Human enteric virus removal from wastewater: Design and operational monitoring of multiple-barrier system and virus removal efficiency improvement using specific interaction

Submitted by

Kalahe Panditha Koralage Mohan Amarasiri

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Examination committee

Supervisor: Professor Satoshi Okabe (Hokkaido University)

Member: Associate Professor Katsuki Kimura (Hokkaido University)

Member: Associate Professor Daisuke Sano (Tohoku University)

Water Quality Control Engineering Laboratory

Division of Environmental Engineering

Graduate School of Engineering, Hokkaido University

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ABSTRACT

Municipal wastewater is considered as a valuable resource to obtain reclaimed wastewater for non-potable reuses like agricultural irrigation, recreational activities and aquifer recharge. However it contains many pathogens including enteric viruses and therefore treatment is necessary before it is being used for another beneficial purpose. Even though solid removal techniques such as filtration, coagulation, flocculation and sedimentation, and disinfection techniques such as UV irradiation and ozonation are regarded as effective for reducing viruses in wastewater and producing virologically safe reclaimed water, virus removal efficiency for these processes are heavily dependent on operation conditions, such that efficiencies can vary greatly even within the same reactor for a given treatment. Under the concept of multiple-barrier system, which is a global concept for the management of microbial risks in wastewater reclamation and reuse, it is required for water engineers to know the "average" virus removal efficiency of each wastewater treatment unit as a log reduction value (LRV). We employed a meta-analysis approach to achieve this objective and obtained a 3.35 $\log_{10}$ [95% CI between 2.39 and 4.30] for human norovirus GII by MBR process, while the enterovirus LRV by MBR was 2.71 $\log_{10}$ [1.52, 3.89]. In the activated sludge process, LRV of rotavirus, norovirus GI and GII were 0.87 $\log_{10}$ [0.20, 1.53], 1.48 $\log_{10}$ [0.96, 2.00] and 1.35 $\log_{10}$ [0.52, 2.18], respectively.

During the operation of wastewater reclamation systems, there is a necessity to monitor the plant performance to make sure the particular plant provides recycled water of expected quality as stipulated by guidelines. Bacteriophages have been the most widely used microbial indicators for the validation and operational monitoring stages with respect to virus reduction efficiency in wastewater treatment processes. Selection of bacteriophage removal as an indicator for the human enteric virus is supported by less time, cost and labor involved in obtaining results, while the inability to always obtain a clear correlation between LRVs of bacteriophages and human enteric virus is reported. Even though there is no strong correlation between LR values of bacteriophages and human viruses in wastewater treatment unit processes, bacteriophage may be used as an indicator for human viruses given that bacteriophage LRVs are almost always lower than those of human viruses, under the concept of multiple-barrier system. Therefore, we evaluated the suitability of bacteriophages as a tool to monitor and validate the performance of human enteric virus reduction at wastewater treatment plants, based on a meta-analysis of published bacteriophage LRVs. The calculated LRVs of bacteriophages were then compared with reported human enteric virus LRVs. MS2 coliphage LRV in MBR processes were shown to be lower than those of norovirus GII and enterovirus, suggesting it as a possible validation and operational monitoring tool. The other bacteriophages provided higher LR values compared to human viruses. The data sets on LRVs of human viruses and bacteriophages are scarce except for MBR and conventional activated sludge processes, which highlights the necessity of investigating LRVs in multiple treatment unit processes.

During the wastewater treatment process, the interaction between enteric virues and wastewater solids plays a major role in virus removal from all types of treatment unit processes. However, previous studies have not paid attention on the contribution of specific interaction of enteric viruses with wastewater solids to the virus removal from wastewater. Therefore, we focused on the specific binding of human enteric viruses and how these interactions can affect the removal of viruses in wastewater. For the experimental study, *Enterobacter cloacae* SENG-6 bearing histo-blood group antigen (HBGA)-like substances in extracellular polymeric substance (EPS) and human norovirus like particles (NoVLPs) of GII.3, GII.4 and GII.6 were used because HBGA can specifically interact with human noroviruses. To analyze the effect of the location of HBGA-like substances on virus removal and survival, *Escherichia coli* O86:K61:B7 which bears HBGAs in LPS was also used. *Staphylococcus epidermidis* was used as an HBGA negative strain. Using a 0.45μm nominal pore size microfiltration membrane, GII.3, GII.4 and GII.6 NoVLPs were filtered in the presence of each bacterial strain. All NoVLP genotypes were rejected by the MF membrane in the presence of *Enterobacter cloacae* SENG-6, which excreted HBGA-like substances in EPS. This MF membrane removal of NoVLPs was not significant when EPS was removed from cells of *Enterobacter cloacae* SENG-6. GII.6 NoVLP was not rejected with the MF membrane in the presence of *E. coli* O86:K61:B7, but the removal of EPS of *E. coli* O86:K61:B7 increased the removal efficiency due to the interaction of NoVLPs with the exposed B-antigen in lipopolysaccharide (LPS) of *E. coli* O86:K61:B7. No MF membrane removal of all three genotypes was observed when *S. epidermidis* was mixed and filtered with NoVLPs. These results demonstrate that the location of HBGAs on bacterial cells is an important factor in determining the genotype-dependent removal efficiency of norovirus particles with the MF membrane. The presence of HBGAs in mixed liquor suspended solids from a membrane bioreactor (MBR) pilot plant was confirmed by immune-transmission electron microscopy, which implies that bacterial HBGA-like substances can contribute to the genotype-dependent removal of human noroviruses with MBR using MF membrane.

To evaluate the importance of specific interaction of human enteric viruses with wastewater solids on the virus removal in a larger scale, we performed the same filtration experiment with a bench scale cross-flow membrane system using a 0.2μm nominal pore size PTFE membrane. Instead of human norovirus, human rotavirus HAL1166 strain which is reported to interact with A-type HBGA was used with *Enterobacter cloacae* SENG-6. Proteolytic cleavage of rotavirus VP4 protein was done to generate VP8* which is responsible to recognize HBGA. In the presence of HBGA-positive bacteria, trypsin-treated rotavirus HAL1166 concentration reduced with time (R^2>0.75, all 3 trials) while the non-trypsin treated rotavirus HAL1166 concentration reduction had a lower correlation (R^2<0.5, all 3 trials). Specific interactions between the HBGA-positive bacteria and trypsin-treated rotavirus HAL1166 have shown to be responsible for lower effluent concentration of rotavirus HAL1166. A strong correlation was obtained for the log reduction of trypsin treated rotavirus HAL1166 with the bacterial deposition on the membrane (Pearson's r = 0.60) explaining the

effect of the interaction between HBGA-like substances and trypsin treated rotavirus HAL1166 on the virus removal. A further analysis on the environmental factors which can lead to improved specific interactions can contribute to the higher human enteric virus removal and provide a better understanding on the life cycle of human enteric virus associated with wastewater solids.

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ABBREVIATIONS

DALY: disability-adjusted life years

ELISA: enzyme-linked immunosorbent assay

EPS: extracellular polymeric substance

HBGA: histo-blood group antigen

HuNoV: human norovirus

IgG: immunoglobulin G

IgM: immunoglobulin M

LB-EPS: loosely-bound extracellular polymeric substances

LPS: lipopolysaccharide

MF: microfiltration

MBR: membrane bioreactor

MLSS: mixed liquor suspended solids

NoVLP: norovirus-like particle

SMP: soluble microbial product

TB-EPS: tightly-bound extracellular polymeric substance

TEM: transmission electron microscopy

ATSE: Australian Academy of Technological Sciences and Engineering

CAS: conventional activated sludge process

CI: confidence interval

df: degree of freedom

DO: dissolved oxygen

E. coli: *Escherichia coli*

GRRP: Groundwater Replenishment Reuse Project

HRT: hydraulic retention time

LRV: $\log_{10}$ reduction value

MBR: membrane bioreactor

MF: microfiltration

MLSS: mixed-liquor suspended solids

NTU: nephelometric turbidity unit

PFU: plaque forming unit

SRT: solids retention time

SD: standard deviation

TMP: transmembrane pressure

UF: ultrafiltration

USEPA: United States Environmental Protection Agency

WHO: World Health Organization

WWTP: Wastewater treatment plant

Chapter 1 : INTRODUCTION

1.1 RESEARCH BACKGROUNDS

Even though 71% of earth surface is covered by water, 96.5% of the total water volume is located in oceans and therefore not suitable for human consumption. More than half of the fresh water is located in glaciers and ice caps making it also unusable for humans. Seven billion people rely on the remaining groundwater and freshwater resources for their domestic, agricultural, industrial and many other needs which make fresh water a very valuable resource. However, about 663 million people do not have accesses to proper drinking water while 2.4 billion people still lack improved sanitation facilities (1). Consumption of improperly treated drinking water and poor hygienic conditions can lead to disease outbreaks and deaths of many people including young children. According to the UNICEF, 16% of post neonatal deaths and 9% of < 5 years old deaths in 2015 are accounted for diarrhoea. These numbers emphasize the importance of clean drinking water and hygiene practices on the health. However, global water sources are unevenly distributed and the quantities per capita are not increasing as the population increases. Therefore, to preserve the water resources necessary for potable use, use of reclaimed wastewater for non-potable uses is suggested as a sustainable solution.

However, wastewater contains many harmful microorganisms. In the case of pathogenic viruses, more than 100 human viruses including norovirus, rotavirus and enterovirus excreted with human faeces and can be transmitted via water (2). Exposure to these viruses can cause diseases like diarrhoea, vomiting, hepatitis and so on and a number of large scale outbreaks have been reported in the recent years (3–5). Virus removal methods like filtration, coagulation, flocculation, sedimentation, and disinfection are widely used in wastewater treatment. By combining above methods which is called as the multiple-barrier system, the required virus log removal performance targets are obtained in wastewater treatment plants (6). However, the studies have shown that even for the same virus, log reduction value (LRV) by a particular unit process can vary depending on the operational conditions (7, 8). Therefore, it is difficult for wastewater treatment plant design engineers to select a specific value as the reference value of a specific virus LRV by a particular unit process. It is a necessity to develop a methodology to calculate a reference LRV for a specific virus by a particular unit process.

Operational monitoring of human enteric virus removal by a wastewater treatment plant is a must to ensure the designed water reclamation system is functioning properly and provide reclaimed water as stipulated by the guidelines. However, due to the cumbersome procedures involving higher costs, labour and time, routine evaluation of virus LRV by a treatment process chain is rare (9). Instead, evaluation of indicator log removal like bacteriophages is preferred (10, 11). There are many studies recommending different bacteriophages as suitable indicators for the evaluation of human

enteric virus log reduction. However, similar to the viruses, bacteriophage LRVs also vary depending on the unit process, operational conditions and bacteriophage characteristics (12–16). A systematic study which shows the log removal performances of different bacteriophages by different treatment unit processes and the suitability of bacteriophages as operational monitoring indicators is therefore necessary.

The successful removal of human enteric viruses from wastewater depends upon the virus removal mechanisms of different unit processes. It can be observed that, the adsorption to solids plays a major role in virus removal from all types of treatment unit processes. Gerba et al. (1978) reported that the percentage of animal enteric viruses associated with solids in the treated sewage effluents varied from 3-100% (17). Norovirus GI and GII were attached to large settleable particles (>180µm), smaller suspended particles (>0.45µm) and colloidal particles in a waste stabilization pond (18). Rotavirus was detected from suspended solids and estuarine sediments and the attached quantities were different depending on the solids (19). The study by Miura et al. (2015) described enteroviruses are less associated with activated sludge than calicivirus (7). In contrast, interactions between wastewater solids and enteric viruses are reported to be responsible for the survival of human enteric viruses from inactivation. According to the studies by Winward et al. (2008), microorganisms associated with wastewater solids are more resistant to chlorine than the free microorganisms (20). Based on the observations by Hejkal et al. (1979), compared to free or secondarily adsorbed poliovirus, fourfold increase of combined chlorine was necessary to achieve the same degree of inactivation in faecal particle associated or occluded poliovirus (21). However, none of the above mentioned studies had focused on the contribution of specific interaction of human enteric viruses with wastewater solids on the removal and survival of human enteric viruses. Therefore, in this study, the contribution of specific interaction of human enteric viruses with wastewater solids to the virus removal and survival in wastewater has been analyzed since it is possible to expect the life cycle of human enteric viruses which are attached to wastewater solids can be different from freely moving virus particles.

1.2 OBJECTIVES

- Calculate the reference LRV for a specific virus by a specific unit process using meta-analysis approach.
- Evaluation of the suitability of bacteriophages as a validation and operational monitoring tool of human enteric virus reduction at wastewater treatment plants.
- Identification of the effects of specific human enteric virus – wastewater solids interactions on the removal of human enteric viruses in wastewater treatment processes using model microorganisms.

1.3 STRUCTURE OF THE DISSERTATION

1.3.1 Chapter 1

Chapter one summarizes the broad background and the research questions addressed in this study. A brief summary of each chapter contents is provided.

1.3.2 Chapter 2

Chapter two provides the basic background information on human enteric viruses present in wastewater including the treatment unit processes and removal mechanisms. Presence of human enteric viruses in wastewater effluent is briefly discussed and the importance of designing of multiple-barrier systems to achieve the stipulated log reductions by guidelines is emphasized. Suitability of bacteriophages as indicators for operational monitoring of virus removal by wastewater treatment plants is highlighted with the current knowledge which lacks agreement on a particular bacteriophage. Importance of human enteric virus – wastewater solids interactions on virus removal and survival is discussed and the lack of information on the specific human enteric virus – wastewater solids interactions and their effects on virus life cycle is described.

1.3.3 Chapter 3

As explained in the literature review, currently there are no information on the average log reduction values obtainable by different wastewater treatment unit processes for specific viruses. However, water treatment plant design engineers need virus removal performance details of different treatment unit processes for the successful design of multiple-barrier systems. A systematic review and a meta-analysis was performed to evaluate the average log reduction value of human norovirus GI, GII, enterovirus and human rotavirus obtainable by MBR and CAS processes.

1.3.4 Chapter 4

Human enteric virus removal performance evaluation using the real virus log reduction requires lot of time, money and labour. However, operational monitoring is a must for any wastewater treatment plant and many studies have recommended different bacteriophages as suitable indicators for virus log reduction evaluation. However, other studies have reported the inability to obtain correlations between the human enteric virus log removal and bacteriophage log removal as the biggest drawback of using bacteriophages as operational monitoring indicators. A systematic review and meta-analysis is performed to evaluate the average log reduction of somatic coliphages, F-specific phages, MS2 coliphage and T4 phage log reduction by MBR, CAS, constructed wetlands, pond systems, MF and UF. Obtained average bacteriophages LRVs are compared with the previously calculated human enteric virus log reduction values and concluded that MS2 coliphage is a suitable operational monitoring indicator to evaluate human enteric virus log reduction by MBR systems.

1.3.5 Chapter 5

To elucidate the importance of specific interaction of human enteric viruses with wastewater solids on the removal of human enteric virus, a microfiltration study was performed using HBGA-positive *Enterobacter cloacae* SENG-6 and human norovirus as model organisms. Genotype dependent removal of human noroviruses was observed in the presence of HBGA-like substances of *Enterobacter cloacae* SENG-6 whereas the removal of bacterial EPS which includes HBGA-like substances resulted in the virus removal efficiency. Usage of *E. coli* O86 which has HBGA-like substances in the LPS didn't contribute to the removal of human norovirus until the surrounding EPS layer is removed. The results demonstrated that the location of HBGAs on bacterial cells is an important factor in determining the genotype-dependent removal efficiency of norovirus particles with the MF membrane.

1.3.6 Chapter 6

A study is conducted to evaluate the importance of specific interaction of human enteric viruses with wastewater solids on the removal human enteric virus by a cross-flow membrane system using *Enterobacter cloacae* SENG-6 and rotavirus HAL1166 as model microorganisms. In the presence of HBGA-positive bacteria, trypsin-treated rotavirus HAL1166 concentration reduced with time while the non-trypsin treated rotavirus HAL1166 concentration reduction had a lower correlation. There was a correlation between the deposition of HBGA-positive bacteria in the membrane and the virus log reduction.

1.3.7 Chapter 7

This chapter summarizes the current study conclusions and future directions for further research.

Chapter 2 : LITERATURE REVIEW

2.1 WATER REUSE

Limitation of fresh water resources and increasing world population has caused the water scarcity in most of the geographic areas. It is estimated that two thirds of the world's population currently lives in areas that experience water scarcity at least one month. Population of 500 million lives in areas where water consumption exceeds the locally renewable water sources by a factor of two (22). Usage of reclaimed water and desalination are considered as major alternatives to overcome the water scarcity (23). However, desalination is not practiced all over the world due to the cost concerns and non-accessibility to the sea water (24). Moreover, the desalinated water is mainly used for industrial and human needs (24). Therefore, other water reuse categories like agricultural irrigation, landscape irrigation, urban non-irrigation uses, environmental and recreational, groundwater recharge, indirect and direct potable reuse depends on reclaimed wastewater (25). **Figure 2.1** displays the global water reuse after tertiary treatment by application (26). According to current estimates, 70% of the generated municipal and industrial wastewater is treated in high-income countries, with reducing percentages of 38% and 28% in upper and lower-middle income countries while only 8% of the wastewater undergoes any kind of treatment in low-income countries. Globally, 80% of the wastewater is discharged without any treatment explains the huge potential of wastewater as an important source of alleviating the water scarcity (22).

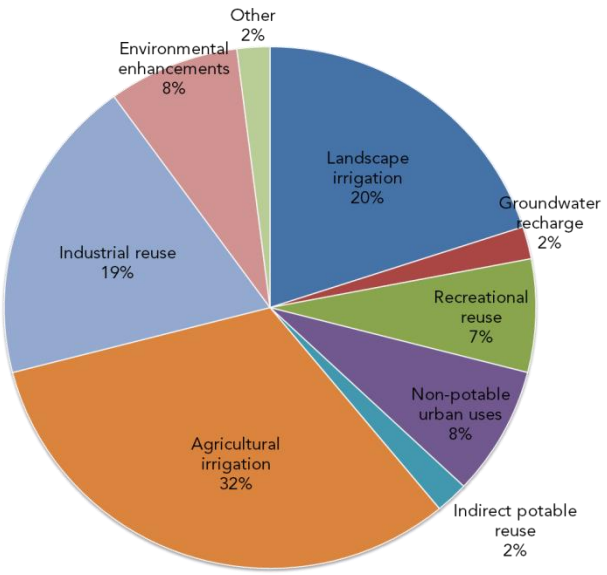

Figure 2.1: Major water reuse categories and the percentage of reclaimed water used in each category (26)

2.2 VIRUSES IN WASTEWATER

More than 150 types of human enteric viruses are excreted with faeces and suggested to be present in contaminated water (27) and the concentration of human enteric viruses in contaminated

waters can be as high as 10^3-10^5 copies/ml (28). Currently, adenoviruses, enteroviruses, coxsackieviruses, echoviruses, hepatitis A viruses and caliciviruses are reported in the EPAs contaminant candidate list 4 (2016). Exposure to human enteric viruses can cause diseases like gastroenteritis, vomiting, hepatitis, muscle pains and cardiomyopathy (3) and it can be fatal for sensitive populations like children, elders and immune-compromised patients (27). In the following section, human norovirus which is the major cause of non-bacterial acute gastroenteritis and human rotavirus which is responsible for the gastroenteritis among children < 5 year old are briefly described (29, 30).

2.2.1 Norovirus

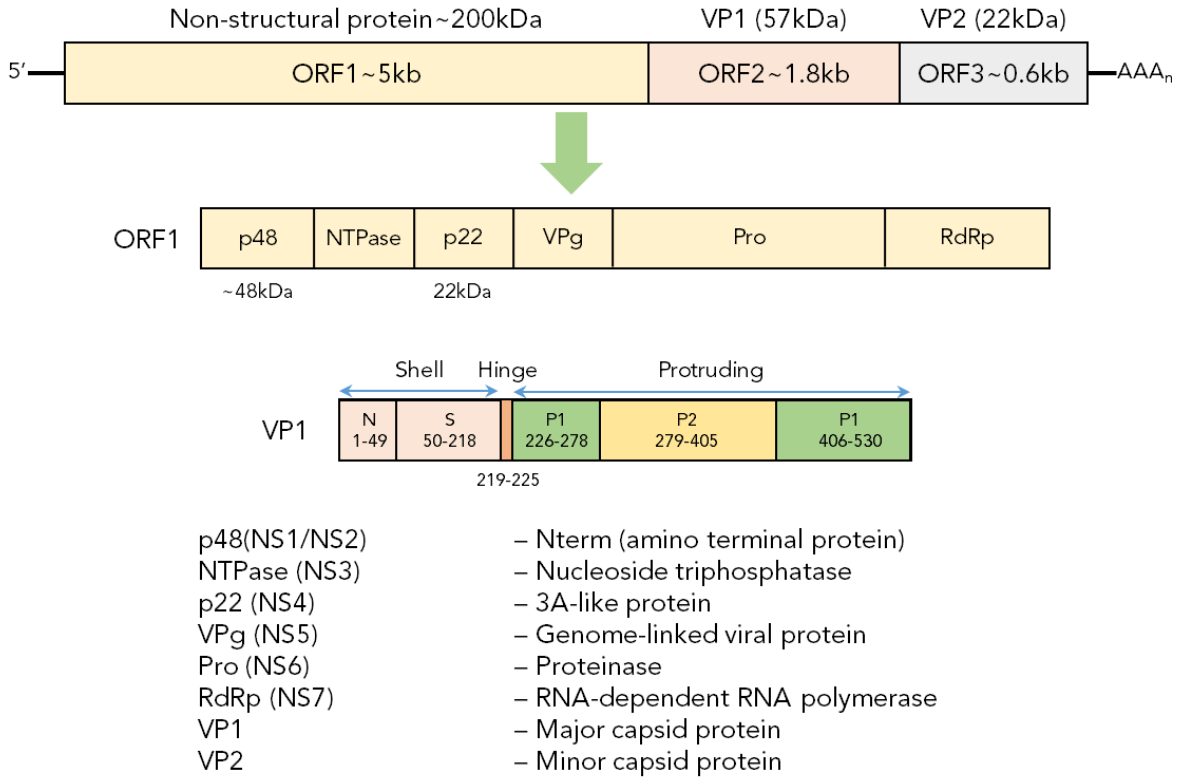

Figure 2.2: The norovirus genome. Reconstructed from references (31–33).

Norovirus (NoV) is a small (27-40nm), icosahedral, non-enveloped, positive-sense, single-stranded RNA (ssRNA) virus belongs to family *Caliciviridae* (34, 35). Norovirus genome consists of 3 open reading frames (ORFs) **(Figure 2.2)**. Proteolytic cleavage of large polyprotein encoded in ORF 1 mediated by Pro (NS6) generates non-structural proteins which are involved in virus replication (36). Among the non-structural proteins, functions of p48 and p22 are still unknown (32). VPg is covalently connected to the 5' end of the genome. ORF2 and ORF3 contains the major and minor capsid proteins (31). The VP1 (~60,000 D) is present in 180 copies (90 dimers) per virion. It is divided in to conserved shell (S) domain and protruding (P) domain which is more variable (37). P

and S domains are linked by a flexible hinge and P domain is further divided in to P1 and P2 sub domains where P2 hypervariable domains is critical in determining the antigenic phenotype of the virus and host-cell interactions (36). Two to three copies of the VP2 (12,000-29,000 D) are present per virion and the function is largely unknown (32). Norovirus exhibits the T=3 icosahedral symmetry and the capsid contains 90 dimers of VP1 that form a shell (32).

Noroviruses are categorized in to 6 major genogroups (GI-GVI) with a proposed seventh genogroup based on amino acid sequence diversity in the complete VP1 capsid protein (36) and more than 40 capsid genotypes are recognized (38). GI, GII and GIV consist of human viruses with some GII strains are exclusively reported from porcine samples and some GIV strains are reported in feline/ canine samples (39). GIII represents bovine norovirus samples and GV represents murine norovirus. GVI and GVII genogroups consist of canine noroviruses (40). In addition, noroviruses have been detected from sheep, sea lions, rodents, bats and harbour porpoises (41).

Human norovirus is reported to be responsible for more than 90% of non-bacterial epidemic gastroenteritis cases and attributable to 18% of acute gastroenteritis patients worldwide (42, 43). A recent meta-analysis on the gastroenteritis reported that norovirus is associated with 1 out of 6 acute diarrhea hospitalizations among the children younger than 5 years old in Latin America (44). Community settings like military camps, cruise ships, hospitals, universities and retirement homes are more vulnerable to norovirus outbreaks (31, 45, 46). Spreading of human norovirus can be from direct person to person contact (47), contaminated food, water or wastewater (48). Norovirus has a very low infectious dose (around 18-1000 particles) (49), a very high attack rate and environmental stability (50). A recent systematic review reported that wastewater influents contain overall mean norovirus density of $4.9\log_{10}$ genome copies/litre for GII and $4.\ 4\log_{10}$ genome copies/litre for GI norovirus which is significantly higher than the infectious dose (51). Direct health costs of $4.2 billion (95% UI: $3.2-5.7 billion) and societal costs of $60.3 billion (95% UI: $44.4-83.4 billion) per year is estimated to be due to the norovirus worldwide (52). Even though there are number of studies on the development of a norovirus vaccine (53), the studies are hampered by the rapid evolution and emergence of novel norovirus strains (54).

