTCAGCA**C**ATGGACAGTTCCACGTCCGCC
GCTTCTTCTTCGAATTATTGTAATCAAATGATGAAGTCTAGAAACCTCACCAAGGACCGTTGCAAGCCCGTTAACACTTTTGTGCACGAATCCT
TAGCGGATGTGCAAGCCGTTTGCAGCCAAAAAAACGTTGCATGCAAGAATGGCCAAACAAACTGTTACCAATCGTACTCAACTATGTCGATCA
CAGACTGCAGGGAGACTGGAAGCTCAAAATATCCAAACTGCGCATATAAAACTACCCAGGCAAACAAACACATCATCGTCGCGTGTGAAGGT
AACCCCTATGTCCCGGTTAACTTTGACGCATCTGTCTAATAAgcttctctctc

Figure A.7. Vector assembly of RNAse A H119N with his-tag on N terminus in pT7-7 plasmid.

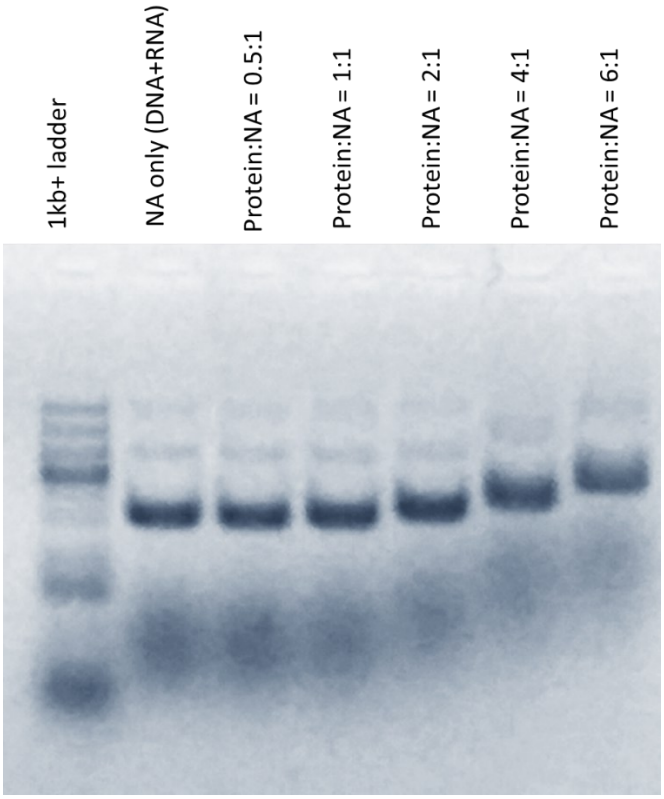


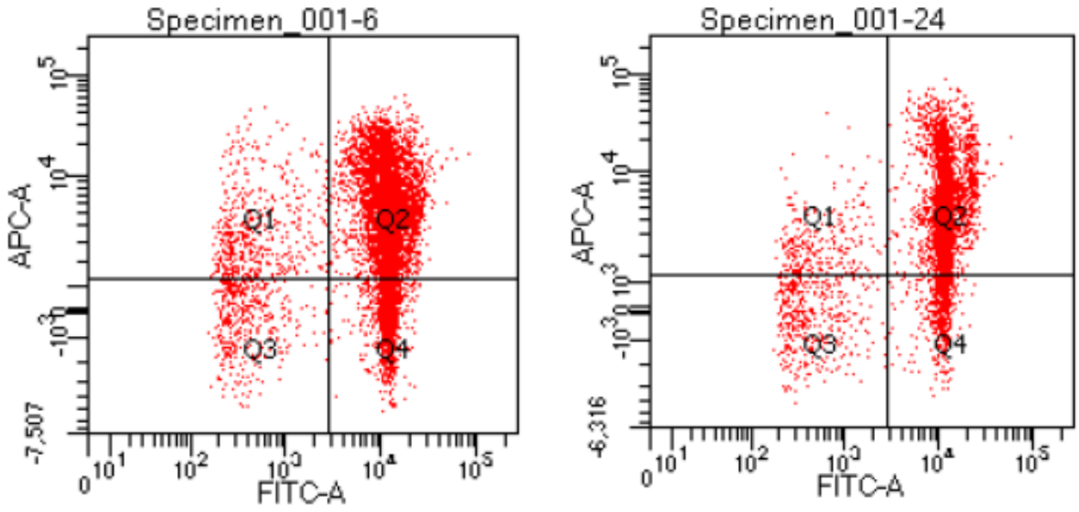
Figure A.8. RNAse A H119N binds to both DNA and RNA and retards their electrophoretic movement. No RNA degradation is observed.

The recombinant protein fluorescent labeling and flow cytometric detection of viruses stained with nucleic acid-binding protein.

The Sso7d protein was labeled with Cys-specific Atto 647 maleimide reagent (Sigma Aldrich) according to the manufacturer's instructions, except for the pH of the PBS buffer was decreased from 7.4 to 6.4 to protonate amino groups in the active site of the protein and prevent non-specific binding of the dye. Then the stained protein was dialysed against 10 mM sodium phosphate + 150 mM NaCl, pH 6.4 in a 3.5 kDa cut off membrane cassette (Thermofisher #2160728).

Bacteriophage λ, stained at 80℃ with Atto-labelled Sso7d, has not yielded any distinct and resolvable FCM signal still (Figure A.9).

In conclusion, the fluorescently labelled recombinant DNA-binding protein generates artificial fluorescent signal when analyzed by flow cytometry and is no better than SYBR stains used.



PBS+SYBR+stained Sso7dM

λ +SYBR+stained Sso7dM

Figure A.9. Use of fluorescently labeled DNA-binding protein creates artificial signal and does not improve bacteriophage λ resolution.

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