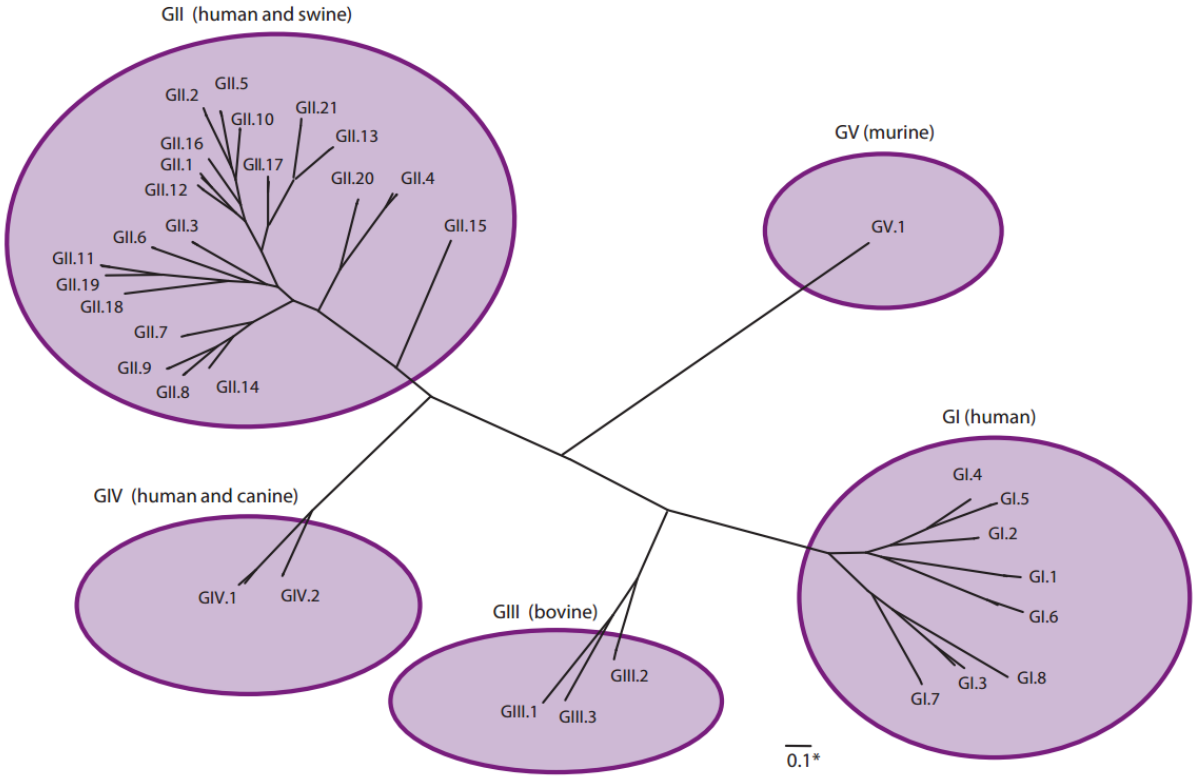

Figure 2.3: Norovirus classification into genogroups. The scale bar reflects the number of amino acid substitutions per site (55).

2.2.2 Rotavirus

Rotaviruses belong to the genus *Rotavirus* within *Reoviridae* family. It is a double-stranded RNA (ds-RNA) virus which possesses a triple-layer icosahedral capsid and about 100nm in size including the spikes (56). The single layered core is formed by 120 molecules of the VP2, arranged as 60 dimers in T=1 symmetry. The core is surrounded by 260 trimers of VP6 and the outer layer contains 260 trimers of VP7 and 60 spikes of VP4 trimers to form the triple layered particle (TLP). Rotavirus genome consists of 11 segments of dsRNA contained within the core capsid (56). Each segment codes for one protein except for the segment 11 which codes two proteins (57). Virus protein 1 (VP1) is the RNA-dependent RNA polymerase needed for ss-RNA binding. VP2 is the core protein required for the replicase activity of VP1. VP4 is protease sensitive and cleavage will generate VP5 and VP8* which is responsible for virus attachment and VP7 is the G-type neutralization antigen (56).

Rotavirus nomenclature is done based on a comprehensive nucleotide sequence-based system developed for RVAs in 2008 (58). According to this system, a specific genotype is assigned for each of the genome segment based on established nucleotide percent cut-off values. Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx(x= number starting from 1) abbreviations are used to describe VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 genes of rotavirus strains (56). Up to now at least 27G types (based on nt sequence of VP7) and 37P types (based on nt sequence of VP4) are named in RVA (59).

Rotavirus consists of 7 distinct groups (RVA-RVG) where RVA, RVB and RBC are found in both animals and humans while D, E, F and G groups are found in animals (56). Human rotavirus is the major cause of severe diarrheal disease and dehydration in young children less than 5 years old (60). In 2013 alone, estimated number of rotavirus related deaths among young children was 215,000 (range 197,000-233,000) (37.3% of the total diarrheal deaths)(61). Majority of these deaths occur in Asia and sub-Saharan Africa where India, Nigeria, Pakistan and Democratic Republic of Congo accounts for 49% of child deaths due to rotavirus infection (61).

2.3 ENTERIC VIRUS REMOVAL FROM WASTEWATER

To achieve the efficient removal of viruses from wastewater, number of treatment methods like filtration, coagulation and flocculation, sedimentation and disinfection are employed in wastewater treatment plants. Considering the virus removal mechanisms in different treatment unit processes, adsorption to solids and sedimentation, predation by organisms of higher trophic levels, and sunlight mediated inactivation are the major mechanisms of virus removal in wastewater treatment pond systems (62). In the case of activated sludge, it is reported that the influent viruses are rapidly adsorbed to the mixed-liquor suspended solids (MLSS) (7, 63, 64). For MBR systems, size separation, attachment to the gel cake layer and attachment to mixed liquor suspended solids and inactivation by mixed liquor suspended solids are considered as crucial mechanisms for virus removal (64–66).

Number of studies have reported the presence of pathogenic viruses in the wastewater treatment plant effluents and are summarized in **Table 2.1** (8, 67–72) and number of outbreaks related to waterborne transmission of human enteric viruses are reported over the years (73–75). Agricultural irrigation using the improperly treated wastewater is one of the major routes we can get exposed to enteric viruses in wastewater. In 2011, there was a multi-state norovirus outbreak in Europe (more than 11000 cases)(74). The major reason for the outbreak was consumption of imported strawberry cultivated using improperly treated wastewater containing norovirus (76). Contamination of shellfisheries areas by sewage can lead to accumulation of enteric viruses in the shellfish digestive tissues and consequently can lead to outbreak situations. A systematic review on the shellfish-borne virus outbreaks identified 368 outbreaks of which 83.7% was related to human norovirus followed by hepatitis A (5). Discharging improperly treated wastewater in to water bodies can contaminate the water source downstream from the discharging point. A recent study in France reported significantly increased detection frequency and virus load for adenovirus, norovirus GI, norovirus GII and rotavirus-A between the samples collected upstream and most downstream of Paris urban area and it was attributable to the wastewater treatment plant (WWTP) effluent discharges to the Seine river (77).

Table 2.1: Presence of human enteric viruses in wastewater effluents

Virus name	Number of virus positive effluent samples						Ref:
	Activated sludge	Biofilm process	MBR process	Waste stabilization pond	Primary treatment	UASB reactor	
Adenovirus	9/9	6/7					(71)
Adenovirus	5/7					3/4	(72)
Adenovirus			2/16				(8)
Astrovirus	17/24	16/20			6/13		(71)
Enterovirus	42/50						(70)
Hepatitis A						5/7	(72)
Norovirus	12/47	19/47			5/24		(71)
Norovirus GI	9/40		2/11	7/24			(67)
Norovirus GI	49/49						(68)
Norovirus GII	5/40		0/8	5/23			(67)
Norovirus GII	5/7					2/4	(72)
Norovirus GII			9/16				(8)
Norovirus GII	49/49						(68)
Rotavirus	21/31	15/27			5/14		(71)
Rotavirus	17/24						(69)
Rotavirus-A	7/7					1/4	(72)
Sapovirus			1/16				(8)

2.3.1 Design of wastewater treatment plants using multiple-barrier system

Water reclamation guidelines have been developed by regulating authorities of many countries which stipulate pathogen removal targets needed to be achieved by wastewater treatment before the reclaimed water are used for another beneficial purpose (6, 78–80). However, the achievable virus $\log_{10}$ reduction by a single wastewater unit process is not enough to obtain the stipulated performance targets. Therefore, by combining different treatment unit processes which is called as the multiple-barrier system **(Figure 2.4)**, the required virus $\log_{10}$ removal performance targets are obtained in WWTPs (6). Since the system is a combination of $\log_{10}$ removals by different treatment unit processes, the most important information regarding the design of a multiple-barrier system is the virus log reduction achievable by each unit process. However, current data suggests that the reported $\log_{10}$ removal value of a specific virus by a particular unit process varies with the study. For example, Sima et al. (2011) reported a 3.42 norovirus GII $\log_{10}$ removal value by MBR while Miura et al. (2015) achieved only 0.96 $\log_{10}$ (7, 8). El-Senousy et al. (2015) achieved a rotavirus $\log_{10}$ reduction value of 2.15 whereas Qiu et al. (2015) reported 1.04 $\log_{10}$ (81, 82). These variations can be due to many reasons including the enteric virus of concern, removal mechanisms of different treatment unit processes and operating conditions. Chaudhry et al. (2015) analysed the effect of virus surface characteristics on virus removal mechanisms inside MBRs and found that virus attachment to biomass was the most influential mechanism depending on the virus type. One influencing factor was

the number of hydrophobic amino acids in the external capsid surface. Depending on the number of hydrophobic amino acid groups in the external capsid of the bacteriophage, it can interact more freely with the hydrophobic portions of the bacterial flocs and extracellular polymeric substance (EPS) in MLSS which in turn leads to a higher LRV (12). As an example of effects of operational conditions on virus removal, Nokes et al. (2003) reported that vegetated wetlands have shown identical or higher LRVs compared to unvegetated wetlands (83). A recent validation and operational monitoring protocol developed by the WaterVal^{TM} program also emphasizes the importance of operational conditions on virus reduction, in which a conservative virus LRV of 1.5 is assigned for MBR systems which have nominal pore sizes between 0.04 - 0.1 µm and operates at pH (6 - 8), DO (1 - 7 mg/l), temperature (16 - 30 ˚C), SRT (> 11d), HRT (> 6h), MLSS (> 3g/l), TMP (> 3kPa), flux (< 30 litres/m^2/h) and turbidity (0.2 NTU) after investigating full-scale WWTPs involving more than 200 data points for each parameter (84).

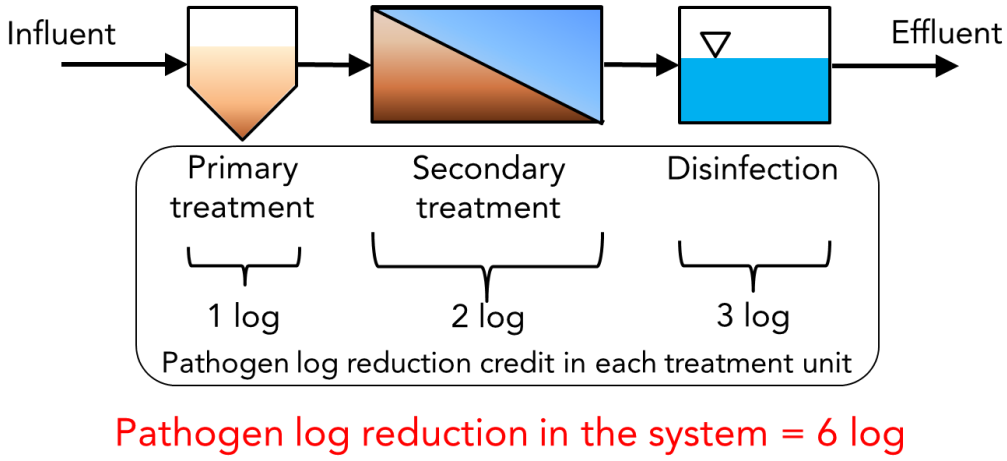

Figure 2.4: Multiple-barrier system for water reclamation (6) (Reproduced with permission from ELSEVIER, **Appendix A-2**)

From the WWTP design point of view, variations in the virus LRV affects the decision making process of identifying the suitable treatment unit processes necessary to achieve guideline stipulated performance targets. If it is possible to develop a methodology to evaluate the average log reduction of a specific virus by a particular unit process incorporating the variations due to operational conditions and other factors, those values can be used as references in designing future WWTPs under multiple-barrier system.

2.3.2 Operational monitoring of human enteric virus removal during wastewater treatment

Operational monitoring of wastewater reclamation systems is performed to make sure the particular plant provides recycled water of expected quality as stipulated by guidelines (80, 85). There are four major stages of a wastewater reclamation system; namely, 1) baseline monitoring to assess risks and impacts of the recycled water on the environment; 2) performance monitoring to validate

performance required by the water quality management plan; 3) operational monitoring to ensure the system is operating within the design specification; and 4) verification monitoring at the end to confirm that the quality of recycled water complies with the water quality management plan and whether any modifications are necessary (86). The most widely used microbial parameter for the second (validation) and third (operational) monitoring stages with respect to virus reduction efficiency in wastewater treatment processes are bacteriophages. According to the USEPA guideline, MS2 coliphage is to be used for on-site validation (85). The Australian guidelines recommend that indigenous *Escherichia coli* be monitored mandatorily and coliphages or other pathogens such as adenovirus or enterovirus be also monitored as representatives of viral contaminants (86). The state of Victoria, Australia provides guidelines on the usage of indigenous somatic coliphages or F-specific RNA (FRNA) bacteriophages, or seeded MS2 coliphage as suitable surrogates for enteroviruses when indigenous or seeded enteroviruses are not used in the validation process (80). MBR validation protocol published by WaterVal^{TM} program suggests that both somatic coliphages and FRNA bacteriophages must be used to validate MBR systems (84).

Selection of bacteriophages for the monitoring over the usage of human enteric viruses is based on many reasons. Some investigations reported that bacteriophages were present in similar or higher numbers in water environments when enteric viruses were present (10, 11). Pouillot et al. (2015) reported a strong positive correlation ($R^2 = 0.8$) between LRVs of male-specific coliphages and human norovirus GII (87). Bacteriophage enumeration results can be obtained within 12 hrs with simpler techniques as opposed to enteric virus enumeration techniques that requires more time, cost and labor (9, 13). Recent research efforts aimed at reducing the process time required for bacteriophage enumeration up to 4 hrs by generating tailored host strains that can detect bacteriophage-induced cell lysis more quickly (88).

However, suitability of the bacteriophages from being a suitable validation and monitoring indicator for enteric virus removal in wastewater treatment is questioned in several studies. Inability to obtain a clear correlation between LRVs of bacteriophages and human enteric virus has been highlighted (89). According to the study performed by Ottoson el al., (2006), enterovirus genome reduction at a wastewater treatment plant was least correlated with the coliphages reduction efficiency (90). A probable reason for this discrepancy is the difference between the quantification methods for bacteriophages and viruses in wastewater. Bacteriophages are commonly quantified with plaque assay using small volumes (1-5 mL) of diluted or undiluted samples, whereas enteric viruses are quantified by genome amplification using concentrated samples from large volumes (91, 92). The recovery efficiencies of viruses during concentration can vary depending on the methods used (91, 93). These methodological differences can in turn add considerable uncertainties and lead to weak correlations between LRVs of bacteriophages and human enteric viruses (88).

Even though there is no strong correlation between LRVs of bacteriophages and human viruses in wastewater treatment unit processes, bacteriophages may be used as an indicator for human viruses given that bacteriophage LRVs are almost always lower than those of human viruses, under the concept of multiple-barrier system. Human virus LRVs have been reviewed (6, 27, 62), however bacteriophage LRVs in multiple unit processes of wastewater treatment have not been reviewed so far. Therefore, it is a necessity to evaluate the suitability of bacteriophages as a tool to monitor and validate the performance of human enteric virus reduction at wastewater treatment plants as it can be performed routinely, with lower time and labour.

2.4 SOLIDS IN WASTEWATER

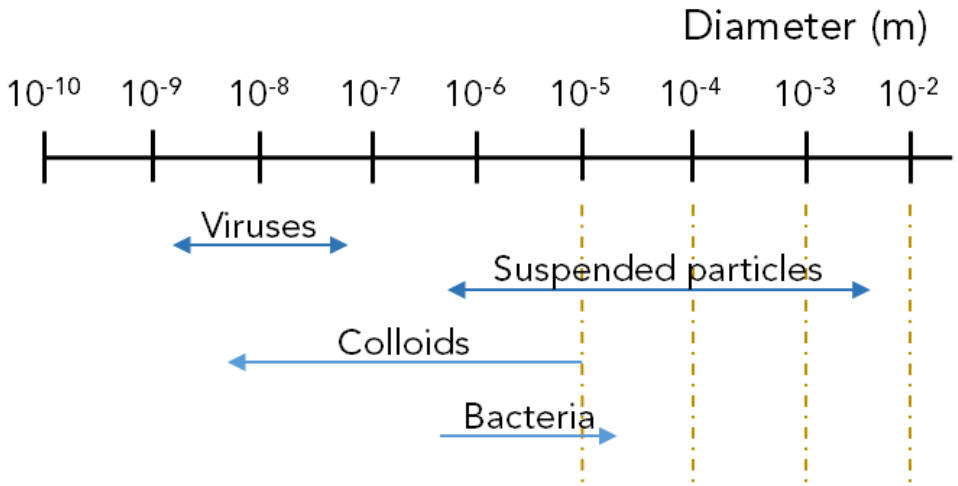

Figure 2.5: Size distribution of water-borne particles. Modified from (94)

Wastewater contains solids of many sizes and characteristics. **Figure 2.5** describes the particle size distribution in water (94). Suspended solids in wastewater consist of organic and inorganic solids. Inorganic particles consist of silt, clay and iron oxides produced by natural weathering of minerals. Colloidal particles include the microorganisms like algae, bacteria and viruses even though there is no agreement on the cut-off value of the size (95).

2.5 INTERMOLECULAR INTERACTIONS INVOLVED IN VIRUS ADSORPTION TO WASTEWATER SOLIDS

Viruses have been considered as colloidal solids and therefore the theories describing the behaviour of colloidal solids in wastewater can be applied to describe the behaviour of viruses (96). This section focuses on the intermolecular interactions which can be used to describe the adsorption of viruses in to wastewater solids.

2.5.1 Derjaguin, Landau, Verwey, Overbeek (DLVO) Theory

DLVO theory explains about the stability of colloids in suspension by the balance between electrostatic repulsive forces and attractive van der Waals forces (2). Surface charges developed by immersed colloids in aqueous solutions due to the adsorption of ions in the surface will be neutralized by the diffused layer of counter-ions around the particle. Diffusive layer thickness can be changed by the addition of cationic salts or by increasing pH to facilitate the attractions between two particles (2).

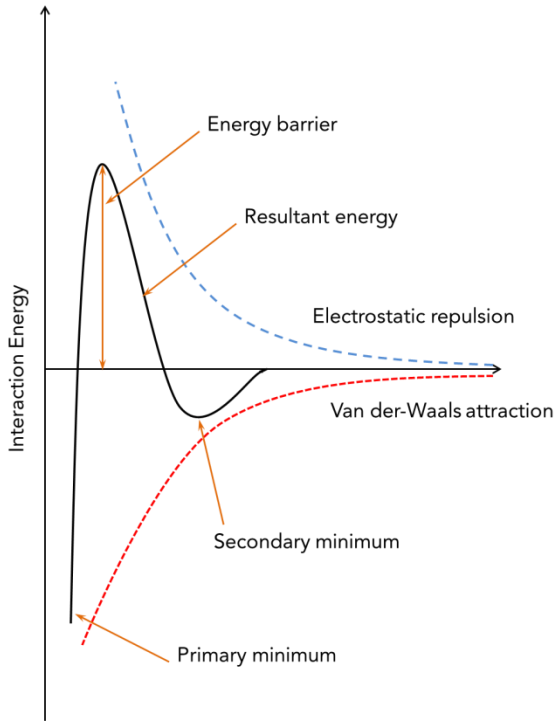

Figure 2.6: Relationship between repulsive and attractive forces with separation distance

2.5.2 Electrostatic interactions

Electrostatic interactions occur between charged or polar molecules. They can be either attractive or repulsive based on the charges of the molecules of concern. For example, carboxylic acids and amine groups in amino acid side chains are negatively and positively charged around pH value of 7.4 respectively and therefore can attract to each other (97). Biopolymer building blocks are mainly polar and therefore dipole-dipole interactions are also possible to occur. Due to electronegativity, molecules can be polar and these polar molecules possess a permanent electric dipole moment (97). By introducing an electric field, charge distribution of a molecule can be changed and a dipole moment can be induced. Typical energy involved in these interactions varies and the values are summarized in **Table 2.2**. Each type of interactions are briefly described in the following section.

Table 2.2: Summary of different molecular interactions (97, 98)

Interaction type	Distance dependence of potential energy	Typical energy/ (kJ/mol)	Comment
Ion – ion	$1/r$	>150	Only between ions
Ion-dipole	$1/r^2$	>50	
Dipole-dipole	$1/r^3$	2	Between stationary polar molecules
	$1/r^6$	0.6	Between rotating polar molecules
London (dispersion)	$1/r^6$	0.1-30	Between all types of molecules
Hydrogen bond		10-40	Interaction of the type A—H···B, with A, B=O,N, or F

Ion-ion interactions are the strongest electrostatic interactions and they occur between two oppositely charged atoms/atom groups. However, dissolving salts in water can reduce the electrostatic interactions because of the shielding effect of water. Ion-dipole interactions occur when a permanent dipole interacts with a cation or an anion where the cations are attracted to the negative end of the dipole while the anions are attracted to the positive end of the dipole. Dipole-dipole interactions (Keesom interactions) are the interactions between permanent dipoles in molecules. They are particularly important when molecules are located in the close proximity. Dipole-induced dipole interactions (Debye interactions) occur due to the polarization of non-permanent dipole molecule as a result of the presence of a permanent dipole molecule in the vicinity. Induced dipole-induced dipole interactions (London dispersion forces) are generated as a result of the resonance of molecules. The movement of electrons in a molecule can influence the movement of electrons in neighbouring molecules and dipoles can be induced. These instantaneous dipoles can cause the atoms to attract to each other. Dispersion forces are significant when the molecules are located in the close proximity. Polarizability (the easiness of distorting the charge distribution of a molecule) decides the strength of dispersion forces i.e. molecules with higher polarizability have larger dispersion forces.

Hydrogen bonds occur between the hydrogen atoms attached to electronegative atoms (F, O or N) and the neighbouring molecules or chemical groups bearing F, N or O. They can be considered as dipole-dipole interactions since the bonds with highly electronegative F, N and O molecules are polar.

2.6 SPECIFIC INTERACTIONS BETWEEN HUMAN ENTERIC VIRUSES AND RECEPTORS

Outer components of enteric viruses are proteins called capsid protein. Capsid proteins are reported to interact with specific receptors in host cells (99, 100). Human norovirus recognition by histo-blood group antigens (HBGAs) is a protein – carbohydrate interaction where the protruding domain of the capsid protein forms an interface with the oligosaccharide side-chains of the antigens, which varies among different strains (101). Human rotavirus recognizes sialic acid and HBGAs as receptors for cell attachment (102). Specific interactions between host cells and viral capsids, is attributable to hydrogen bonds and van der Waals forces **(Figure 2.7)** (103).

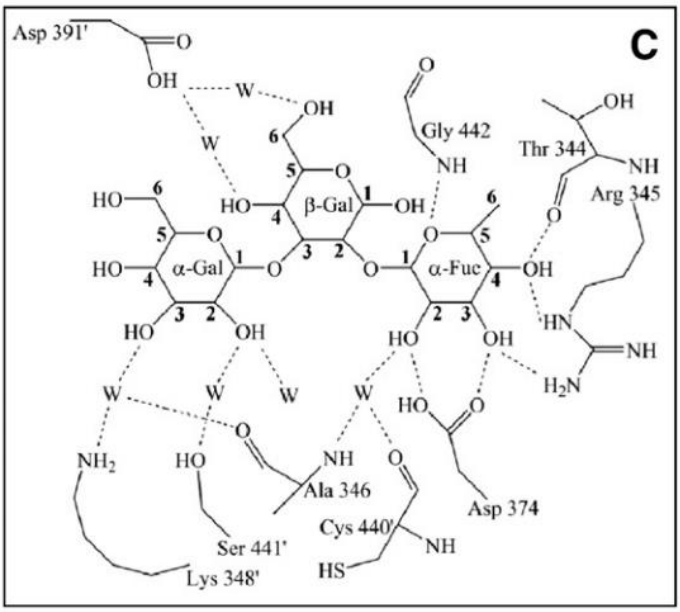

Figure 2.7: Schematic representation of hydrogen bonds (dotted lines) between the individual saccharides of the B-trisaccharide and the nearby amino acids of the capsid of norovirus GII.4 (VA 387 strain). The waterbridged hydrogen bonds are indicated by W (103). (Reproduced with permission from ELSEVIER, **Appendix A-5**)

2.6.1 Histo-blood group antigens (HBGAs)

Human histo-blood group antigens (HBGAs) are complex carbohydrates which can be found in the faces of red blood cells, mucosal epithelial cells etc. (100, 104, 105). They have been suggested to be important factors for human norovirus and human rotavirus infection of intestinal epithelial cells (101, 102, 106). The association between human HBGAs and human norovirus infection has been extensively studied using in-vitro binding assays, human volunteer challenge studies, and outbreak investigations (100, 107, 108). In the case of human rotavirus, proteolysis of spike protein VP4 generates VP5* and VP8* proteins. The binding to cellular glycans is mediated by the VP8* domain of VP4 (102). One such glycan is HBGA and human rotavirus recognizes A-type HBGAs for cell attachment in a strain dependent manner (60).

The biosynthesis pathway of HBGA starting from Type-1 precursor is explained in **Figure 2.8**. The synthesis starts with the addition of a monosaccharide to the disaccharide precursor. By adding a a fucose residue to the disaccharide precursor at an α-1,3 or α-1,4 linkage using FUT3, an α-1,3- or α-1,4-fucosyltransferase, will result a non-secretor type trisaccharide (Le^a). H type 1 trisaccharide is produced by adding a fucose residue to the disaccharide precursor at an α-1,2 linkage by FUT2, another fucosyltransferase. The FUT2 phenotypes are called secretor or secretor positive, whereas the FUT2-inactivating phenotypes are non-secretor or secretor negative. Lewis b (Le^b) antigen is resulted by the further addition of a fucose residue to the H type 1 antigens by the FUT3 enzyme. Tetrasaccharide A type 1 and B type 1 are the result of adding GalNAc or a galactose at α-1,3 linkage of the H type 1 with the help of Enzymes A and B glycosyltransferases. They can be further developed into pentasaccharide A and B antigens (ALe^b and BLe^b), respectively by adding a fucose catalysed by the α-1,3/4-fucosyltransferase (FUT3) (109).

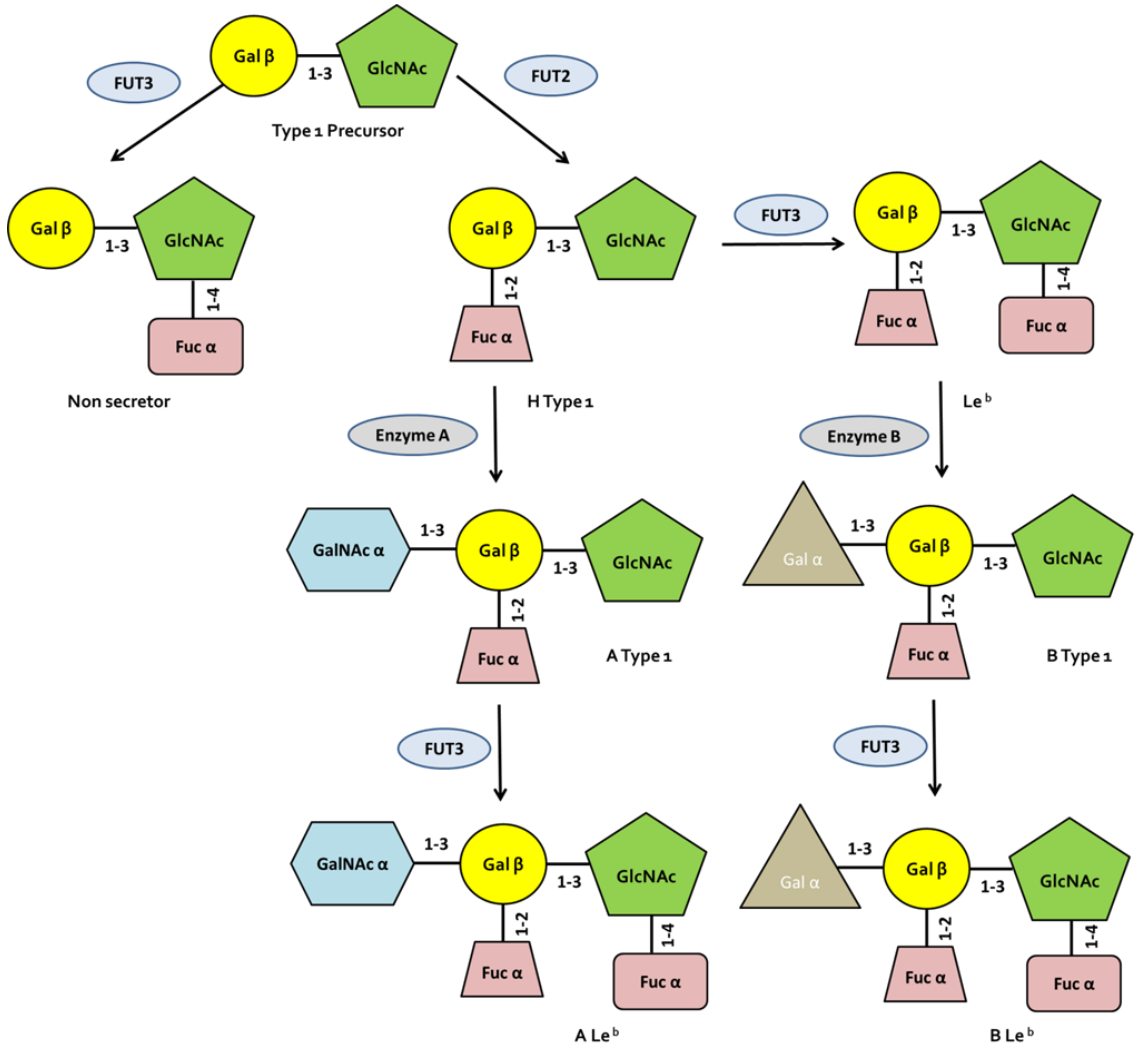

Figure 2.8: The biosynthesis pathways of the human ABO and Lewis histo-blood group antigens (HBGAs) (109)**. Abbreviations**: Enzyme A, N-acetylgalactosamine transferase; enzyme B, galactosyltransferase; Fuc, l-fucose; FUT2, α-1,2-fucosyltransferase; FUT3, α-1,3/4-fucosyltransferase; Gal, d-galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine.

2.6.2 Binding specificity of human norovirus to HBGAs

Different norovirus strains have different HBGA-recognition profiles and up to now there are 8 distinct binding patterns recognized (104, 110, 111). Some of the HBGA-recognition profiles of GI and GII genogroup noroviruses are summarized in **Table 2.3** and **Table 2.4**. P2 subdomain of the norovirus capsid is directly responsible for receptor recognition (112). A study was conducted to determine the precise locations and binding modes of HBGAs on the viral capsids using a recombinant P protein of GII.4 VA387 strain cocrystallized with synthetic A or B trisaccharides (112). Results revealed that both A and B saccharides strongly interacted with P protein of VA387 **(Figure 2.9)** (103). α-fucose plays a central role in norovirus – receptor interactions while β-galactose may not be crucial (103). The cavity which binds with α-fucose is formed by β5 strand and residues S441, G442 and Y443 in one monomer and S343, T344, R345 and D374 of the other monomer (103, 111). The interface between the P protein and fucose was dominated by hydrogen bonds. A recent study reported that not only the presence of a binding epitope but also the orientation of the receptor is critical for norovirus binding (113).

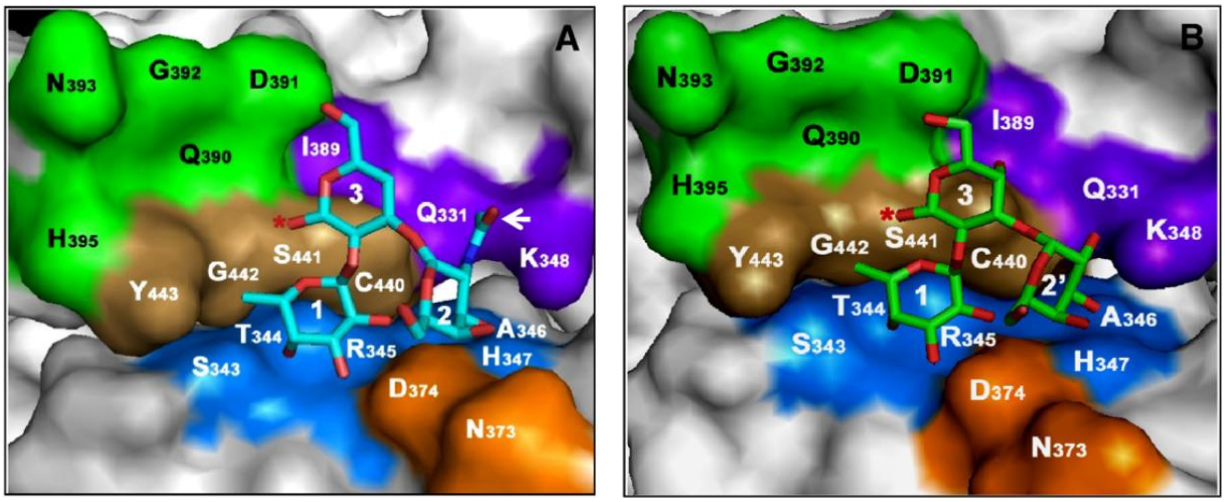

Figure 2.9: The HBGA-binding interface of the norovirus capsid of VA387 (GII-4) interacting with the type A- and B-trisaccharides. The binding interfaces in (A) and (B) are in surface representation. The A- and B-trisaccharides are shown in stick representation, with nitrogen, oxygen, and carbon atoms colored blue, red, and cyan (A-antigen) or green (B-antigen), respectively. The saccharides are indicated as 1=α-1,2-fucose, 2=α- 1,3-N-acetylgalactoseamine, 2′=α-1,3-galactose, 3=β-1,3-galactose. The star symbol indicates the junction linking to the remaining portion of the antigen. The white arrow shows acetamide group in (A). The nearby open pocket is shown in green at the top-left corner (103). (Reproduced with permission from ELSEVIER, **Appendix A-5**)

Table 2.3: Interactions of some genogroup II norovirus VLPs with synthetic HBGAs and/or saliva with defined ABO, secretor and Lewis blood types (114)

			Binding to synthetic oligosaccharides									Binding to Saliva			
Strains	Genotype	Year	H_1	H_2	H_3	A	B	Le^a	Le^b	Le^x	Le^y	O	A	B	N
HV[3]	GII-1	1971	-	-	-	+	+	-	+	-	-	-	-	-	-
HV[3]	GII-1	1971	-	/	-	+	-	-	-	-	-	/	/	/	/
HV[3]	GII-1	1971	-	-	-	-	-	-	-	/	/	-	-	-	/
Noda485	GII-1	2000	-	-	-	-	-	-	-	/	/	-	-	-	/
Weisbaden	GII-1	2001	-	/	-	-	-	-	-	-	-	/	/	/	/
SMV[3]	GII-2	1976	-	-	+	-	+	-	-	/	/	-	-	+	-
SMV[5]	GII-2	1976	-	/	+	-	-	-	-	-	-	/	/	/	/
BUDS[3]	GII-2	2002	-	/	-	-	-	-	-	-	-	/	/	/	/
BUDS[3]	GII-2	2002	-	-	-	+	+	-	-	-	-	-	+	-	-
Ina	GII-2	2002	-	/	-	-	-	-	-	-	-	/	/	/	/
MxV	GII-3	1998	-	-	-	+	-	-	+	-	-	+	+	+	-
Toronto virus	GII-3	1999	-	/	+	+	-	-	-	-	-	/	/	/	/
Kashiwa336	GII-3	2000	-	-	+	-	-	-	-	/	/	-	+	+	/
Mutsudo18	GII-3	2000	-	-	+	-	-	-	-	/	/	-	+	+	/
PiV	GII-3	2003	-	-	-	+	+	-	+	-	-	-	+	+	-
GII-4 1987	GII-4	1987	-	/	+	-	-	-	-	-	+	+	+	+	-
GII-4 1997	GII-4	1997	-	/	+	+	+	-	-	-	+	+	+	+	-
Narita104	GII-4	1997	+	+	+	+	+	-	+	/	/	+	+	+	-
VA387	GII-4	1998	+	-	+	+	+	-	+	+	-	+	+	+	-
M7	GII-4	1999	-	/	-	-	-	-	-	-	-	/	/	/	/
GII-4 2002a	GII-4	2002	-	/	-	+	-	+	-	+	-	+	+	+	+
GII-4 2002	GII-4	2004	-	/	+	-	-	-	-	-	+	+	+	+	-
GII-4 2004	GII-4	2004	-	-	-	-	-	-	-	-	-	-	-	-	-
GII-4 2005	GII-4	2005	-	-	-	-	-	-	-	-	-	-	-	-	-
GII-4 2006	GII-4	2006	-	/	+	+	+	-	-	-	-	/	/	/	/
New Orleans[#]	GII.4	2009	+	+	+	+	+	-	+	-	+	/	/	/	/
Sydney[#]	GII.4	2012	+	-	+	+	+	-	+	-	+	/	/	/	/
MOH	GII-5	1999	-	-	-	+	+	-	-	-	-	-	+	+	-
Ichikawa754	GII-5	1998	-	-	-	+	-	-	-	/	/	-	+	+	/
Ueno7k	GII-6	1994	-	+	+	-	+	+	+	/	/	+	+	+	/
Snbu445	GII-6	2000	-	-	+	-	-	-	-	/	/	+	+	+	/
Osaka 10-25	GII-7	1999	-	-	+	-	+	+	+	/	/	+	+	+	/
VA207	GII-9	1997	-	-	-	-	-	-	-	+	+	+	+	+	+
Chitta/Aichi 76	GII-12	1996	-	-	-	-	-	-	-	/	/	-	-	+	/
OIF	GII-13	2003	-	-	-	-	-	+	-	-	-	+	-	-	+
Kashiwa47	GII-14	1997	-	-	-	-	-	-	-	-	-	-	-	-	/
DG-42*	GII.17	2014	/	/	/	/	/	/	/	/	/	+	+	+	-

- added from (115), * - added from (116)

Table 2.4: Interactions of some genogroup I norovirus VLPs with synthetic HBGAs and/or saliva with defined ABO, secretor and Lewis blood types (114)

			Binding to synthetic oligosaccharides									Binding to Saliva			
Strains	Genotype	Year	H_1	H_2	H_3	A	B	Le^a	Le^b	Le^x	Le^y	O	A	B	N
Norwalk	GI-1	1968	+	-	+	+	-	-	+	-	+	+	+	-	-
Aichi124	GI-1	1989	+	+	+	+	-	-	+	/	/	+	+	-	/
West Chester	GI-1	2001	-	/	+	-	-	-	-	-	-	/	/	/	/
Funabashi258	GI-2	1996	+	-	+	+	-	+	-	/	/	+	+	-	/
SoV	GI-2	1999	-	/	+	-	-	+	-	-	-	/	/	/	/
DSV[32]	GI-3	1999	-	/	-	+		+	-	-	-	-	-	-	-
DSV[3]	GI-3	1999	-	-	-	-	-	-	-	-	-	/	/	/	/
Kashiwa645	GI-3	1999	-	+	-	+	-	+	-	/	/	+	+	-	/
VA115	GI-3	1997	-	-	-	-	-	-	-	-	-	-	-	-	-
Chiba407	GI-4	1987	-	-	-	+	-	+	+	/	/	+	+	-	/
Chiba	GI-4	2000	-	/	-	-	-	+	-	-	-	/	/	/	/
Boxer	GI-8	2002	-	-	-	-	-	-	+	-	+	+	+	+	+
WUG1	GI-8	2000	-	-	-	+	-	+	+	/	/	+	+	+	/

2.6.3 Binding specificity of rotavirus to HBGAs

Cell attachment protein VP8* exhibits a fold with two twisted β-sheets separated by a shallow cleft **(Figure 2.10)**. The VP8* of P[4] human rotavirus DS-1 and P[8] strain Wa reacts with Lewis b and H-type I HBGA. VP88 of neonatal strain ST-3 (P[6]) bounds to H-type I HBGA (117). Rotavirus P[14] VP8* specifically recognizes the glycans with a terminal oligosaccharide sequence typical to A-type HBGAs (118).

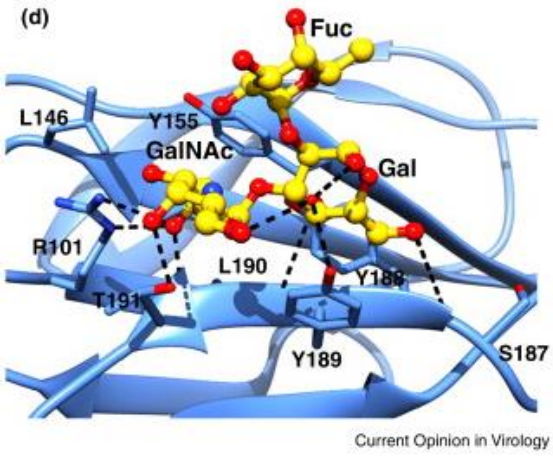

Figure 2.10: Interactions between the P[14] VP8* of sialidase insensitive human strain rotavirus HAL1166 with A-type HBGA. The P[14] VP8* structure is presented in blue ribbon with the amino acid residues interacting with A-HBGA shown as yellow sticks with oxygen and nitrogen atoms coloured. Network of hydrogen bond interactions (dashed lines) are shown (118). (Reproduced with permission from ELSEVIER, **Appendix A-6**)

2.7 IMPORTANCE OF VIRUS ADSORPTION TO WASTEWATER SOLIDS

It can be observed that, the adsorption to solids plays a major role in virus survival and distribution in nature, removal during sewage treatment, land treatment, drinking water treatment and concentration from environmental samples (119). Gerba et al.(1978) quantified the animal enteric viruses in the sewage effluents from 2 activated sludge plants and reported that 49% and 100% of the viruses were associated with wastewater solids while 3-20% of the of the animal enteric viruses from the trickling filter effluent was associated with wastewater solids (17). Norovirus GI and GII were attached to large settleable particles (>180µm), smaller suspended particles (>0.45µm) and colloidal particles in a waste stabilization pond (18). Rao et al. (1984) quantified the enteroviruses in the Galveston bay (Texas, USA) which is reported to be contaminated by the secondary treated and chlorinated sewage effluents and found that 72% of the suspended solids samples contained enterovirus while 14% of the samples without solids were positive. Moreover, 47% of the top layer sediments also contained Enterovirus whereas only 5% of the bottom layer sediments were positive (120). The study by Miura et al. (2015) on the virus removal by a pilot-scale MBR revealed that compared to caliciviruses, enteroviruses are less associated with activated sludge (7).

Current wastewater treatment facilities utilize chlorination and UV disinfection as the principal means of disinfection (95). Chlorine exposure can cause irreversible cell damages or alter the cell functions (121). Chlorine diffuses through the wastewater solids by radial diffusion in a two-step process which consists of different boundary layers (122). As the diffusion rate is governed by the initial concentration, chlorine deactivation of pathogens is proportional to the initial concentration (20). Wastewater solids can increase the chlorine demand of wastewater and the free chlorine available for the virus inactivation is decreased (121). The phenomenon is called as tailing where the increased chlorine dose doesn't increase the $\log_{10}$ reduction of viruses. According to the studies by Winward et al. (2008), microorganisms associated with wastewater solids are more resistant to chlorine than the free microorganisms (20). Based on the observations by Hejkal et al. (1979), compared to free or secondarily adsorbed poliovirus, four-fold increase of combined chlorine was necessary to achieve the same degree of inactivation in fecal particle associated or occluded poliovirus (21). Another study also reported that the increment of organic matter content in wastewater significantly reduced the MS2 coliphage $\log_{10}$ reduction (123).

UV disinfection of viruses occurs via the DNA damage which obstructs the replication cycle (124). The recent review by Chahal et al. (2016) described that wastewater solids can shield microorganisms in different ways by providing shading or partial absorption of UV energy to reduce the effective dose, or by scattering the light (121). Templeton et al. (2005) study on the inactivation of viruses by UV light compliments the fact as humic acid and activated sludge flocs shielded MS2 and T4 by UV inactivation to a statistically significant degree (with >99%CI) relative to the particle free conditions (125).

Hoff and Akin (1986) reported that microorganisms associated with organic matter are protected from inactivation to a higher degree compared to the microorganisms attached to inorganic particles (126). A study on the influence of physicochemical parameters of microbial flocs on UV inactivation of viruses in sequencing batch reactor effluent reported that extracellular polymeric substances (EPS) absorb UV light at 254 nm with an absorbance of 250 cm^{-1} and that EPS may therefore also prevent the penetration of UV light through microbial floc (127).

2.8 SPECIFIC INTERACTIONS BETWEEN WASTEWATER SOLIDS AND VIRUSES

The studies which focus on the interactions between the wastewater solids and human enteric viruses and their effects on virus removal and persistence has overlooked the fact that there are specific interactions between the wastewater solids like bacteria and viruses. There were no studies on this phenomenon until 2013 when Miura et al. (2013) reported the successful isolation of *Enterobacter cloacae* SENG-6 bearing histo-blood group antigens (HBGAs) like substances which can specifically capture human norovirus particles **(Figure 2.11)**. Li et al. (2015) reported that HBGA-expressing *Escherichia coli* maintained the antigen integrity and mucin-binding ability of NoVLPs after heat treatment **(Figure 2.12)**.

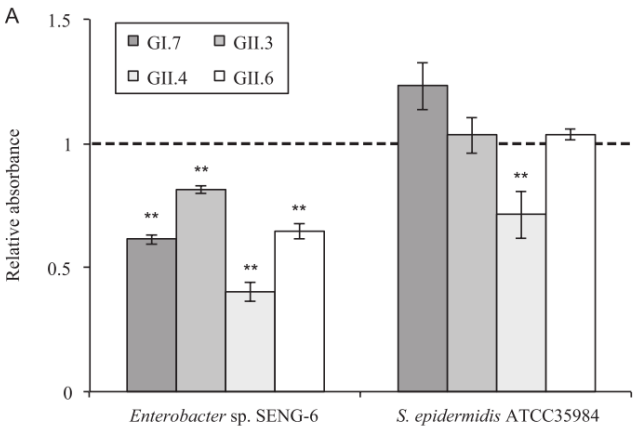

Figure 2.11: NoVLP binding to bacterial cells. Cells of *Enterobacter cloacae* SENG-6 and *Staphylococcus epidermidis* (ATCC 35984) were mixed with each NoVLP genotype (GI.7, GII.3, GII.4, and GII.6) in PBS. Unbound NoVLPs were recovered by filtration and detected by ELISA. Suspended NoVLPs in PBS that were not mixed with bacterial cells were filtered and used as controls. The results were expressed as the absorbance relative to the control; therefore, values of <1 (dashed line) indicate the significant binding of NoVLPs to bacterial cells. The error bars represent the standard deviations of triplicate independent measurements. **, $P < 0.01$ (*t* test) (128). (Open access article)

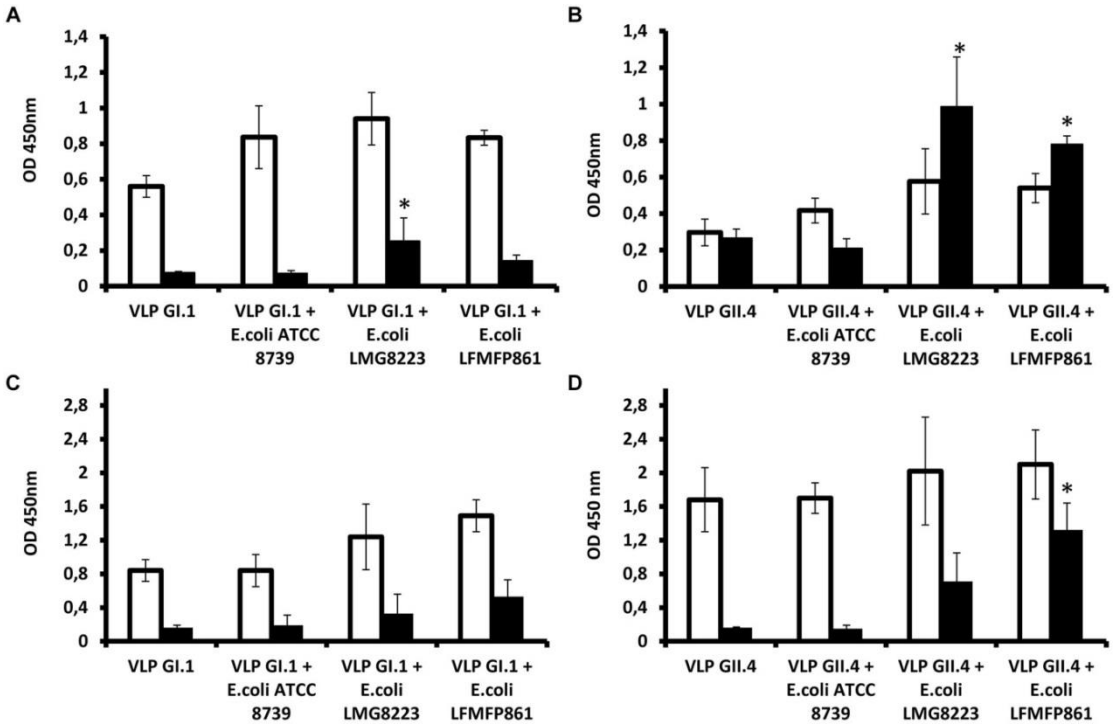

Figure 2.12: Protection of histo-blood group antigen (HBGA) expressing bacteria on human norovirus (NoV) virus-like particles (VLPs) toward heat treatment. (A, B) Antigenicity detection of NoV GI.1 and GII.4 in the absence of bacteria, in the presence of non-HBGA expressing *E. coli* ATCC8739 or HBGA-expressing *E. coli* LMG8223 and LFMFP861 before (white bars) or after (black bars) heat treatment at 90°C for 2 min. **(C,D)** Mucin-binding ELISA results of NoV GI.1 and GII.4 in the absence of bacteria, in the presence of non-HBGA expressing *E. coli* ATCC8739 or HBGA expressing *E. coli* LMG8223 and LFMFP861 before (white bars) or after (black bars) heat treatment at 90°C for 2 min. * $p<0.05$. Each data point is an average of three independent tests, and each error bar represents the data range (129). (Open-access article distributed under the terms of the Creative Commons Attribution License (CC BY))

2.9 SUMMARY AND CONCLUSION

Wastewater reclamation and reuse has been identified as a major solution for water scarcity. However, it is a necessity to make sure the reclaimed water is devoid of disease causing pathogens. Among the pathogens of concern, human enteric viruses are important due to their lower infectious dose, environmental stability and high attack rate. Many studies have reported that interactions between wastewater solids-viruses affect the removal and survival properties of viruses. Previous studies haven't focused on the contribution of specific virus – wastewater solids interactions on the on the life cycle of human enteric viruses, including the environmental persistency and removal properties in water and wastewater treatment processes. A deeper understanding on the specific virus – wastewater solids interactions can help in developing more efficient water reclamation systems which provide microbiologically safe water.

Chapter 3 : VIRUS REMOVAL EFFICIENCY OF WASTEWATER TREATMENT TECHNOLOGIES

Environment International (2016), 91, 220-229

(Reproduced with permission from ELSEVIER, **Appendix A-2**)

3.1 INTRODUCTION

Solid removal techniques such as filtration, coagulation and flocculation, and sedimentation, and disinfection techniques such as UV irradiation and ozonation are regarded as effective for reducing viruses in wastewater and producing virologically safe reclaimed water. However, the values of virus removal efficiency for these processes are heavily dependent on operation conditions, such that efficiencies can vary greatly even within the same reactor for a given treatment (130). For example, Katayama et al. (2008) reported that a $\log_{10}$ reduction value (LRV) of 1.94 was obtained for norovirus GII while the study by Flannery et al. (2012) reported a 0.77 LR (68, 131). However, for the efficient design of wastewater reclamation systems under the multiple-barrier system, it is a necessity for water engineers to know the “average” virus removal efficiency of each wastewater treatment unit as a virus LR.

In this study, we employed a meta-analysis approach to obtain the representative values of virus removal efficiency for wastewater treatment process units which is important in designing wastewater reclamation plants.

3.2 META-ANALYSIS METHODOLOGY

3.2.1 Study Selection

Google scholar, PubMed and Web of Knowledge were considered as search engines in this study. Different keyword combinations are analyzed to obtain appropriate number of results. Finally, "Xvirus" + "removal efficiency" + "wastewater treatment" is selected as the search criteria and all three databases were searched for articles between 2000 and 2015 (Xvirus = Norovirus, rotavirus and enterovirus). The results obtained from PubMed and Web of Knowledge coincided with the google scholar results. Therefore, hereafter all the results mentioned can be considered as the results obtained from google scholar. Norovirus, rotavirus and enterovirus resulted in 261, 397 and 281 results respectively in google scholar between years 2000 and 2015.

3.2.2 Identification of relevant literature

Since there are no guidelines for systematic reviews and meta-analysis processes in engineering, data obtained from the google scholar is processed roughly according to the preferred reporting items for systematic reviews and meta-analysis (PRISMA) flow diagram used in clinical medicine with some modifications (**Figure 3.1**) (132). The method explains the process of selecting

relevant articles among the records obtained via web search. After identification of records, they were screened to eliminate records published in different than English, articles without virus data or quantitative virus data, doctoral dissertations, book chapters, review papers, posters, conference proceedings and other technical reports. The total number of articles screened, eliminated and selected for the quantitative analysis is mentioned in **Table 3.1**.

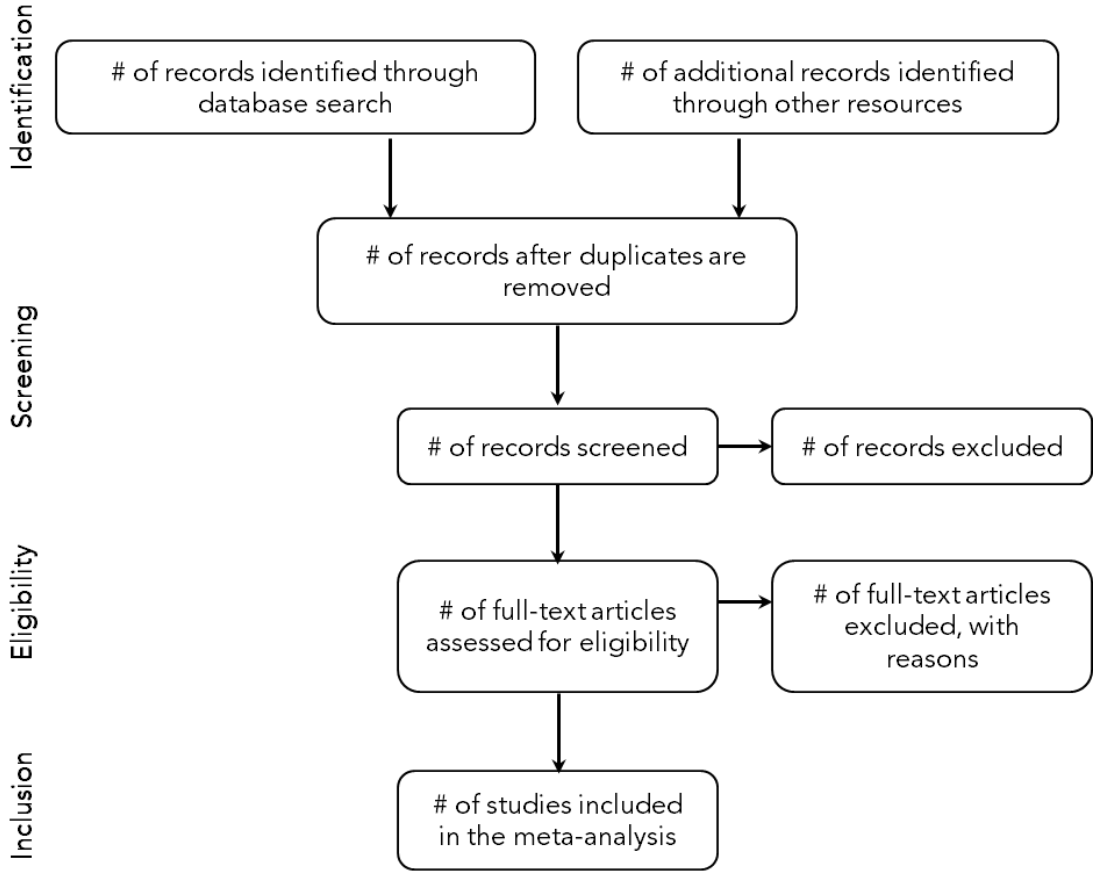

Figure 3.1: Article selection procedure according to PRISMA methodology (132)

3.2.3 Data extraction

After the screening, selected full text articles were analyzed for log removal of viruses by different wastewater treatment processes. Major information expected to be obtained from the articles were treatment method, number of virus positive samples in the plant effluent and quantitative reduction of viruses (average and standard deviation (SD)). Articles which implicitly or explicitly mentioned these data were selected for the final step and the remaining were eliminated. Attention was paid to the articles which clearly expressed the type of treatment system employed in the wastewater treatment plant as it was expected to analyze the efficiency of different treatment processes on virus removal.

Articles which mentioned the influent and effluent virus concentrations or log reductions (LR) and SD were used directly. If the data were presented in figures, they were extracted using the ImageJ software (version 1.48) developed by National Institutes of Health, USA. Arithmetic mean

and standard deviation values of LRs were calculated from the available data using the **equations A and B**, when these values were not specified explicitly in the articles. In the equations, μ_{i-e} and σ provides the LR value and standard deviation where μ_i, σ_i, μ_e and σ_e are the influent and effluent virus concentrations and standard deviations respectively. The complete article lists which were selected representing membrane bioreactor (MBR) process and conventional activated sludge process (CAS) and the data extraction method used are described in **Tables 3.2 and 3.3**.

$$\mu_{i-e} = \mu_i - \mu_e \qquad \textbf{(A)}$$

$$\sigma = \sqrt{\frac{\sigma_i^2}{n} + \frac{\sigma_e^2}{n}} \qquad \textbf{(B)}$$

Table 3.1: Number of selected and rejected articles representing each virus based on the PRISMA method

Virus name	Norovirus	Rotavirus	Enterovirus
Selected articles	29	23	16
Articles for meta-analysis	**7**	**4**	**3**
Rejected articles			
No virus data	91	130	97
No quantitative data	16	13	7
Book chapters	16	45	28
Dissertations	39	87	42
Review papers	36	47	39
Posters	1	0	1
Proceedings	7	14	10
Reports	15	30	23
Different languages	11	8	18
Total articles	261	397	281

Table 3.2: Data extraction, sample number and treatment methods applied in selected publications about virus removal in membrane bioreactor

Publication	Process Chain	Analyzed virus	Sample number	HRT	Data extraction procedure
Ottoson (2006) (133)	Drum filter + Membrane Bioreactor	Enterovirus	5		Direct extraction from the manuscript
Simmons and Xagoraraki (2011) (134)		Enterovirus	8	11-12 hrs	Mean from manuscript $\mu_{i-e} = \mu_i - \mu_e$ $\sigma = \sqrt{\frac{\sigma_i^2}{n} + \frac{\sigma_e^2}{n}}$
Sima (2011) (8)		Norovirus GII	5	N/A	Calculation of monthly log reductions from positive data
Miura (2015) (7)	Anoxic /oxic MBR process	Enterovirus	4	7.2 hrs	Calculation from data in the manuscript
		Norovirus GII	5		
Chaudhry (2015) (65)	Screening and grit removal + Anoxic oxic MBR process + Chlorine/UV + Permeate after backwash	Norovirus GII	4	N/A	Data extraction from Figure 2 in the manuscript using ImageJ
	Screening and grit removal + Anoxic oxic MBR process + Chlorine/UV + Permeate after 3hr of backwash	Norovirus GII	3	N/A	
	Screening and grit removal + Anoxic oxic MBR process + Chlorine/UV + Permeate after 1day of backwash	Norovirus GII	2	N/A	

Table 3.3: Data extraction, sample number and treatment methods applied in selected publications about virus removal in activated sludge processes

Publication	Process Chain	Analyzed virus	Sample number	HRT	Data extraction procdure
Katayama (2008) (131)	Activated sludge process	Enterovirus	41		Calculation from data
		Norovirus GI	65		
		Norovirus GII	63		
Nordgren (2009) (135)	Primary sedimentation+ Activated sludge process+ Secondary sedimentation	Norovirus GI	4		Calcualtion of monthly log reduction from positive data and calculation of average log reduction
		Norovirus GII	6		
Li (2011) (136)	Plant G: Aerated grit chamber + Primary sedimentation + Activated sludge process + secondary settling + coagulation + sand filtration	Rotavirus	4	5 hrs	Data extraction from figure 2 in manuscript using imageJ
	Plant Q: Aerated grit chamber + Primary sedimentation + A2O activated sludge process + secondary settling + 0.02µm ultrafiltration		2	6 hrs	
	Plant X: Aerated grit chamber + Primary sedimentation + A2O activated sludge process + secondary settling + RO membrane ultra-filtration		2	6 hrs	
Prado (2011) (72)	Activated sludge process with extended aeration+ Chlorination	Rotavirus	4		Direct extraction from publication
		Norovirus GII	3		
Flannery (2012) (68)	Primary sedimentation+ Activated sludge process+ Secondary sedimentation	Norovirus GI	49		Mean from manuscript $\mu_{i-e} = \mu_i - \mu_e$ $\sigma = \sqrt{\frac{\sigma_i^2}{n} + \frac{\sigma_e^2}{n}}$
		Norovirus GII			
El-Senousy (2015) (81)	Primary sedimentation+ Activated sludge process+ Secondary sedimentation	Rotavirus	3		Direct extraction from publication

3.2.4 Calculation of representative log reduction

Meta-analysis calculation and Forest plot construction is performed using Microsoft Excel software package. An excel sheet is developed as described in Neyeloff et al. (2012) with some modifications for calculation purposes (137).

3.2.5 Step-by-step calculation procedure

First, study name, number of samples, log reduction and standard deviation data were entered to the spreadsheet. Standard error is calculated using **equation 1**.

$$\text{Standard Error} = \frac{\text{Standard Deviation(SD)}}{\sqrt{\text{Number of samples(n)}}} \qquad \textbf{(1)}$$

Then, inverse variance weights (w) are calculated for each studies using **equation 2**.

$$w = \frac{1}{SE^2} \qquad \textbf{(2)}$$

Then weighted effect size of each study is calculated using w×log reduction (LR). The representative log reduction is calculated by using the **equation 3**.

$$\text{Representative log reduction} = \frac{\sum w \times LR}{\sum w} \qquad \textbf{(3)}$$

Analysis of heterogeneity among different studies is performed using the calculation of Q statistic. All studies are equal is considered as null hypothesis. To confirm that, Q is calculated with **equation 4** and the obtained value is compared with the Chi-square distribution at α=0.05 and df=n-1. If the obtained Q value is higher than the critical value, we consider the studies are not similar.

$$Q = \sum (w \times LR^2) - \frac{[\sum (w \times LR)]^2}{\sum w} \qquad \textbf{(4)}$$

To accommodate the heterogeneity of results, a random effects model is applied. In that case, the weight of each study is adjusted with a constant (v) which can be represented by **equation 5**.

$$v = \frac{Q - (k-1)}{\sum w - \left[\frac{\sum w^2}{\sum w}\right]} \qquad \textbf{(5)}$$

Finally, the weights are adjusted using the calculated constant using **equation 6**.

$$w_n = \frac{1}{SE^2 + v} \qquad \textbf{(6)}$$

The analysis is repeated using the calculated new weights to obtain representative log reduction. To calculate the representative standard error, **equation 7** has been used.

$$\text{Standard Error(SE)} = \sqrt{\frac{1}{\sum w_n}} \qquad \textbf{(7)}$$

Finally, Forest plots with 95% confidence intervals are constructed using Microsoft Excel to graphically represent the representative log reductions.

3.3 RESULTS AND DISCUSSION

3.3.1 Norovirus genogroup II log reduction by MBR

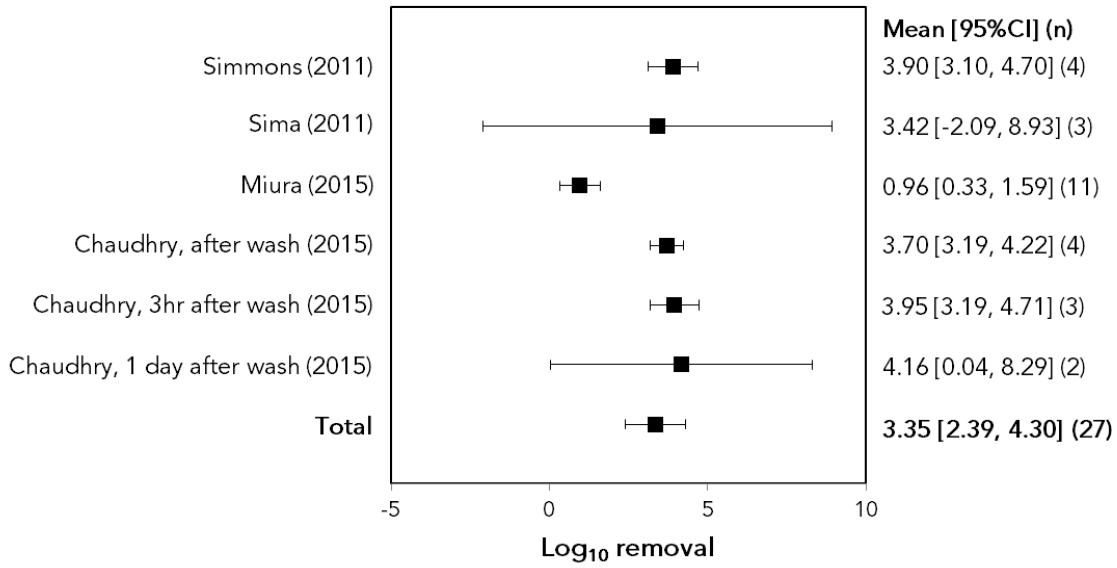

Figure 3.2: Reduction efficiency of norovirus genogroup II by membrane bioreactors (MBR). Black square is the mean value of virus log reduction (LR) obtained in each study, and a bar indicates 95% confidence interval (CI). The sample number in each study is indicated in a parenthesis next to 95% CI values. The total LR value is obtained by the calculation procedure indicated above.

The forest plot of LR values of norovirus GII by MBR with 95% confidence interval (CI) values is shown in **Figure 3.2**. Four articles were selected based on the availability of quantified LR data (7, 8, 65, 138). These studies investigated virus concentrations in the influent and effluent of an MBR process by quantitative reverse-transcription PCR (RT-qPCR) and LR values, each calculated as a ratio of virus concentration in influent to that in effluent at each sampling event, were presented. Since heterogeneity was detected via Q-statistics (equation 4) between studies of norovirus GII reduction in MBR, a random effect model utilized by Neyeloff et al. (2012) was applied (see equations 5 to 7) in order to calculate a representative value of 3.35 LR (95% CI between 2.39 and 4.30, **Figure 3.2**). The mean values of norovirus GII LR varied between 0.96 (7) and 4.16 (65), which may reflect differences between these MBR configurations and their operational conditions **(Table 3.2)**. Since the PCR for norovirus does not discriminate genotypes within a genogroup, the fluctuation of virus removal efficiency between studies may also reflect that different genotypes or strains of norovirus GII were removed dissimilarly, although genotype- or strain-dependent removal properties have not been thoroughly investigated (7). Chaudhry et al. (2015) reported the gradual increase of LR

when the time after membrane wash was increased from 0 to 24 hours, which implies that the level of membrane fouling is one of the determining of factors of virus LR. The hydraulic retention time (HRT) is another influential factor for virus reduction in MBR(139), but not all the publications specified the details of operational conditions including HRT. Among the retrieved papers, Sima et al. (2011) and Chaudhry et al. (2015) did not include HRT of the reactors, because these studies did not focus on the relationship between virus removal efficiency and operational conditions.

3.3.2 Enterovirus log reduction by MBR

We retrieved three articles related to the reduction of enteroviruses in MBR processes (7, 90, 134). Q-statistics detected heterogeneity between these studies as with norovirus GII; therefore, the random effect model was again employed to calculate a representative virus removal value of 2.71 LR with a 95% CI between 1.52 and 3.89 **(Figure 3.3)**. The broad CI may be attributable to different operational conditions **(Table 3.2)**, and/or different removal efficiencies among viral species within the genus Enterovirus. The RT-qPCR assay used for enterovirus in these studies targets the 5' non-coding region, which is highly conservative among enteroviruses and includes more than 100 serotypes. This means that the virus reduction efficiency obtained in each study is the representative reduction efficiency of multiple enterovirus types in each MBR process.

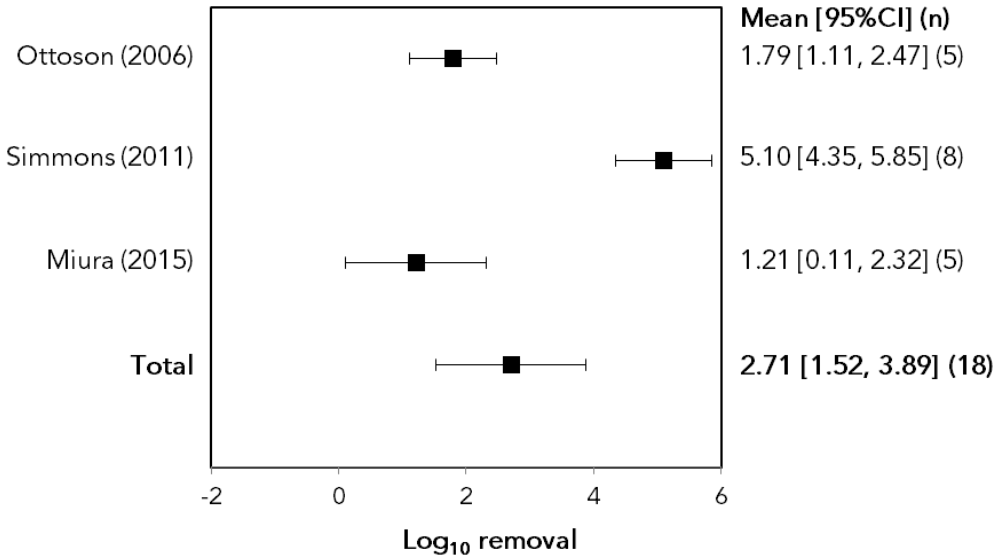

Figure 3.3: Reduction efficiency of enteroviruses by membrane bioreactors. Black square is the mean value of virus log reduction (LR) obtained in each study, and a bar indicates 95% confidence interval (CI). The sample number in each study is indicated in a parenthesis next to 95% CI values. The total LR value is obtained as explained above.

3.3.3 Rotavirus log reduction by activated sludge process

Four articles were selected for rotavirus reduction in AS processes (72, 81, 82, 136). The mean values obtained in each study ranged between -1.31 (Prado et al. 2011) and 2.15 (El-Senousy et al. 2015) (**Figure 3.4**). Q-statistics detected heterogeneity between these studies as well, and a representative removal efficiency of 0.87 LR with 95% CI between 0.20 and 1.53 was calculated.

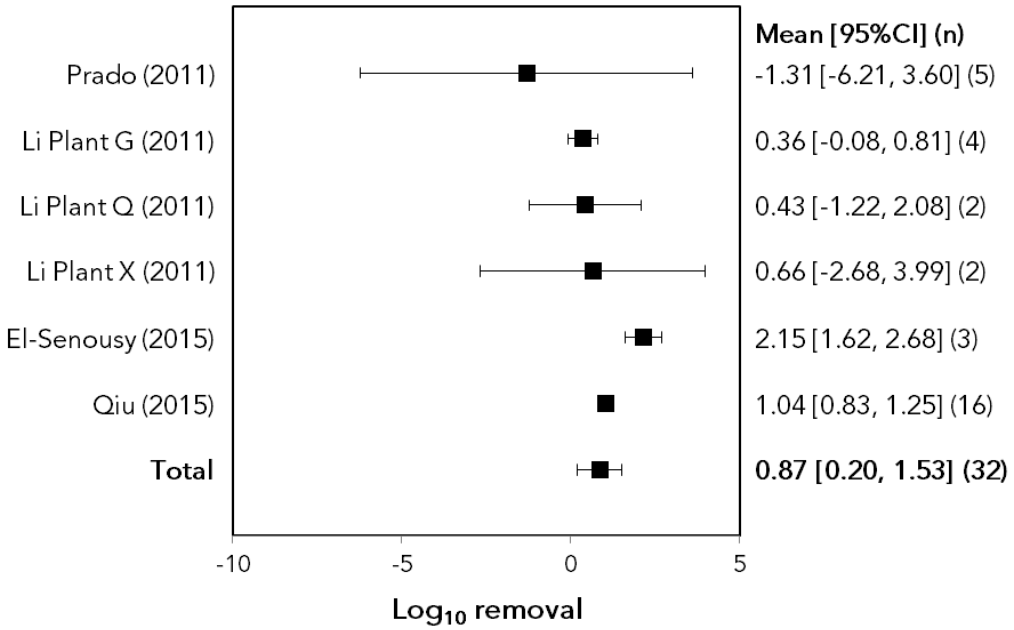

Figure 3.4: Reduction efficiency of rotaviruses by activated sludge processes. Black square is the mean value of virus log reduction (LR) obtained in each study, and a bar indicates 95% confidence interval (CI). The sample number in each study is indicated in a parenthesis next to 95% CI values. The total LR value is obtained by the calculation procedure indicated above.

3.3.4 Norovirus genogroups I and II log reduction by activated sludge process

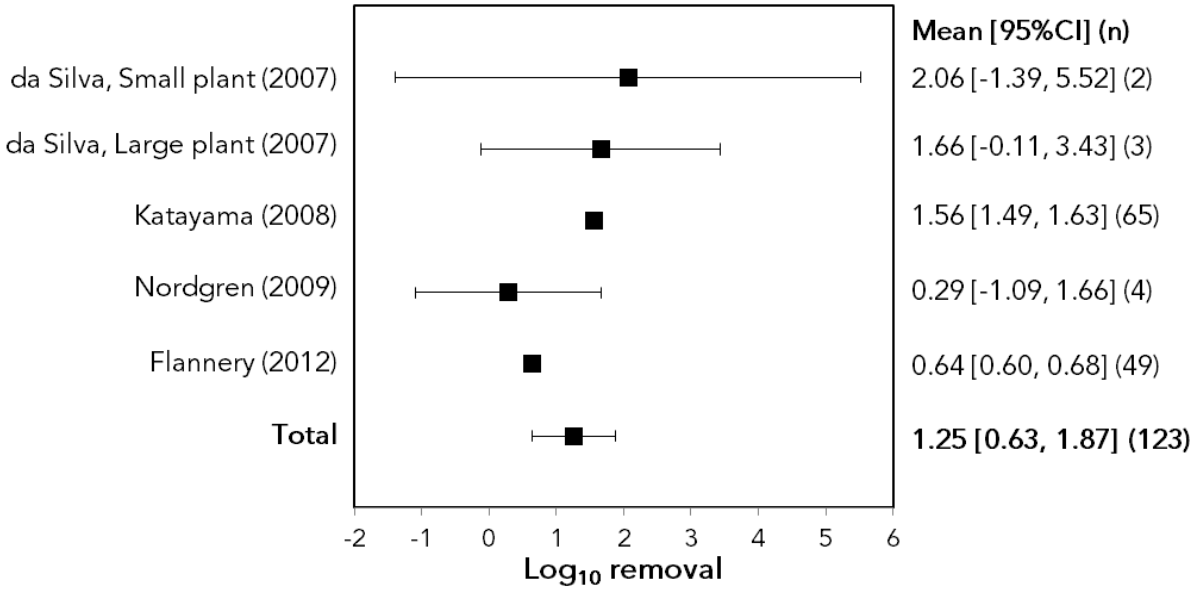

Figure 3.5: Reduction efficiency of norovirus genogroup I by activated sludge processes. Black square is the mean value of virus log reduction (LR) obtained in each study, and a bar indicates 95% confidence interval (CI). The sample number in each study is indicated in a parenthesis next to 95% CI values. The total LR value is obtained by the calculation procedure indicated above.

Four articles were obtained pertaining to norovirus GI and GII removal in AS processes (67, 68, 135, 140). The representative reduction efficiency of norovirus GI in AS processes was 1.25 with 95% CI between 0.63 and 1.87 (**Figure 3.5**), while that of norovirus GII was 1.35 with 95% CI between 0.52 and 2.18 (**Figure 3.6**). These results indicate that the removal efficiencies of gastroenteritis viruses (rotavirus, norovirus GI and GII) by AS are very comparative, and about 1 to 1.5 LRs are expected on average.

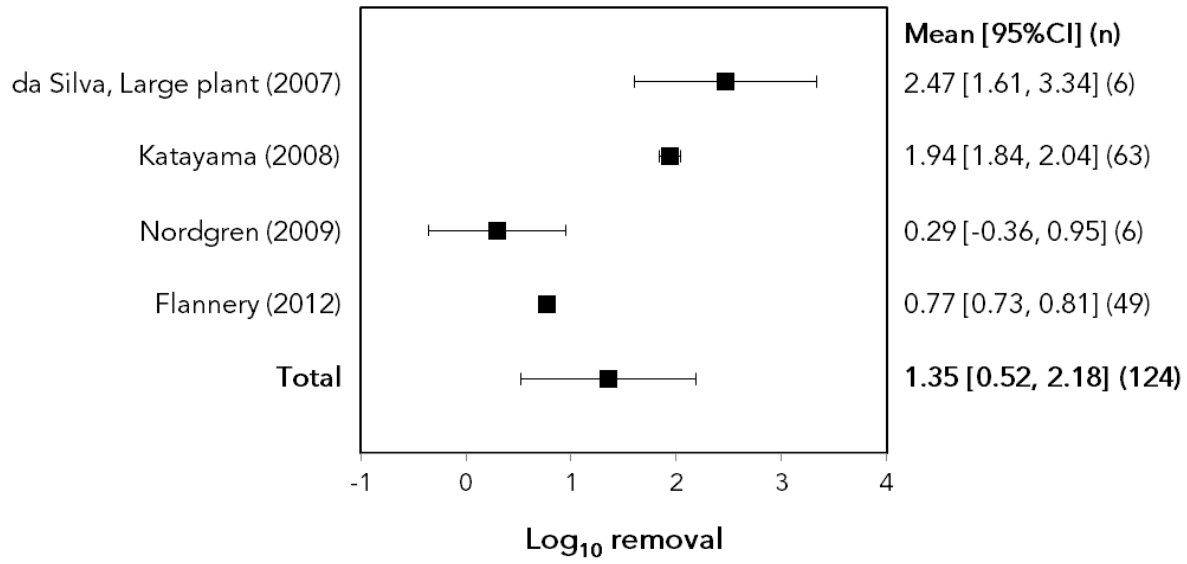

Figure 3.6: Reduction efficiency of norovirus genogroup II by activated sludge processes. Black square is the mean value of virus log reduction (LR) obtained in each study, and a bar indicates 95% confidence interval (CI). The sample number in each study is indicated in a parenthesis next to 95% CI values. The total LR value is obtained by the calculation procedure indicated above.

3.4 LIMITATIONS AND SUGGESTIONS

3.4.1 Addressing the left-censored data

Any pathogenic virus can be removed by wastewater treatment to some extent, the quantification datasets of viruses in treated wastewater commonly include a significant number of non-detects. Even for untreated wastewater samples, pathogenic viruses are frequently not detected because of diel and annual variability. For example, noroviruses are frequently detected in untreated wastewater during the epidemic season, but not during other times of year (141). These non-detects do not allow us to calculate the virus removal efficiency and, therefore, the meta-analysis examples above employed only datasets where viruses in untreated and treated wastewater samples were both quantified and could be used to calculate the log-ratio of virus concentrations before and after a given treatment. This approach, in general, may underestimate the true virus removal efficiency value because viruses are not detected from an effluent sample when highly efficient virus removal is achieved as described above. Substitution of the non-detect data with specific values such as the limit of quantification, the half value of quantification limit, or zero has been used as a classical approach

for dealing with non-detects, but the substitution gives inaccurate estimation of distribution parameters when the distribution of concentration is predicted (142). It has been proposed that datasets including non-detects or so called “left-censored” datasets can be analyzed by Bayesian estimation of the posterior predictive distribution of pollutant concentrations in environmental samples (Paulo et al. 2005). We have extended the Bayesian model to estimate the posterior predictive distribution of virus-removal efficiency (143, 144). These models employ a parametric probabilistic distribution such as a log-normal distribution for expressing pollutant concentrations, which may give inaccurate estimations when the observed data is skewed (145). It is necessary to test the robustness of these statistical models and their applicability to environmental survey data.

3.4.2 Unavailability of the necessary data required for the calculation

Several articles were excluded from this representative value calculation even though they have provided LR values of human enteric viruses in different wastewater treatment unit processes. For example, Zhou et al. (2015) investigated the concentrations of multiple virus types including norovirus GII and enterovirus before and after an MBR process by RT-qPCR, but no information about sample size in the calculation of average LR was described, thereby making it impossible to include the presented LR data in the meta-analysis (146). Da Silva et al. (2007) also analyzed the reduction efficiency of norovirus GII in MBR, but all effluent samples were norovirus GII-negative and could not be included in the current meta-analysis calculation method. Francy et al. (2012) reported the reduction efficiency of enterovirus in MBR, but there was only one enterovirus-positive sample in the MBR effluent, which did not allow to include this study in the meta-analysis (147). Kitajima et al. (2014) reported the reduction efficiency of rotavirus in two wastewater treatment plants employing AS processes, but the reduction efficiency was obtained for the combined process of AS and chlorine treatment. Since this study aimed to calculate the virus LR in each treatment unit, we did not use those datasets in the calculation (148). Campos et al. (2013) reported the norovirus concentration in influent and effluent samples of AS processes, but did not provide the sample number and SD values of the concentration, which are indispensable for the meta-analysis (139). La Rosa et al. (2010) also reported the norovirus concentration in influent and effluent samples of AS processes, but did not provide SD values (149).

All manuscripts excluded in the meta-analysis because of the lack of required information in the meta-analysis are trustworthy and useful as a case study for the virus removal from wastewater, but not available for calculating the average value of virus LR by meta-analysis in the present study. It is strongly recommended that arithmetic mean and standard deviation values of LR as well as sample size, which are necessary for meta-analysis calculations, be clearly described in the publication, otherwise LR datasets cannot be included in the calculation of representative values of virus reduction efficiency.

In addition, it was noted that operational conditions were not mentioned in several publications.

Since the determination factors of virus removal efficiency in wastewater treatment units must be beneficial information for wastewater engineers, it is recommended that future publications with regards to virus LR include detail operational conditions (HRT, time after membrane wash, etc) of the wastewater treatment units.

3.4.3 Unavailability of human enteric virus log reduction data for wastewater treatment unit processes

We could not retrieve sufficient number of publications regarding human virus reduction in MBR that met the systematic review criteria except norovirus GII and enterovirus. Similarly, we could not retrieve sufficient number of publications regarding human virus reduction in AS processes that met the systematic review criteria except rotavirus and noroviruses. We found that there was very limited number of, or completely no publications about the other wastewater treatment processes. In the multiple-barrier system, wastewater engineers have to connect wastewater treatment units to establish a wastewater reclamation system that can achieve the target LR values, which means that the accumulation of the virus LR information in the other wastewater treatment units, such as reverse osmosis (RO) and disinfection practices, that includes all required information for the meta-analysis is very critical and mandatory in the future study.

Chapter 4 : BACTERIOPHAGE REMOVAL EFFICIENCY AS A VALIDATION AND OPERATIONAL MONITORING TOOL FOR VIRUS REDUCTION IN WASTEWATER RECLAMATION: REVIEW

Water Research (2017), 121, 258-269

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ABSTRACT

The multiple-barrier concept is widely employed in international and domestic guidelines for wastewater reclamation and reuse for microbiological risk management, in which a wastewater reclamation system is designed to achieve guideline values of the performance target of microbe reduction. Enteric viruses are one of the pathogens for which the target reduction values are stipulated in guidelines, but frequent monitoring to validate human virus removal efficacy is challenging in a daily operation due to the cumbersome procedures for virus quantification in wastewater. Bacteriophages have been the first choice surrogate for this task, because of the well-characterized nature of strains and the presence of established protocols for quantification. Here, we performed a meta-analysis to calculate the average $\log_{10}$ reduction values (LRVs) of somatic coliphages, F-specific phages, MS2 coliphage and T4 phage by membrane bioreactor, activated sludge, constructed wetlands, pond systems, microfiltration and ultrafiltration. The calculated LRVs of bacteriophages were then compared with reported human enteric virus LRVs. MS2 coliphage LRVs in MBR processes were shown to be lower than those of norovirus GII and enterovirus, suggesting it as a possible validation and operational monitoring tool. The other bacteriophages provided higher LRVs compared to human viruses. The data sets on LRVs of human viruses and bacteriophages are scarce except for MBR and conventional activated sludge processes, which highlights the necessity of investigating LRVs of human viruses and bacteriophages in multiple treatment unit processes.

Keywords: bacteriophage, human virus, $\log_{10}$ reduction value, validation and operational monitoring, wastewater reclamation and reuse

4.1 INTRODUCTION

Reported outbreaks of viral infectious diseases caused by insufficiently treated wastewater (4, 74, 150) emphasizes the importance of wastewater treatment as a barrier for the virus transmission, especially for reclamation and reuse (28, 151). To ensure the microbiologically safe usage of reclaimed wastewater, multiple-barrier concept has been employed in international and domestic guidelines for wastewater reclamation (85, 152, 153). In the multiple-barrier concept, each unit process of wastewater treatment is assigned a credit value of pathogen reduction efficiency, and the total pathogen reduction efficiency of a treatment process chain is calculated as the sum of $\log_{10}$ reduction values (LRVs) of each process (6). For example, in the Groundwater Replenishment Reuse Project (GRRP) of the state of California, USA, performance target LRVs of 12 for viruses and 10 for both *Giardia* cysts and *Cryptosporidium* oocysts, are required when treated wastewater is used for groundwater recharge intended for indirect potable reuse (78). The Texas Water Development Board has proposed that water reclamation plants achieve LRV of 12 for viruses for direct potable reuse and has assigned 6 $\log_{10}$ upper end reduction of viruses by UV disinfection combined with advanced oxidation process (79). Wastewater engineers are required to combine a number of unit processes to exceed these performance target values when designing wastewater reclamation systems under the multiple-barrier concept.

During the operation of wastewater reclamation systems, there is a necessity to monitor the plant performance to make sure the particular plant provides recycled water of expected quality as stipulated by guidelines (80, 85). Four stages of a wastewater reclamation monitoring system are: 1) baseline monitoring to assess risks and impacts of the recycled water on the environment; 2) performance monitoring to validate performance required by the water quality management plan; 3) operational monitoring to ensure the system is operating within the design specification; and 4) verification monitoring at the end to confirm that the quality of recycled water complies with the water quality management plan and whether any modifications are necessary (86). Bacteriophages have been the most widely used microbial parameter for the second (validation) and third (operational) monitoring stages with respect to virus reduction efficiency in wastewater treatment processes. The USEPA guideline suggests that MS2 coliphage be used for on-site validation (85). The Australian guidelines recommend that indigenous *Escherichia coli* be monitored mandatorily and coliphages or other pathogens such as adenovirus or enterovirus be also monitored as representatives of viral contaminants (86). The state of Victoria, Australia provides guidelines on the usage of indigenous somatic coliphages or F-specific RNA (FRNA) bacteriophages, or seeded MS2 coliphage as suitable surrogates for enteroviruses when indigenous or seeded enteroviruses are not used in the validation process (80). MBR validation protocol published by WaterVal^{TM} program suggests that both somatic coliphages and FRNA bacteriophages must be used to validate MBR systems (84).

There are compelling reasons for the selection of bacteriophages for the monitoring over the usage of the human enteric viruses. Some investigations reported that bacteriophages were present in similar or higher numbers in water environments when enteric viruses were present (10, 11). In a previous meta-analysis study, Pouillot et al. (2015) reported a strong positive correlation (R^2 = 0.8) between LRVs of male-specific coliphages and human norovirus GII (87). Bacteriophage enumeration results can be obtained within 12 hrs with simpler techniques as opposed to enteric virus enumeration techniques that requires more time, cost and labor (9, 13). Recent research efforts aimed at reducing the process time required for bacteriophage enumeration up to 4 hrs by generating tailored host strains that can detect bacteriophage-induced cell lysis more quickly (88).

On the other hand, several studies have argued the drawbacks of bacteriophages from being a suitable validation and monitoring indicator for enteric virus removal in wastewater treatment. The most critical drawback is the inability to always obtain a clear correlation between LRVs of bacteriophages and human enteric virus (89). As an example, Ottoson et al. (2006) reported that the reduction efficiency of coliphages correlated least among tested microbial indicators with enterovirus genome reduction at a wastewater treatment plant (133). This is probably attributed to the difference between the quantification methods for bacteriophages and viruses in wastewater. Bacteriophages are commonly quantified with plaque assay using small volumes (1-5 mL) of diluted or undiluted samples, whereas enteric viruses are quantified by genome amplification using concentrated samples from large volumes (91, 92). The recovery efficiencies of viruses during concentration can vary depending on the methods used (91, 93). These methodological differences can in turn add considerable uncertainties and lead to weak correlations between LRVs of bacteriophages and human enteric viruses (88).

Even though there is no strong correlation between LRVs of bacteriophages and human viruses in wastewater treatment unit processes, bacteriophages may be used as an indicator for human viruses given that bacteriophage LRVs are almost always lower than those of human viruses, under the concept of multiple-barrier system. Human virus LRVs have been reviewed (6, 27, 62), but bacteriophage LRVs in multiple unit processes of wastewater treatment have not been reviewed so far. Therefore, we evaluated the suitability of bacteriophages as a tool to monitor and validate the performance of human enteric virus reduction at wastewater treatment plants, based on a meta-analysis of published bacteriophage LRVs. Here we present our analysis on inter- and intra-process variations between LRVs of bacteriophages and discuss influence of the variations on the usability of bacteriophages for validation and operational monitoring in a wastewater reclamation system.

4.2 METHODOLOGY

4.2.1 Screening of articles

Article screening for the meta-analysis was performed using Google Scholar and PubMed search engines. Relevant articles published before 2016 were searched using the keywords *bacteriophages*, *removal efficiency* and *wastewater treatment* with appropriate combination of Boolean connectors. We obtained 873 search results (May 2, 2016) and PubMed search results coincided with Google Scholar search, and therefore, the article numbers mentioned hereafter are based on the search results from Google Scholar.

Further screening of articles for the meta-analysis was performed according to the preferred reporting items for systematic reviews and meta-analyses (PRISMA) flow diagram used in clinical medicine (132). First, non-English articles were eliminated. Dissertations, book chapters, review articles, proceedings and other technical reports were also excluded from the analysis as shown in **Figure 4.1**.

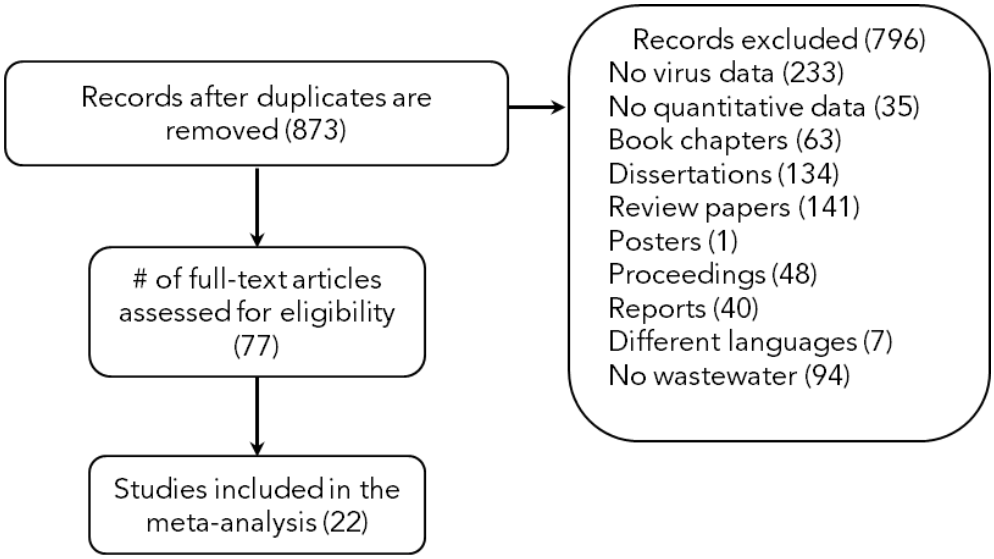

Figure 4.1: Article screening process according to PRISMA methodology. Numbers inside the parentheses expresses the number of articles selected for the next step or eliminated based on the criteria indicated in the methodology section. Finally, 22 articles were selected and used for the average LRV calculation.

Altogether 796 articles were eliminated in the first stage. In the second stage, 77 articles were further screened to confirm the availability of required parameters (peer-reviewed articles, paired influent and effluent bacteriophage concentrations, average, SD and number of samples). Finally we identified 22 articles satisfying the requirements and used them for the meta-analysis. These studies were performed mainly in North America and Europe with a few studies in East Asia. This geographical skewness is one of the properties of the datasets to note.

4.2.2 Extraction of data and calculation of average LRV

For the calculation of average LRVs achieved by each unit process, average LRV obtained in each study as well as SD and sample size were necessary. If these values were provided in a table (i.e., numerical form), the values were directly used. Data extraction from a figure was carried out if necessary using the Image J program provided by the National Institutes of Health. When the LRV or SD was not explicitly specified in a paper, LRVs are calculated using the available influent and effluent virus concentrations. SD is calculated using the variance sum law.

Plaque-forming unit (PFU) data were considered for the calculation of average LRV in this study as they outnumber RT-qPCR based data. Extracted data were classified based on treatment process types: membrane bioreactor process (MBR), conventional activated sludge process (CAS), constructed wetlands, pond systems, microfiltration (MF) and ultrafiltration (UF). Meta-analysis calculation was performed and the forest plot containing the average LRV and 95% confidence interval (CI) by each unit process was generated on Microsoft Excel package as described elsewhere (6, 137). Q test was employed in evaluating the heterogeneity among LRVs from individual studies. Q test was followed by χ^2 square distribution with k-1 degrees of freedom (k = number of studies used in a particular calculation) (154). The Q value larger than $Q_{critical}$ for the given degrees of freedom suggests heterogeneity of data and a correction is made by adjusting the weights of each study (6).

4.2.3 Evaluation of intra-process and inter-process LRV variations

Intra-process LRV variations between bacteriophage types and inter-process LRV variation of each bacteriophage type were evaluated using Welch's adjusted F ratio, because the assumption of homogeneity of variances was not supported for any LRV datasets by Levene's test (155). Measure of association was calculated using ω^2 (equation 1).

$$\text{est.}\omega^2 = \frac{df_{between}(F-1)}{df_{between}(F-1)+N_T} \quad \textbf{(1)}$$

If any groups provided a statistically significant F-ratio at $p < 0.05$ level, pairwise comparisons were performed using Games-Howell post-hoc test using equations 2, 3 and 4 to calculate q value and it is compared with the $q_{critical}$ value at $\alpha < 0.01$ using the studentized range distribution table. Pairwise comparisons with $q > q_{critical}$ were considered significantly different at $p < 0.01$ level (156).

$$q = \frac{\left|\overline{x_i} - \overline{x_j}\right|}{SE} \quad \textbf{(2),}$$

$$SE = \sqrt{\frac{1}{2}\left(\frac{var_i}{n_i} + \frac{var_j}{n_j}\right)} \quad \textbf{(3),}$$

$$df = \frac{\left(\frac{var_i}{n_i} + \frac{var_j}{n_j}\right)^2}{\frac{\left(\frac{var_i}{n_i}\right)^2}{n_i - 1} + \frac{\left(\frac{var_j}{n_j}\right)^2}{n_j - 1}} \quad \textbf{(4),}$$

where

$\overline{x_i}$ and $\overline{x_j}$: group means of concern,

var_i and $var_{j:}$: variance of group i and j,

n_i and n_j : number of samples in group i and j.

For all significant pairwise comparisons, Cohen's *d* was calculated considering d = 0.2, 0.5 and 0.8 as small, moderately and largely significant effect sizes, respectively. Welch's t-test was used to evaluate the statistical significance of somatic coliphages and F-specific phages LRVs by CAS process, vegetated wetlands, and non-vegetated wetlands.

Table 4.1: Data extraction, sample number and treatment methods applied in selected publications about bacteriophage removal in membrane bioreactor

Publication	Process chain	Analyzed bacteriophage	Sample number	HRT/ SRT	Data extraction procedure	Remarks
Zhang and Farahbakhsh (2007)(13)	Fine screen+ Aeration basin+ Membrane tank	Somatic coliphages F-specific bacteriophages	19	HRT = 6 h SRT = 18 d	Extraction from Figure 10 using Image J	
Wu et al. (2010) (157)		Somatic coliphages	26	HRT = 13.3 h SRT = 10 d	Extraction from Figure 8(b) using Image J	Surface area = 0.2 m^2
Zanetti et al. (2010)(158)	Screen + MBR + Disinfection	Somatic coliphages	20		Direct extraction from Table 1	Surface area = 0.8 m^2
De Luca et al. (2013)(159)	Screen + MBR	Somatic coliphages	18		Direct extraction from Table 1	Surface area = 0.8 m^2
Chaudhry et al. (2015)(65)	Screen + Anoxic/Oxic MBR + Disinfection	F-specific phages	14		Extraction from Figure 2 using Image J	Flow 5700 m^3/d
Fox and Stuckey (2015)(14)	MBR unit	MS2 coliphage/ T4 phage	28	HRT = 12 h	Extraction from Figure 2 and 3 using Image J	Surface area = 0.1 m^2
Zheng et al. (2005)(160)	MBR unit	T4 phage	5	HRT = 10.8 h	Extraction from Figure 4 using Image J	
Lv et al. (2006) (160)	MBR unit	T4 phage	21	HRT = 10.8 h	Extraction from Figure 3 using Image J	
Zheng and Liu (2007)(64)	MBR unit	T4 phage	5		Extraction from Figure 4 using Image J	

Table 4.2: Data extraction, sample number and treatment methods applied in selected publications about bacteriophage removal in conventional activated sludge process (CAS)

Publication	Process chain	Analyzed bacteriophage	Sample number	HRT/ SRT	Data extraction procedure	Remarks
Funderburg and Sorber (1985)(63)	Primary sedimentation + Flow equalization + Activated sludge process + Secondary sedimentation + Chlorination and Rapid sand filtration	Somatic coliphages	33		Direct extraction from Table 1 and 2	
Zhang and Farahbakhsh (2007)(13)	Primary clarifier + Aeration tank + Secondary clarifier + RBC + Sand filtration + Chlorination	Somatic coliphages	10	HRT = 7 h	Direct extraction from Figures 6 and 7	
		F-specific phages	10			
De Luca et al. (2013)(159)	Activated sludge process	Somatic coliphages	18		Direct extraction from Table 1	
		F-specific phages	18			
Ulbricht et al. (2014)(16)	Primary sedimentation + Activated sludge process + Secondary clarifier	Somatic coliphages	11	SRT = 12.1 d (winter) 10.9 d (summer)	Extraction from Figure 3 using Image J	Flow = 78983 m^3/d (winter), 62401 m^3/d (summer)
Flannery et al. (2012)(68)	Screening + Grit removal + Primary settlement + Aeration + Secondary settlement	F-specific phages	49		Direct extraction from Table 1	Flow = 45000 m^3/d
Hata et al. (2013)(91)	Activated sludge process + Chlorination + Sand filtration	F-specific phages		HRT = 9.3 h	Provided by author M. Kitajima	Flow = 450000 m^3/d

Table 4.3: Data extraction, sample number and treatment methods applied in selected publications about bacteriophage removal in constructed wetlands and pond systems

Publication	Process chain	Analyzed bacteriophage	Sample number	HRT	Data extraction procedure	Remarks
Nokes et al. (2003)(83)	Septic tank + Constructed wetland	Somatic coliphages	7		Direct extraction from Tables 1, 2 and 3	Wetland 1 (55.6 m^2) Wetland 2 (80 m^2) Wetland 3 (80.4 m^2)
Olson et al. (2005)(161)	Sub surface flow CW	Somatic coliphages	31	13 d	Direct extraction from Table 3	Area (148m^2)
Nasser et al. (1994)(162)	Anaerobic pond + Stabilization pond	F-specific phages	13	6 -10 d	Extraction from Figure 2 using Image J	Area (1500m^2) Depth (1m)
Lucena et al. (2004)(11)	Stabilization ponds	F-specific phages	20	49 d		4 ponds Total Area (11800m^2), Volume (13400m^3), 6-8 ˚C (Winter), 20-26 ˚C (Summer)

Table 4.4: Data extraction, sample number and treatment methods applied in selected publications about bacteriophage removal by microfiltration and ultrafiltration

Publication	Process chain	Analyzed bacteriophage	Sample size	Data extraction procedure	Remarks
Iranpour (1998)(163)	Activated sludge + Microfiltration + Reverse Osmosis	MS2 coliphage	37	Extraction of data from Figures 2, 3, 4 and 5 using Image J	Memcor MF unit (Model 3M10C)
Jolis et al. (1999) (164)	Microfiltration + UV Disinfection	MS2 coliphage	17	Extraction of data from Figure 3 using Image J	Memcor MF unit (Model 60M10)
Lu et al. (2013)(165)	Ultrafiltration	MS2 coliphage	9	Extraction of data from Figure 2 (a) using Image J	PVDF membrane from ZW-10 unit (Zenon)
Frohnert et al. (2015)(166)	Ultrafiltration	MS2 coliphage	16	Extraction of data from Figure 5	Membrane area 0.2m^2 Volume flow 16 l/h

4.3 RESULTS AND DISCUSSION

4.3.1 Intra-process LRV variation

MBR process

Average LRVs obtained by MBR process for somatic coliphages, F-specific phages, MS2 coliphage and T4 phage with 95% CI values are shown in **Figure 4.2.** In total, 9 articles provided data on LRVs of bacteriophages by MBR process (13, 14, 64, 65, 157–160, 167) (**Table 4.1**). Q-statistic detected heterogeneity among the data sets, and therefore, the random effects model suggested by Neyeloff et al. (2012) was used for the calculation of the average LRV.

There are two types of intra-process LRV variation, which are a virus type-specific variation and a variation among virus types. A virus type-specific variation, shown in each panel of **Figure 4.2**, is attributable to operational conditions in MBR systems, including membrane pore size, membrane integrity failures, solution environment, membrane charge, pH, foulant layer and membrane imperfections (160, 168, 169). A recent validation and operational monitoring protocol developed by the WaterVal^{TM} program also emphasizes the importance of operational conditions on virus reduction, in which a conservative virus LRV of 1.5 is assigned for MBR systems which have nominal pore sizes between 0.04 - 0.1 μm and operates at pH (6 - 8), DO (1 - 7 mg/l), temperature (16 - 30 ˚C), SRT (> 11d), HRT (> 6h), MLSS (> 3g/l), TMP (> 3kPa), flux (< 30 liters/m^2/h) and turbidity (0.2 NTU) after investigating full-scale WWTPs involving more than 200 data points for each parameter (84).

The cake layer formed on the surface of membrane and the irreversible fouling were shown to be influential for the variation in LRVs as particle passing efficiency depends on thickness of cake layer and membrane pore clogging (65). LRV of MS2 coliphage displayed an inverse relationship with the gas sparging rate where increasing the gas sparging rate from 2 liters/min to 10 liters/min resulted in a reduction of LRV from 2.67 to 1.75 **(Figure 4.2(c))** (14). Membrane fouling mitigation by the gas sparging may be the main reason for this observation because the higher gas sparging rates can decrease the growth rate of membrane foulant layer responsible for the virus retention. The other studies using the virgin membranes also provided a virus LRV of only less than 1 compared to 2 - 3 obtained by the Chaudhry et al. (2015) for a full-scale MBR system which had been in operation for more than 10 years (12, 65, 157, 170, 171). Irreversible fouling is described as the plausible cause and it is further strengthened by the study of ElHadidy et al. (2014) which reported an increase of both MS2 coliphage and φX174 phage LRVs by 2.5 after the hydraulically irreversible fouling of the membrane was developed (172).

Nominal pore size of the membrane is apparently an important factor for virus reduction. In the Lv et al. (2006) study, a LRV of 4.59 was obtained for T4 phage using a 0.22 μm membrane while

the LRV of T4 phage was improved to 6.05 by using a membrane with nominal pore size of 0.1μm. The effect of membrane pore size was also confirmed in previous studies by filtering *E. coli* phages using 0.2 μm, 0.1 μm and 13000 Da molecular weight cut-off membranes (173).

Bacteriophage diversity can also significantly affect the virus type-specific LRV variation (15, 174). In this study, MS2 coliphage, genogroup-I (GI) FRNA coliphage, displayed much lower CI compared to the overall GI FRNA coliphages (**Figure 4.2(b)** and **(c)**). FRNA bacteriophages has been classified into four genogroups GI-GIV (175). Hata et al. (2013) quantified LRVs of these F-specific phages by RT-qPCR using genogroup specific assays and observed the LRVs in GIII > GII > GI order. Haramoto et al. (2015) calculated genogroup-dependent LRVs using the plaque assay and also observed the lowest LRVs with GI phages. The genogroup-dependency of the bacteriophage reduction resembles the genotype-dependent human norovirus reduction by microfiltration, in which specific interactions between norovirus particles and histo-blood group antigen-like bacterial substances were influential (176). It is possible to consider the interactions of varying degrees between wastewater solids and bacteriophages of different genogroups can lead to different LRVs during the treatment process.

The other type of intra-process variation in LRVs is observed when the average LRVs are compared among panels in **Figure 4.2**. The lowest average LRV (1.99 with 95% CI between 1.76 and 2.22) was obtained for MS2 coliphage, in which membranes with pore sizes larger than 0.1 μm were used. This was followed by somatic coliphages [3.96 (3.57, 4.35)], F-specific phages [4.63 (4.07, 5.19)] and T4 phages [5.54 (5.33, 5.75)]. Welch's adjusted F ratio (3, 78.95) is 97.53 ($p < 0.001$), which means that not all the bacteriophages are equally removed by MBR process. Estimated ω^2 of 0.63 indicates that approximately 63% of the total variation in the average LRV by MBR is attributable to the difference of bacteriophage type. Pairwise comparisons by Games-Howell post hoc test revealed that there are significant differences in LRVs between somatic coliphages and MS2 coliphage, somatic coliphages and T4 phage, F-specific phages and MS2 coliphage, and MS2 coliphage and T4 phage, while LRVs between "somatic coliphages and F-specific phages" and "F-specific phages and T4 phage" displayed no significant differences in LRVs by MBR at $p < 0.01$ level **(Table 4.5)**. Cohen's *d* values calculated for the significantly different pairs show significant effect sizes for all pairs.

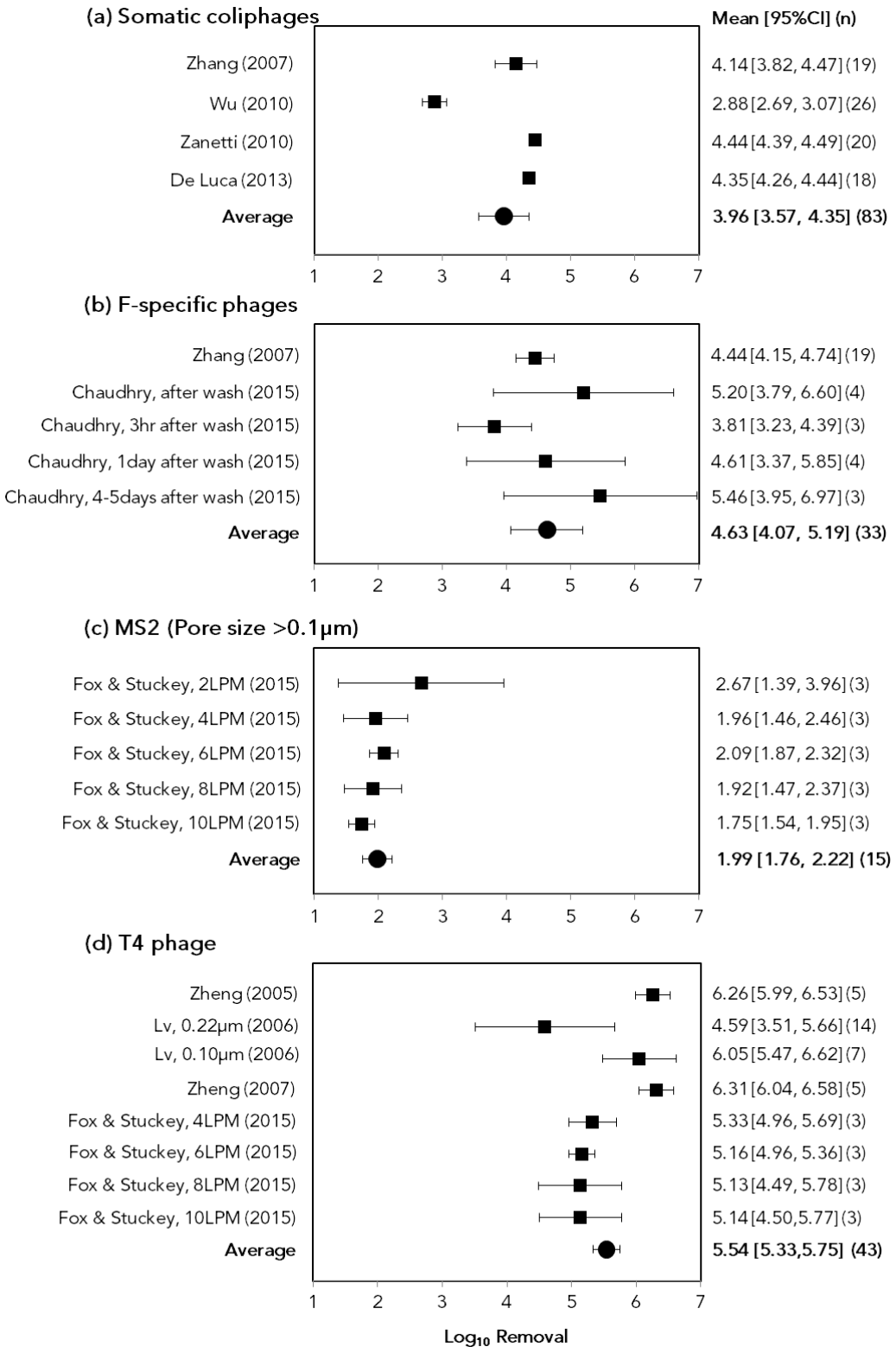

Figure 4.2: Reduction of (a) somatic coliphages (b) F-specific phages (c) MS2 coliphage and (d) T4 phage by MBR process. Black square is the average LRV obtained in each study, and the bar indicates 95% confidence interval (CI). Black circle shows the average LRV calculated using the meta-analysis approach. Sample size in each study is indicated in parenthesis next to the 95% CI values. LPM stands for liter per minute.

Table 4.5: Pairwise comparison scores obtained by Games-Howell post hoc procedure on the significance between the average LRV of different bacteriophages by MBR process

Pair	df	q	$q_{critical}$	Significance at $p < 0.01$	Cohen's *d*
Somatic coliphage : F-specific phage	65.21	2.71	4.58	No	
Somatic coliphage : MS2 coliphage	92.51	12.29	4.53	Yes	1.17
Somatic coliphage : T4 phage	108.94	7.74	4.51	Yes	0.95
F-specific coliphage : MS2 coliphage	39.56	12.25	4.70	Yes	1.84
F-specific coliphage : T4 phage	61.46	3.65	4.59	No	
MS2 coliphage : T4 phage	55.39	21.63	4.61	Yes	3.04

One reason for the variation in LRVs among virus types can be the virus type-dependent properties of bacteriophage particles, including surface characteristics. Chaudhry et al. (2015) analyzed the effect of virus surface characteristics on virus removal mechanisms inside MBRs and found that virus attachment to biomass was the most influential mechanism depending on the virus type. One influencing factor was the number of hydrophobic amino acids in the external capsid surface. Depending on the number of hydrophobic amino acid groups in the external capsid of the bacteriophage, it can interact more freely with the hydrophobic portions of the bacterial flocs and extracellular polymeric substance (EPS) in MLSS which in turn leads to a higher LRV (12).

Ueda and Horan (2000) observed a positive correlation between the bacteriophage removal efficiency and the increment of bacteriophage concentration in the bulk solution inside the MBR (171). Hirani et al. (2012) studied the removal of MS2 coliphages in 9 different MBR systems located in two wastewater treatment plants (WWTPs). The influent concentrations of indigenous MS2 coliphages for these WWTPs were different (95% of the samples showed less than 4.7 $\log_{10}$ and less than 5.8 $\log_{10}$ PFU/100ml of MS2 coliphages in the influent). There was a general trend of increasing LRV with the increment of coliphage concentration in influent, and the correlation coefficient between the influent concentration of indigenous MS2 coliphage and the LRV in the MBR systems was 0.81 (66). However, a clear reason for the increment of bacteriophage reduction with the increasing influent bacteriophage concentration is not explained explicitly in the literatures and therefore may not be conclusive.

Somatic coliphage and F-specific phage LRVs by CAS process

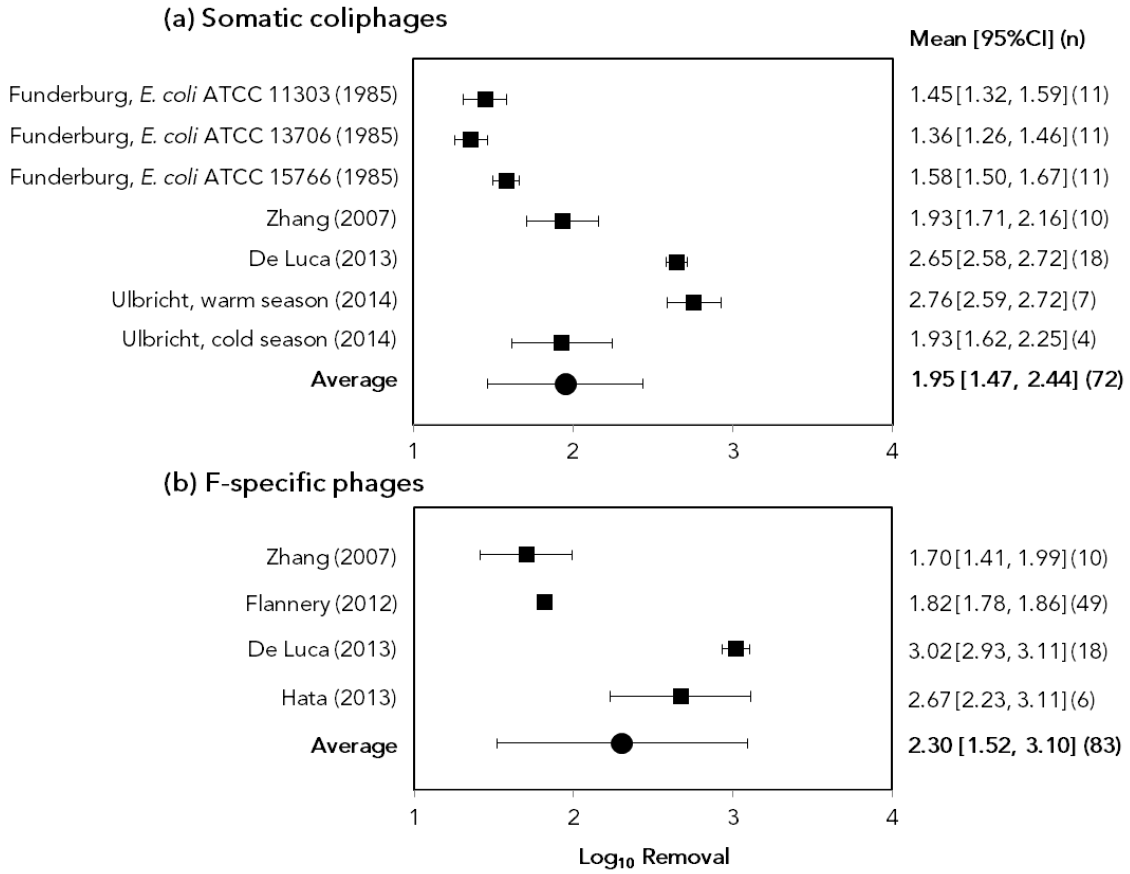

Figure 4.3: Reduction of (a) somatic coliphages and (b) F-specific phages by CAS process. Black squares is the average LRV obtained in each study, and the bar indicates 95% confidence interval (CI). Black circle shows the average LRV calculated using the meta-analysis approach. Sample size in each study is indicated in parenthesis next to the 95% CI values. *E. coli* strain number is associated with Funderburg (1985).

Six articles provided the required quantitative data and were used for the calculation process of average LRV by CAS process (13, 16, 63, 68, 91, 159). Similar to the MBR process data (**Figure 4.2**), heterogeneity was observed among the studies and therefore random effects model was used for the calculations. Average LRV of 1.95 with 95% CI between 1.47 and 2.44 was obtained for somatic coliphages while F-specific phages displayed an average LRV of 2.30 (1.52, 3.10) **(Figure 4.3)**.

There was not a statistically significant difference between these average LRVs with a Cohen's d value of 0.12. Meanwhile, virus type-specific variations were observed in the panels of **Figure 4.3**. As well as the case for MBR systems, operational conditions can be considered responsible for this intra-process LRV variation. One of the operational conditions that affect LRV is the concentration of mixed liquor suspended solids (MLSS). Shimohara et al. (1985) observed higher

LRVs for poliovirus 1 with increasing MLSS levels (177). However, several studies included in the meta-analysis didn't provide the data on MLSS levels in the studied CAS systems which hamper the confirmation of MLSS concentration as a factor for intra-process LRV variation.

In addition to the LRV variations owing to the operational conditions, a seasonal variation of the LRV of somatic coliphages was observed ($p < 0.0001$) **(Figure 4.3(a))** (16). In CAS process, lower concentrations of somatic coliphages are detected in the secondary clarifier during the summer season which leads to higher LRVs (16). Possible reasons are the viral capsid damage due to higher temperatures and the increased rate of production of harmful metabolic byproducts due to the enhanced microbial metabolism (178).

Somatic coliphage and MS2 coliphage LRVs by constructed wetlands

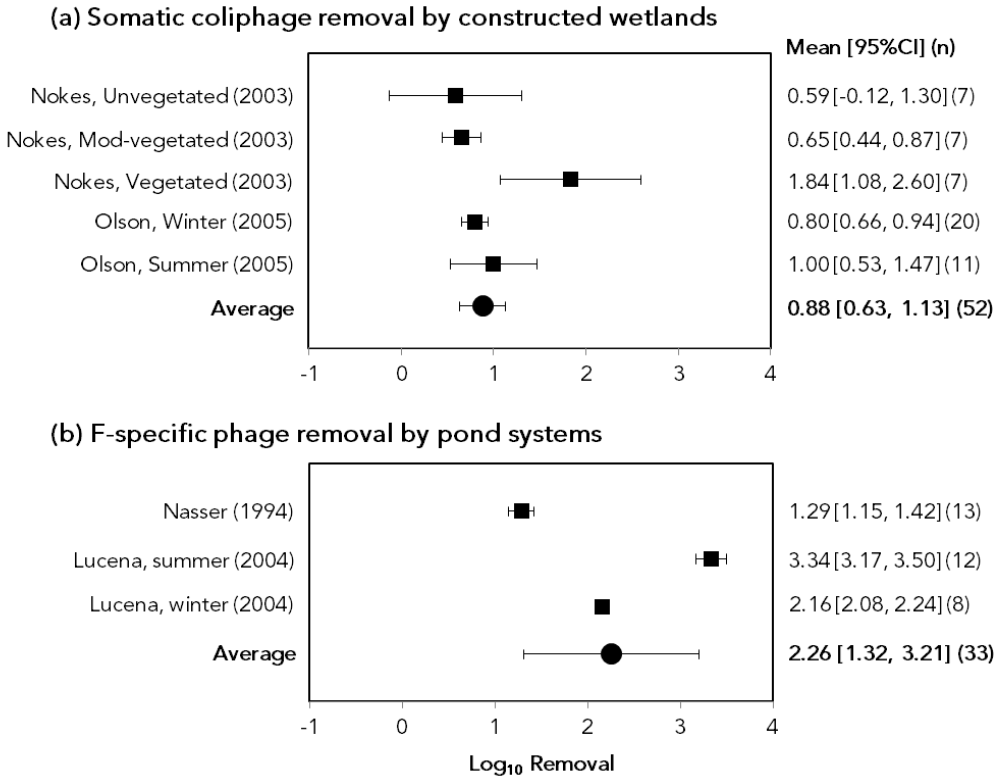

Figure 4.4: Reduction of (a) somatic coliphage by constructed wetlands and (b) F-specific phages by pond systems. Black square is the average LRV obtained in each study, and the bar indicates 95% confidence interval (CI). Black circle shows the average LRV calculated using the meta-analysis approach. Sample size in each study is indicated in parenthesis next to the 95% CI values.

The LRV of somatic coliphages by constructed wetlands was analyzed in two studies considering the vegetation and seasonal conditions **(Figure 4.4(a))** (83, 161) (Table 4.3). Q-statistic calculation displayed heterogeneity among the studies and therefore random-effects model was used for the LRV calculation. An average somatic coliphage LRV of 0.88 was obtained with 95% CI

between 0.63 and 1.13. MS2 coliphage LRV is not shown in Figure 4.4(a), however there is only one study about the MS2 coliphage removal by constructed wetlands (179). This study investigated LRVs of MS2 coliphage at three different wetlands and reported that the constructed wetlands provided an average LRV of 3.18 with 95% CI between 2.85 and 3.50 (179).

Vegetated wetlands have shown identical or higher LRVs compared to unvegetated wetlands as confirmed by the Welch's t-test ($p < 0.05$) (83). Higher removal of bacteriophages is due to filtering or adsorption to the root-substrate complexes and associated biofilms (180). Comparison between the summer LRVs obtained by Nokes et al. (2003) and Olson et al. (2005) provides clear evidence on the importance of temperature on virus removal by constructed wetlands. Nokes et al. (2003) did in Arizona with an average summer temperature of 25.6 ˚C and obtained almost 2 times higher LRVs than Olson et al. (2005), which studied in Minnesota with an average summer temperature of 15.4 ˚C. Temperature effects on bacteriophage removal in constructed wetlands is further strengthened by Olson et al. (2005) which failed to provide significant differences in LRVs of somatic coliphages between the summer and winter seasons where the temperature difference was only 6.6 ˚C.

F-specific phage LRVs by pond systems

Two studies reported on the reduction of F-specific phages using pond treatment systems (11, 162) (**Table 4.3**). Lucena et al. (2004) studied the LRV of F-specific phages in summer and winter whereas Nasser et al. (1994) studied only in summer. An average LRV of 2.26 was observed with 95% CI between 1.32 and 3.21. The range of LRV varied between 1.29 and 3.34 **(Figure 4.4(b))**. All the calculations were done using the random effect model because heterogeneity was observed by Q-statistic. The variations between individual studies compared to the calculated average LRV were in the low and moderate range according to the Cohen's *d* calculation.

Similar to wetlands, pond systems also displayed higher reduction of bacteriophages in the summer compared to winter ($p < 0.0001$), in which temperature difference between summer and winter was approximately 18 ˚C (11). However, there is a 2 $\log_{10}$ difference in the summer LRV of pond systems between the studies by Nasser et al. (1994) and Lucena et al. (2004). One possible explanation is the retention time where previous one had only 6-10 days while the latter had 49 days. Due to the shorter retention time, the possibility of exposing to adverse environmental conditions also reduces. Moreover, the summer LRV obtained by Nasser et al. (1994) is even smaller than the winter LRV by Lucena et al. (2004), which may be attributed to the thermal stratification and short circuiting (162).

MS2 coliphage LRVs by microfiltration and ultrafiltration

Four studies provided the information on the LRVs of MS2 coliphage by microfiltration and ultrafiltration (163–166) (**Table 4.4**). The average LRVs obtained for MS2 coliphage by microfiltration and ultrafiltration (UF) membranes are shown in **Figure 4.5**. All the calculations were done using the random effects model as the heterogeneity was observed among the studies. Average LRV of MS2 coliphage by microfiltration membranes was 1.38 with 95% CI between 0.70 and 2.07 (**Figure 4.5(a)**). In the case of UF membranes, the average LRV was 3.69 with 95% CI between 2.87 and 4.52 **(Figure 4.5(b))**.

In the microfiltration of MS2 coliphage, LRVs obtained by Iranpour (1998) using a pristine membrane for the filtration test displayed more than 2 $\log_{10}$ lower LRVs compared to the study by Jolis et al. (1999) (**Figure 4.5(a)**), in which a membrane with an attached biofilm was used (163, 164). Importance of the biofilm on the higher LRV of bacteriophages was strengthened by the results reported by Lu et al. (2013). Importance of the membrane integrity on the LRV is highlighted in the study by Jolis et al. (1999) where the membrane integrity failure (**Figure 4.5(a)**, Test 2) caused a decrease in the virus reduction capability of the membrane.

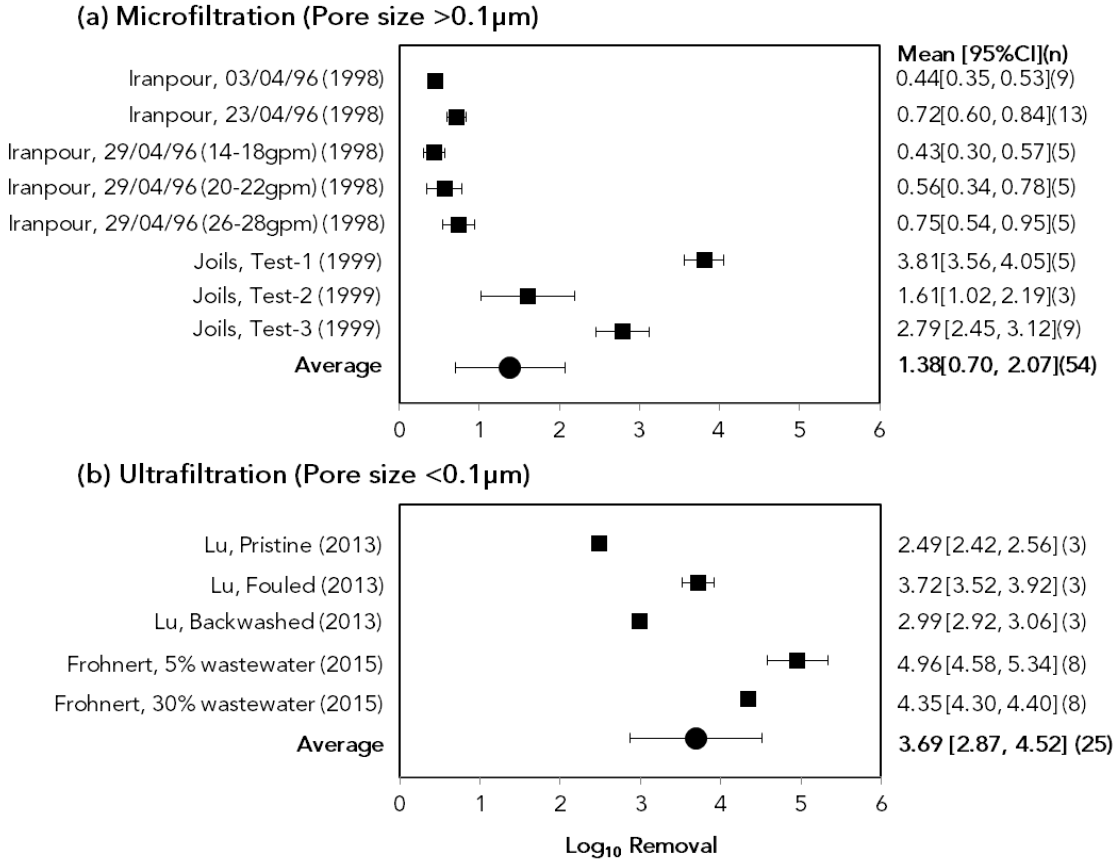

Figure 4.5: Reduction of MS2 coliphage by (a) microfiltration (pore size > 0.1µm) and (b) ultrafiltration (pore size < 0.1µm). Black square is the average LRV obtained in each study, and the

bar indicates 95% confidence interval (CI). Black circle shows the average LRV calculated using the meta-analysis approach. Sample size in each study is indicated in parenthesis next to the 95% CI values.

4.3.2 Inter-process LRV variations

Inter-process LRV variations were observed for each virus type. Somatic coliphage average LRV was 3.96 [95% CI 3.57- 4.35] using MBRs (**Figure 4.2(a)**) whereas LRV of 1.95 [1.47, 2.44] was obtained by CAS systems (**Figure 4.3(a)**). F-specific phage average LRV was 4.63 [95% CI 4.07-5.19] using MBRs (**Figure 4.2(b)**) whereas average LRV of 2.30 [1.52, 3.10] was obtained by CAS systems (**Figure 4.3(b)**). The multiple comparison using Welch's test revealed that there were statistically significant differences in the average LRV of a particular bacteriophage by different unit processes at $p < 0.001$ level (**Table 4.6**).

Table 4.6: Multiple comparison of average log10 reduction values of somatic coliphages, F-specific phages, and MS2 coliphage between wastewater treatment unit processes

	Number of groups	df_1	df_2	F	p	ω^2
Somatic coliphages	3	2	132.61	83.34	< 0.001	0.443
F-specific phages	3	2	79.24	15.39	< 0.001	0.162
MS2 coliphage	3	2	49.16	10.27	< 0.001	0.165

Approximately 44% of somatic coliphage removal, 16% of F-specific phage removal and 17% of MS2 coliphage removal were attributable to the difference in treatment unit types. Significant differences in the LRVs of a particular bacteriophage by different treatment processes were found by pairwise comparisons using Games-Howell post hoc test at $p < 0.01$ except CAS-pond systems and MBR-MF systems (**Table 4.7**). Cohen's *d* calculation displayed moderate to high effect sizes (**Table 4.7**).

Table 4.7: Pairwise comparison scores obtained by Games-Howell post hoc procedure on the significances in the average LRV of bacteriophages by different treatment unit processes

Pair	df	q	$q_{critical}$	Significance at p<0.01	Cohen's *d*
Somatic coliphages					
MBR-CAS	142.18	8.93	4.20	Yes	1.03
MBR-Wetlands	128.12	18.28	4.20	Yes	2.01
CAS-Wetlands	103.83	5.44	4.21	Yes	0.63
F-specific phages					
MBR-CAS	112.30	6.67	4.21	Yes	0.73
MBR-Ponds	52.05	5.98	4.31	Yes	0.98
CAS-Ponds	77.39	0.10	4.25	No	
MS2 coliphage					
MBR-MF	61.13	2.37	4.28	No	
MBR-UF	27.20	5.81	4.49	Yes	1.09
MF-UF	58.97	6.15	4.29	Yes	0.98

The superior LRV of MBR to that of CAS systems is presumably due to the effect of the physical/mechanical removal mechanisms involved in the MBR processes. In CAS systems, the main mechanisms of virus removal are the attachment to solids (63), predation (181, 182) and inactivation (183) while MBRs additionally utilize the mechanisms of size separation and virus attachment to the membrane biofilm, which provides superior effluent quality (157).

For MS2 coliphage, the average LRV for MBR was 1.99 $\log_{10}$ [1.76, 2.22] (**Figure 4.2(c)**), which is relatively lower among bacteriophages indicated in **Figure 4.2**. There were no available datasets for the meta-analysis about LRVs of MS2 coliphage in CAS process, but Wen et al. (2009) performed a laboratory experiment on the removal of MS2 coliphage by CAS process and reported LRVs of 2.1 ± 0.44 and 1.85 ± 0.25, which are comparable with the LRV of MS2 coliphage by MBR (184). One possible reason for the relatively lower LRV of MS2 coliphage in MBR is the gas sparging used in the study by Fox and Stuckey (2015). The gel and cake layer formed on membrane surface contributes to the virus reduction improvement in MBR significantly (65), while at higher gas sparging rates, fouling layer generation rate drops and the virus reduction capability of MBR declines (14). It was further evinced in the same study where the lower gas sparging rate resulted in a higher LRV of MS2 coliphage.

4.3.3 LRV calculation procedure

Calculations of LRVs were performed using only the samples with both detectable influent and effluent virus concentrations in each sampling event. This procedure may result in underestimation of the virus reduction performance (Sano et al. 2016). We obtained the LRVs of 4.59 and 6.05 for T4 phage by membranes with 0.22 μm and 0.1 μm pore sizes, respectively, based on the datasets from Lv et al. (2006) (**Figure 4.2(d))**, while authors claimed that the obtained LRV was about 6.3 (160). This discrepancy is due to the difference in the treatment of non-detected data. In this study, only paired influent and effluent samples which had T4 phage concentrations above detection limit were considered for the LRV calculations. On the other hand, Lv et al. (2006) substituted zero with non-detect in effluent, which would result in the overestimation of LRV. A dataset containing non-detects is called a left-censored dataset, and the substitution of a non-detect with a specific value, such as zero or a detection/quantification limit value, has been regarded statistically non-preferable (142, 185). Some statistical approaches have been proposed to estimate representative values such as mean and SD (143, 186, 187), and the use of Bayesian estimation method is recommended for expressing uncertainty and variability of virus concentration in water (188). Several studies were excluded from the calculation of average LRVs in this study because of the left-censored data issue, even though important datasets about the reduction of bacteriophages by different treatment unit processes were presented. For example, Purnell et al. (2015) and Hirani et al. (2012) studied the removal of bacteriophages by full-scale MBR systems, but bacteriophages were not detected in the effluent due to the superior removal capability of MBR (66, 189). Statistically correct calculation of virus LRVs when no virus is detected in effluent must be a challenging and important topic in future studies. It is strongly recommended for authors that the every point value of virus concentration in influent and effluent be presented even in supplementary materials in a publication of virus reduction efficiency, which enables precious datasets to be included in future meta-analysis studies.

4.3.4 Bacteriophages as validation and operational monitoring indicators of human enteric virus

The main objective of this study is to analyze the suitability of bacteriophages as validation and operational monitoring indicators for human enteric virus log reduction in wastewater reclamation unit processes as suggested by the water reclamation guidelines. We observed intra-process and inter-process LRV variations by unit processes mainly because of the operational conditions, bacteriophage diversity, and surface characteristics of bacteriophages whereas inter-process variations were due to operational conditions and virus removal mechanisms in a particular unit process.

If a statistically significant correlation between the bacteriophage LRVs and human virus LRVs is validated, the obtained correlations can then be used as references to estimate the human virus LRV using the bacteriophage LRV data. However, the universal indicator bacteriophages to be used in the validation and operational monitoring of human enteric viruses have not been identified

(85, 88). In this section, we discuss the suitability of indicator bacteriophages as the validation and operational monitoring parameters based on the results from our meta-analysis.

Bacteriophage LRVs calculated in this study and human virus LRVs (Sano et al. 2016) were compiled in **Figure 4.6**. T4 phages have shown to be removed effectively by MBR treatment process, which is comparable with other studies reporting similar LRV of 5-7 (14). The size of the T4 phage is significantly larger compared to single-stranded RNA bacteriophages (~30nm), which may be reflected in the higher LRVs of T4 phage. These LRVs of T4 phages in MBR are fairly higher than those of human viruses (**Figure 4.6**), which make it difficult to use the T4 phage LRV as an indicator for human virus LRVs in MBR.

Somatic coliphages and F-specific phages provided comparable LRVs of 3.96 and 4.63 respectively by MBR processes (**Figure 4.2(a)** and **(b)**). Somatic coliphages and F-specific phages consist of diverse species, and thus the calculated LRVs must be averaged ones among different species. These LRVs of somatic coliphages and F-specific phages are higher than those of norovirus GII (3.35) and enteroviruses (2.71) in MBR (Sano et al. 2016), as indicated in **Figure 4.6**. This means that the LRVs for bacteriophages do not ensure the same level of reduction of human viruses, even though the difference in LRVs is within 2 $\log_{10}$.

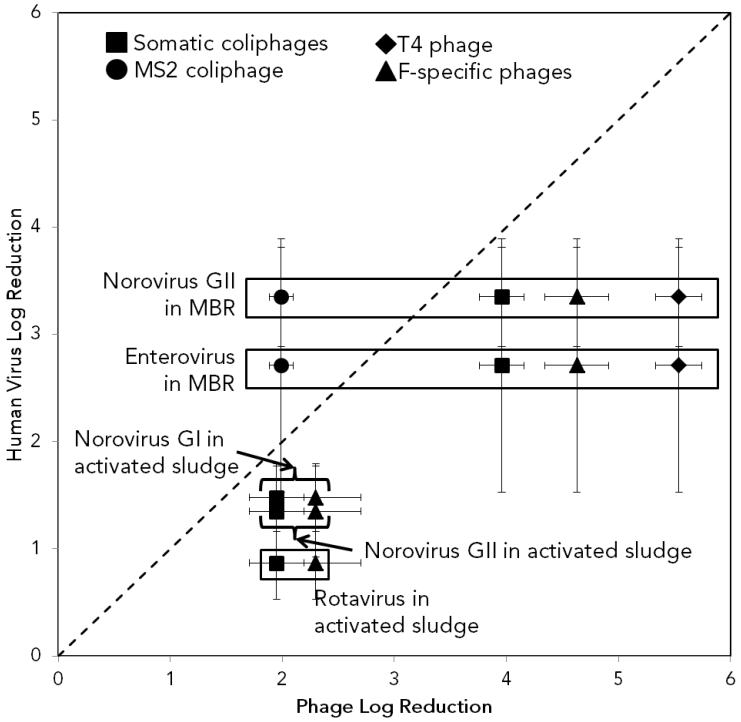

Figure 4.6: Comparison of $\log_{10}$ reduction values (LRVs) between human viruses and bacteriophages. LRVs of bacteriophages were obtained in this study, and those of human viruses were acquired from Sano et al. (2016). The dot line indicates the ratio of 1 between human virus and bacteriophage LRVs.

Several studies have determined the LRVs of each genogroups of FRNA phages. For example, Haramoto et al. (2015) reported that an average LRV of 2.69 was obtained for F-specific phages using plaque assay for a CAS process (15). They analyzed the LRVs of different genogroups of FRNA phages and reported a 0.91 $\log_{10}$ reduction for GI FRNA phages whereas GII and GIII phages showed a higher reduction of greater than 3 $\log_{10}$. Hata et al. (2013) also observed that 0.83 ± 0.25, 2.50 ± 0.35, and 2.86 ± 0.25 $\log_{10}$ reductions were obtained for GI, GII, and GIII FRNA phages, respectively, as determined by genogroup-specific RT-qPCR assays. These observations evince that the virus reduction efficiency of F-specific phages has a genogroup-dependent variation where GI FRNA phages are most persistent in wastewater treatment unit processes (15, 88, 91). Instead of measuring the F-specific phages LRVs in its entirety, monitoring of GI may be preferable as a validation and operational monitoring indicator for human enteric viruses (190, 191).

The reduction efficiency of MS2 coliphage, a member of GI FRNA phage, in MBR was lower than norovirus GII and enteroviruses (**Figure 4.6**). Usage of MS2 coliphage removal data is recommended for validation and operational monitoring of water reclamation processes by several authorities (California State Water Resources Control Board, 2015; Grabow, 2001; US Environmental Protection Agency, 2012; Victoria Department of Health, 2013). Our calculations indicated that an average LRV of 1.99 for infectious MS2 coliphage was obtained in MBR systems using microfiltration membranes, which are preferably used in MBR (193), and it is comparable with the LRV of 2.25 obtained by a full-scale MBR system (189). WaterVal^{TM} MBR validation protocol also provides a conservative LRV of 1.5 for virus removal by MBR systems. Among all the bacteriophage removal studies using MBR systems, MS2 coliphage displayed the lowest LRV, which explains the highest persistence in wastewater and therefore the suitability as an indicator for the human enteric virus removal monitoring (194). Langlet et al. (2008) reported that the aggregation of MS2 coliphage particles did not occur at pH values above the isoelectric point (pI) which is similar to the results obtained by the study of aggregation of norovirus GI.1 and GII.4 virus-like particles (VLPs) (195, 196). Based on the meta-analysis results and previously published knowledge, we can conclude that MS2 coliphage is the best among bacteriophages as a validation and operational monitoring indicator of human enteric virus removal in MBR. As for the other unit processes, it is necessary to accumulate LRV datasets of MS2 coliphage and human viruses to show the availability of this bacteriophage as a universal removal indicator of human viruses.

4.4 CONCLUSIONS

- Meta-analysis results displayed intra- and inter-process variations in bacteriophage reduction efficiency in wastewater treatment unit processes.
- The intra-process LRV variation mainly depends on operational conditions, while bacteriophage diversity and surface characteristics of bacteriophages are additional factors for the LRV variation.
- The inter-process LRV variation is due to the difference in virus removal mechanisms involved in a particular unit process.
- MS2 coliphage shows lower LRVs compared to human viruses and is suggested as a validation and operational monitoring indicator in MBR.

Chapter 5 : BACTERIAL HISTO-BLOOD GROUP ANTIGENS CONTRIBUTING TO GENOTYPE-DEPENDENT REMOVAL OF HUMAN NOROVIRUSES WITH A MICROFILTRATION MEMBRANE

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ABSTRACT

We demonstrated the genotype-dependent removal of human norovirus particles with a microfiltration (MF) membrane in the presence of bacteria bearing histo-blood group antigens (HBGAs). Three genotypes (GII.3, GII.4, and GII.6) of norovirus-like particles (NoVLPs) were mixed with three bacterial strains (*Enterobacter* sp. SENG-6, *Escherichia coli* O86:K61:B7, and *Staphylococcus epidermidis*), respectively, and the mixture was filtered with an MF membrane having a nominal pore size of 0.45 μm. All NoVLP genotypes were rejected by the MF membrane in the presence of *Enterobacter* sp. SENG-6, which excreted HBGAs as extracellular polymeric substances (EPS). This MF membrane removal of NoVLPs was not significant when EPS was removed from cells of *Enterobacter* sp. SENG-6. GII.6 NoVLP was not rejected with the MF membrane in the presence of *E. coli* O86:K61:B7, but the removal of EPS of *E. coli* O86:K61:B7 increased the removal efficiency due to the interaction of NoVLPs with the exposed B-antigen in lipopolysaccharide (LPS) of *E. coli* O86:K61:B7. No MF membrane removal of all three genotypes was observed when *S. epidermidis*, an HBGA-negative strain, was mixed with NoVLPs. These results demonstrate that the location of HBGAs on bacterial cells is an important factor in determining the genotype-dependent removal efficiency of norovirus particles with the MF membrane. The presence of HBGAs in mixed liquor suspended solids from a membrane bioreactor (MBR) pilot plant was confirmed by immune-transmission electron microscopy, which implies that bacterial HBGAs can contribute to the genotype-dependent removal of human noroviruses with MBR using MF membrane.

Keywords: extracellular polymeric substance, histo-blood group antigen, human norovirus, lipopolysaccharide, microfiltration, membrane bioreactor

5.1 INTRODUCTION

Noroviruses are single-stranded, non-enveloped, positive-sense RNA viruses that belong to family *Caliciviridae* (33). Currently there are more than 40 genotypes, which are divided into 6 major genogroups (GI-GVI) with a proposed seventh (36, 38). The GI, GII, and GIV strains infect humans (38). Human noroviruses (HuNoVs) are responsible for more than 90% of non-bacterial epidemic gastroenteritis cases and attributable to 18% of acute gastroenteritis patients worldwide with 15.1 million disability-adjusted life years (DALYs) (42, 43, 197). Outbreaks of HuNoV infection can occur in various community settings such as hospital wards, cruise ships, and restaurants and can affect all age groups, with particularly higher infection risks to organ transplant and immune-compromised patients (29).

Wastewater is a major transmission source of HuNoVs (198, 199). In 2012, a large outbreak of HuNoV infection (more than 10,000 gastroenteritis patients) in eastern Germany was likely caused by frozen strawberries imported from China (74). Multiple genotypes of HuNoVs were found from gastroenteritis patients, which supported the speculation that the imported strawberries were cultivated with untreated wastewater (76). Other previous studies also reported that reclaimed wastewater used for irrigation (73) and recreational activities (75) has caused HuNoV outbreaks. These cases exemplify the importance of the efficient removal of HuNoVs in wastewater treatment for reducing infection risks of HuNoVs through reclaimed wastewater.

The effects of the attachment of viral particles to a variety of wastewater solids, in which one of the main components is organic matter including bacterial cells, on the removal efficiency of enteric viruses from wastewater has been studied (7, 18, 135, 200, 201). Understanding the binding affinity and specificity of NoV particles to wastewater solids is thus crucial for achieving efficient removal of this human pathogen in wastewater treatment processes (202). However, the wide variety of wastewater solids makes it difficult to characterize the attachment of HuNoVs in wastewater, and the factors that affect the binding state of HuNoVs in wastewater have not been very well identified.

In our previous study, *Enterobacter* sp. SENG-6 bearing histo-blood group antigens (HBGAs) was isolated and proven to specifically capture noroviral particles (128). Human HBGAs are carbohydrates determining ABO and Lewis blood types. They have been suggested to be important factors for HuNoV infection of intestinal epithelial cells (101, 106). Human HBGAs are found on mucosal epithelial cells and excreted in body fluids, including the saliva of secretor-positive (Se+) individuals (101). The association between human HBGAs and HuNoV infection has been extensively studied using in-vitro binding assays, human volunteer challenge studies, and outbreak investigations (100, 107, 108). The binding of HuNoV particles with non-human HBGAs has been also investigated, and it has been reported that the genotype-dependent bioaccumulation of HuNoVs in oyster is caused by the attachment to HBGA-like substances expressed on oyster tissue surface

(203, 204). Since *Enterobacter* sp. SENG-6 excretes HBGAs as extracellular polymeric substances (EPS) (128), the binding of HuNoVs to bacterial HBGAs may affect the persistency of HuNoVs in environments. As an example of the effect of HBGA-positive bacteria on the persistent property of HuNoVs, Li et al. (2015) reported that HBGA-expressing *Escherichia coli* maintained the antigen integrity and mucin-binding ability of NoVLPs after heat treatment (129). However, the effect of bacterial HBGAs on the life cycle of HuNoVs, including the environmental persistency and removal properties in water and wastewater treatment processes, has not been fully investigated.

In this study, we tested the removal efficiency of norovirus-like particles (NoVLPs) associated with HBGA-positive enteric bacteria using a microfiltration (MF) membrane. Two HBGA-positive bacteria and one HBGA-negative bacterium were separately mixed with three genotypes of NoVLPs and filtered by an MF membrane installed in a spin column. The reduction of NoVLP in the filtrate was detected with enzyme-linked immunosorbent assay (ELISA). The presence of HBGAs in mixed liquor suspended solids (MLSS) from a membrane bioreactor (MBR) pilot plant was analyzed by immuno-transmission electron microscopy (TEM), and the effect of the localization of bacterial HBGAs on the genotype-dependent removal of noroviral particles with an MF membrane was discussed.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains and norovirus genotypes

Three bacterial strains (*Enterobacter* sp. SENG-6, *E. coli* O86:K61:B7, and *Staphylococcus epidermidis*) and three norovirus genotypes (GII.3, GII.4 and GII.6) were used in this study (Table S1). *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7 (ATCC 12701) have HBGAs in EPS and lipopolysaccharides (LPS), respectively (128, 205). *S. epidermidis* (ATCC 35984) was used as an HBGA-negative control strain (128). Luria-Bertani (LB) agar and broth were prepared according to the manufacturer's instruction manual (206) for the cultivation of *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7, whereas Reasoner's 2A (R2A) agar and broth were used for the cultivation of *S. epidermidis* (Nihon Pharmaceuticals, Japan). HBGA activity of these bacterial strains was tested with the ABOsphia (Kamakura Techno Science Inc., Japan) blood typing kit according to the manufacturer's instructions. NoVLPs of genotypes GII.3, GII.4, and GII.6 were prepared as described previously (207). These genotypes are the prevalent genotypes among recent NoV GII outbreaks (208–210). The information on bacterial strains and norovirus like particles (NoVLPs) used in this study are summarized in **Table 5.1**.

Table 5.1: Bacterial strains and NoVLPs used in the study

	Registration code	Reference
Bacterial strains		
Enterobacter cloacae SENG-6	AB758448	(128)
Escherichia coli O86:K61:B7	ATCC 12701	(205)
Staphylococcus epidermidis	ATCC 35984	(128)
Norovirus genotypes		
Genogroup II, genotype 3 (GII.3)	AB758450	(128)
Genogroup II, genotype 4 (GII.4)	AB668028	(128)
Genogroup II, genotype 6 (GII.6)	AB758451	(128)

5.2.2 Removal of EPS and LPS from bacterial cells

The removal of EPS from bacterial cells was performed in two steps: the separate removal of loosely-bound EPS (LB-EPS) and tightly-bound EPS (TB-EPS) (211). Bacterial cells of *Enterobacter* sp. SENG-6, *E. coli* O86:K61:B7 and *S. epidermidis* were cultivated in relevant broth overnight. On the following day, 25 ml of the bacterial cell suspension including the growth medium were centrifuged at 6000 × g for 5 min to separate the supernatant and cell pellet. The supernatant includes soluble microbial products (SMP). The cell pellet was re-suspended in 5 ml of 0.1 M PBS (pH 6.5) and mixed for 2 min by a vortex machine. Then the cell suspension was shaken using a multi shaker for 10 min at 37 ˚C and again mixed for 2 min by a vortex machine. Finally, the cell suspension was centrifuged at 8000 × g for 10 min, and the supernatant including LB-EPS was removed. The cell pellet was re-suspended in 5 ml of 0.1 M PBS and mixed for 3 min by a vortex machine. The cell suspension was then heated in a water bath at 80 ˚C for 30 min, and the final centrifugation at 12000 × g for 20 min was performed to remove TB-EPS in the supernatant. The heat treatment at 80˚C does not lead to the loss of antigen integrity and the leaching of bacterial intracellular components (Li et al. 2015). Cells after the removal of LB- and TB-EPS were used for the filtration experiment.

A fraction of the cell pellet remained after the TB-EPS removal was used for LPS extraction. LPS extraction was performed in four steps, namely lysis, LPS purification, washing, and elution using the LPS extraction kit (Intron Biotechnology, Inc., Korea) according to the manufacturer's instructions. Briefly, 1 ml of lysis buffer was added and thoroughly mixed using a vortex machine until the cell clump disappeared. Then 200 µl of chloroform was added and mixed vigorously for 10-20 sec using a vortex machine. The mixture was then incubated at room

temperature for 5 min and again centrifuged at 18700 × g for 15 min at 4 ˚C using a Hitachi Rx series centrifuge with T15A33 rotor (Hitachi Corporation, Japan). Next, 400 µl of the supernatant was transferred to a new 1.5 ml centrifugation tube by carefully avoiding the transfer of white sediment, and 800 µl of purification buffer was added. The mixture was incubated at −20˚C for 10 min. The incubated mixture was then centrifuged at 18700 × g for 15 min at 4˚C using the T15A33 rotor. An LPS pellet was obtained by removing the supernatant. The pellet was then washed using 1 ml of 70% ethanol, and the mixture was centrifuged at 18700 × g for 3 min at 4˚C using the T15A33 rotor. The supernatant was removed, and 60 µl of 10 mM Tris-HCl buffer (pH 7.9) was added to the tube. This mixture was then boiled for 2 min until the LPS pellet was completely dissolved. LPS samples were stored at −80˚C until further experiments.

5.2.3 ELISA for the detection of bacterial HBGAs

ELISA using a GII.6 NoVLP-immobilized microplate was performed to confirm the presence of bacterial HBGAs in EPS and LPS of *E. coli* O86:K61:B7. GII.6 NoVLP was used as an adsorbent because this genotype can recognize HBGAs (106) and is less adhesive to non-HBGA substances (128). Firstly, GII.6 NoVLP was serially diluted 100 times with PBS (0.1 M, pH = 6.5) and added in triplicate to the relevant wells of a Microlon 96 well flat bottom, high-binding ELISA plate (Greiner Bio-One, Germany) and kept overnight at 4 ˚C until the NoVLPs were attached to the wells. On the following day, the NoVLP suspension was removed, and the wells were washed twice using 200 µl of PBS (pH=6.5) per well. The well surface was blocked using a 5% bovine serum albumin (BSA) (Wako Chemicals, Japan) in PBS for 2 h at room temperature and washed twice with PBS. After the blocking and washing, 50 µl of LPS solution was added to the well and incubated at room temperature for 1 h. The wells were washed twice with PBS and 50 µl each of anti-A, B, and H mouse monoclonal antibodies (sc-69951, sc-69952, and sc-52372, respectively; Santa Cruz Biotechnology Inc., USA) serially diluted up to 100-fold with 5% BSA in PBS added to relevant wells. The plate was incubated for 1 h at room temperature, and washed twice with PBS. Then, 50 µl of goat anti-mouse immunoglobulin M (IgM) HRP-conjugated antibody (A90-101P; Bethyl Laboratories, USA) diluted 500-fold with 5% BSA in PBS were added to each well. The plate was incubated at room temperature for 1 h, and washed four times with PBS. O-phenylenediamine (OPD) solution was prepared by dissolving 0.52 g of citric acid, 0.71 g of disodium hydrogen phosphate (Na_2HPO_4), one OPD tablet (P-7288; Sigma-Aldrich, Japan), and 30 µl of hydrogen peroxide in 100 ml of MilliQ water. One hundred microliters of the OPD solution was added to each well, and the plate was incubated for 30 min at room temperature. Then 50 µl of 2 M H_2SO_4 was added to stop the coloring reaction. Absorbance at 490 nm in each well was measured for 0.1 sec using a multi-label counter (ARVO MX; Perkin Elmer Inc., Japan). The signal/noise (S/N ratio) was calculated by dividing the absorbance value from GII.6 NoVLP-immobilized well by that from GII.6 NoVLP-

negative well. Student's t test was performed using Microsoft Excel 2010 to compare the S/N ratio values between samples.

5.2.4 Membrane filtration of NoVLPs

All bacteria grown overnight were centrifuged at 3000 × g for 15 min to obtain the cell pellet. The supernatant was removed, 10 ml of 0.1 M PBS was added to the tubes, and the cells were suspended by a vortex machine. Optical density was measured using a spectrophotometer (Bio-Rad Laboratories, USA) at 600 nm, and the value was adjusted to around 1.5 by careful addition of PBS (7.6×10^8–7.9×10^8 cells/ml). NoVLPs of human norovirus GII.3, GII.4, and GII.6 genotypes were prepared by the dilution of the original VLP suspension with PBS. Final particle numbers per milliliter were 2.0×10^{11} particles for GII.3, 1.2×10^{11} particles for GII.4, and 6.0×10^{11} particles for GII.6. Equal volumes of VLP and bacteria solutions were mixed for 15 min at 4 ˚C using a mechanical shaker. Then, 500 µL of the mixture of cells and NoVLP were filtered using NANOSEP MF GHP 0.45 µm filter with the effective filtration area of 0.28 cm^2 (PALL Corporation, Ann Arbor, MI, USA) at 8,000 × g until the total volume passed through the membrane. The filtrate was collected and stored at 4 ˚C for further analysis.

5.2.5 ELISA for the detection of NoVLPs

The mixture of bacterial cells and NoVLP (before filtration) or NoVLP only and its filtrate obtained in the membrane filtration test were added to relevant wells in triplicate and kept overnight at 4 ˚C. Wells without any samples are considered as negative. On the following day, the wells were washed twice with PBS. The well surface was blocked using a 5% BSA in PBS for 2 h at room temperature. Wells were washed twice with PBS, and 50 µl of anti-NoVLP serum from rabbit diluted with 5% BSA in PBS was added to relevant wells. The plate was incubated for 1 h and washed twice with PBS. Goat Anti-Rabbit immunoglobulin G (IgG) pAb HRP conjugate (ADI-SAB-300-J; Enzo Life Sciences Inc., USA) was used as the secondary antibody. The secondary antibody was diluted with 5% BSA in PBS, and 50 µl was added to each well. The plate was incubated for 1 h and washed 4 times with PBS. The coloring reaction was performed using the OPD solution as described above, 50 µl of 2M H_2SO_4 was added to stop the coloring reaction, and absorbance at 490 nm (A490) in each well was measured for 0.1 sec using a multi-label counter (ARVO MX; Perkin Elmer Inc., Japan). The filtration experiment and the ELISA quantification is schematically explained in **Figure 5.1**. The Mixture/Filtrate ratio was then calculated as the A490 of mixture/A490 of filtrate. The major focus of this study is the retention of NoVLPs on the MF membrane due to the interaction with bacterial HBGAs, which can be reflected by the larger values of Mixture/Filtrate ratio. All tests were performed in triplicates and the average data were presented. Student's t-test was performed using Microsoft Excel 2010 to compare the mixture/filtrate ratio values.

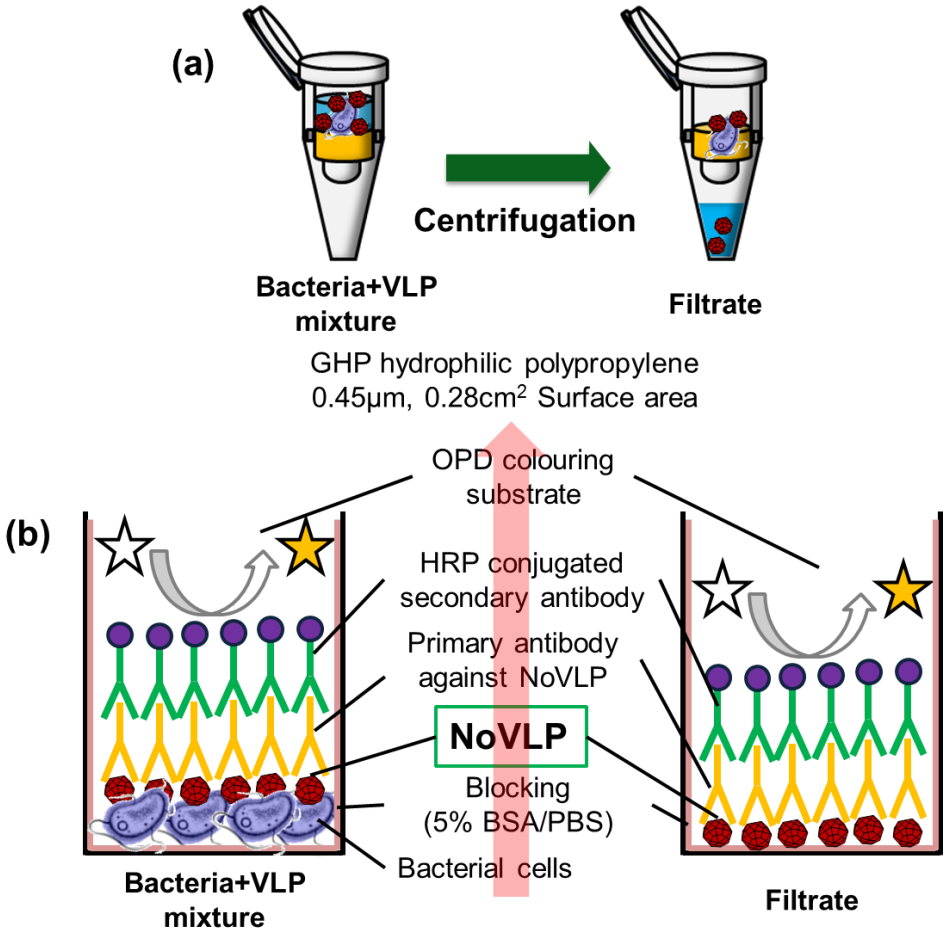

Figure 5.1: Schematic representation of the filtration setup and the ELISA experiment for the detection of NoVLPs in section 5.2.5. (a). Bacteria + NoVLP mixture was filtrated using a 0.45µm pore size GHP hydrophilic polypropylene membrane. (b). Bacteria+VLP mixture and the NoVLP filtrates were analyzed using the ELISA method to quantify the available NoVLP quantity in each fraction. Addition of reagents follows the arrow. (Figure is not to scale).

5.2.6. Immuno-TEM observation of the localization of HBGAs in MLSS from MBR

The localization of HBGAs in MBR MLSS was analyzed by immuno-TEM. Mixed liquor was taken from an MBR pilot plant (7). One hundred microliters of the mixed liquor were placed in a 2-mL tube for 30 min and resulting supernatant was removed. Sedimented MLSS were re-suspended in 2 mL of PBS containing 4% paraformaldehyde and 0.1% glutaraldehyde, and incubated at 4 ˚C for 2 h. The fixed MLSS was collected by sedimentation. The sedimented MLSS were washed by soaking in 2 mL of PBS at 4 ˚C for 10 min. This washing step was repeated twice. The MLSS were dehydrated by soaking in 2 mL of increasing concentrations of ethanol (70, 80, 90, and 95%) and then mixed gently using a rotator at 4˚C for 15 min. The following dehydration and infiltration reactions were also conducted on a rotator at 4˚C. For complete dehydration, the MLSS were soaked in 2 mL of 100% ethanol for 20 min. This final dehydration step was repeated twice. Next, the MLSS were infiltrated with 2 mL of LR white resin (Medium grade; London Resin Company Ltd., England) and 100% ethanol (1:2) mixture for 12 h, followed by 2 mL of LR white resin and 100% ethanol (2:1)

mixture for 12 h. The solution was replaced with 2 mL of pure LR white resin for 1 h, followed by another 2 mL of pure LR white resin for 12 h. The MLSS and pure LR white resin were polymerized using an ultraviolet polymerizer (TUV-100; Dosaka EM Co., Ltd., Japan) at 4 ˚C for 3 days. Ultrathin sections measuring 70 nm in thickness were prepared from the embedded MLSS using a diamond knife on an ultramicrotome (RMC MTXL; Boeckeler Instruments, Inc., USA). The sections were then placed on nickel grids.

The ultrathin sections on grids were washed by floating the grid upside down on a drop of PBS at room temperature for 1 min. The following incubations were also conducted by floating the grid upside down on drops of reagent at room temperature. The sections were blocked with 4% BSA in PBS for 5 min and incubated with anti-blood group A, B, or O(H) antibody diluted 1:30 in PBS containing 1% BSA for 2 h, followed by washing with PBS (6 changes, 1 min each). The bound primary antibodies were localized by incubating the sections on anti-mouse IgM antibody gold conjugate (10 nm, ab39613; Abcam, Japan) diluted 1:10 in PBS containing 1% BSA for 1 h, followed by washing with PBS (6 changes, 1 min each). The sections were fixed with 2% glutaraldehyde in PBS for 15 min, followed by washing with deionized distilled water (DDW; 7 changes, 1 min each). Finally, the sections were stained with 5% uranyl acetate, followed by washing with 50% ethanol for 1 min and DDW (4 changes, 1 min each). Stained sections were observed under TEM. Ultrathin sections without the primary antibodies were used as controls.

5.3 RESULTS

5.3.1 Availability of bacterial HBGAs for NoVLP attachment

HBGAs associated with bacterial cells were detected by the blood typing kit. This kit detects A, B, and O(H) antigens on cells by forming an aggregate of cells and anti-HBGA antibody-coated beads. *Enterobacter* sp. SENG-6 displayed the highest response for all A, B, and O(H) antigens, whereas *E. coli* O86:K61:B7 and *S. epidermidis* gave negative results for all tested cell densities (Table 1, Figure S1). These A, B, and O(H) antigens of *Enterobacter* sp. SENG-6 were not detected when EPS was removed mechanically, which indicates that bacterial HBGAs of *Enterobacter* sp. SENG-6 were included mainly in EPS. On the other hand, the B antigen in LPS of *E. coli* O86:K61:B7 was detected after the EPS removal (**Table 5.2, Figure 5.2**), showing that the B antigen in LPS of *E. coli* O86:K61:B7 can be recognized by the anti-B antibody in the kit, but this antigen-antibody interaction is physically blocked by the presence of EPS covering cells of *E. coli* O86:K61:B7. We also confirmed that A and O(H) antigens of *E. coli* O86:K61:B7 were not detected even after the EPS removal **(Table 5.2, Figure 5.2)**.

Table 5.2: Detection of bacterial histo-blood group antigens by a blood typing kit

	Enterobacter cloacae SENG-6						*E. coli* O86:K61:B7 (ATCC 12701)						*Staphylococcus epidermidis* (ATCC 35984)		
	with EPS			w/o EPS			with EPS			w/o EPS					
Cells/ml	A	B	O(H)	A	B	O(H)	A	B	O(H)	A	B	O(H)	A	B	O(H)
10^9	+++	+++	+++	–	–	–	–	–	–	–	+	–	–	–	–
10^8	+++	+++	+++	–	–	–	–	–	–	–	+	–	–	–	–
10^7	++	++	++	–	–	–	–	–	–	–	–	–	–	–	–

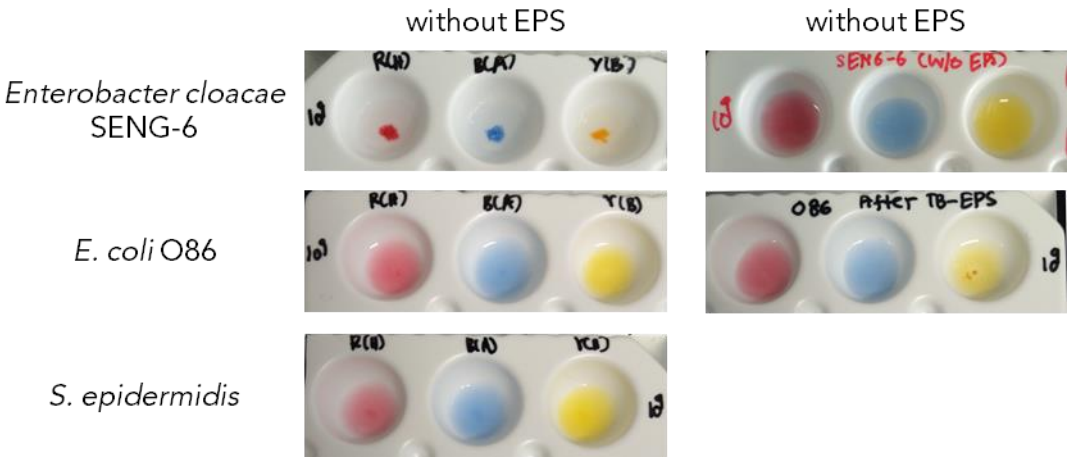

Figure 5.2: Blood typing kit observations for *Enterobacter* cloacae. SENG-6, *Escherichia coli* O86:K61 (B7) (ATCC 12701) and *Staphylococcus epidermidis* (ATCC 35984) before and after the removal of EPS. Red color is derived from anti-H antibody-immobilized beads, while blue and yellow are from anti-A and anti-B antibodies-immobilized beads, respectively.

For confirming the interaction between LPS of *E. coli* O86:K61:B7 and NoV particles, ELISA using a GII.6 NoVLP-immobilized microplate was performed. Extracted LPS from *E. coli* O86:K61:B7 was applied to the GII.6 NoVLP-immobilized well, and bacterial HBGAs in the trapped LPS were detected by anti-HBGA antibodies. The negative control condition (without LPS) gave a signal/noise ratio value larger than 1.0 for the B antigen, but a significantly higher value of the signal/noise ratio was observed under the test condition (with LPS, $p < 0.01$, **Figure 5.3**). Although a signal/noise ratio value larger than 1.0 with LPS was also observed for the O(H) antigen, the statistically significant difference was not obtained between test and negative conditions. These results indicate that the B antigen is the most abundant in LPS of *E. coli* O86:K61:B7 and can bind the GII.6 NoVLP.

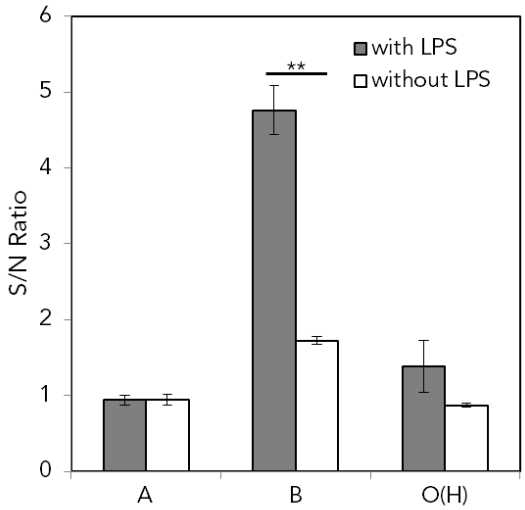

Figure 5.3: Detection of histo-blood group antigens (HBGAs) of *Escherichia coli* O86:K61:B7 (ATCC 12701). A plate was coated by GII.6 norovirus-like particles (NoVLPs), and HBGAs in lipopolysaccharide of *E. coli* O86:K61:B7 attached to NoVLP were detected by anti-A, B, and O(H) antibodies. **, $p < 0.01$.

5.3.2 Membrane filtration of NoVLPs mixed with bacterial cells

NoVLP of GII.3, GII.4, or GII.6 was mixed with suspended cells of *Enterobacter* sp. SENG-6, *E. coli* O86:K61:B7, or *S. epidermidis*. The mixture of NoVLPs and bacterial cells was filtered using NANOSEP MF GHP 0.45 µm filter in a spin column. NoVLPs in the mixture with bacterial cells (before filtration) and those in filtrate (after filtration) were detected by ELISA. As a bacteria-negative control, only NoVLPs (without bacterial cells) were also filtered separately. The mixture/filtrate ratio of ELISA signal was around 1.0 (0.91-1.01) for all genotypes tested under the bacteria-negative control (**Figure 5.4**, VLP only), which explains that NoVLP (diameter: ca. 40 nm) has passed through the MF membrane pore (nominal pore size of 0.45 µm). The mixture/filtrate ratio values were always less than 1.0 when cells of *S. epidermidis* were mixed with NoVLPs (**Figure 5.4**), probably because of an inhibitory effect of *S. epidermidis* cells on the signal detection in ELISA. The presence of *S. epidermidis* did not affect the passing of the majority of NoVLPs through the MF membrane pore as there were no HBGAs to retain the NoVLPs.

When NoVLPs were mixed with cells of *Enterobacter* sp. SENG-6, the mixture/filtrate ratio of ELISA signal was significantly larger than 1.0 for all genotypes tested ($p < 0.01$), showing that a significant amount of NoVLPs were trapped by *Enterobacter* sp. SENG-6 cells and did not pass through the MF membrane (**Figure 5.4**). On the other hand, the behavior of NoVLPs was dependent on the genotype when mixed with cells of *E. coli* O86:K61:B7. The mixture/filtrate ratio of GII.4 NoVLP was significantly larger than 1.0 ($p < 0.01$), but those of GII.3 and GII.6 were not, which means that GII.3 and GII.6 NoVLPs were not trapped by *E. coli* O86:K61:B7 cells very effectively.

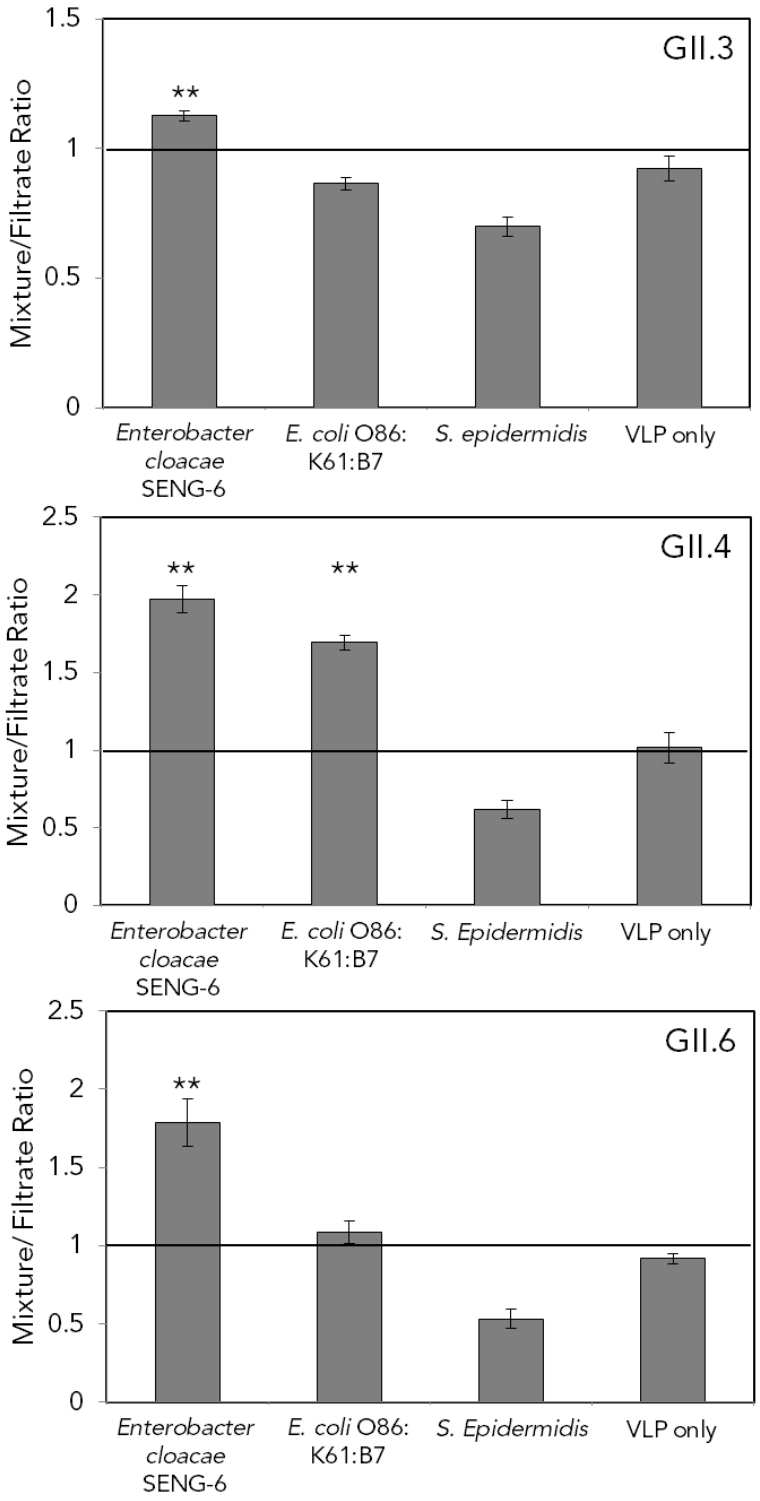

Figure 5.4: Membrane separation of norovirus-like particles (NoVLPs) with bacterial cells. Reduction of (A) GII.3, (B) GII.4, and (C) GII.6 NoVLP by the filtration with a microfiltration membrane (nominal pore size of 0.45 µm) in the presence of Enterobacter cloacae. S SENG-6, *Escherichia coli* O86:K61(B7) (ATCC 12701), and *Staphylococcus epidermidis* (ATCC 35984). NoVLP was filtered without bacterial cells in the condition of VLP only. **, $p < 0.01$.

5.3.3 Impact of the location of bacterial HBGAs on the cell attachment of noroviral particles

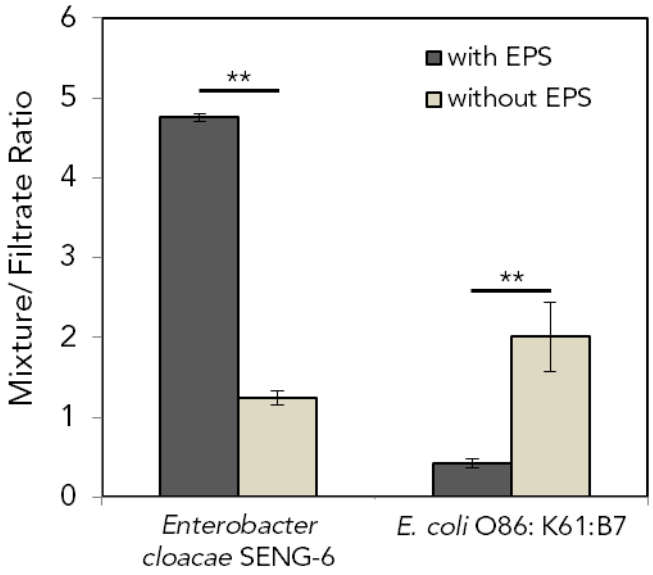

Figure 5.5: Effect of extracellular polymeric substances on membrane separation of norovirus-like particles (NoVLPs). The mixture/filtrate ratio between the quantity of GII.6 NoVLPs in the samples before and after the filtration with a microfiltration membrane (pore size: 0.45μm) in the presence of *Enterobacter* cloacae. SENG-6 and *Escherichia coli* O86:K61 (B7) (ATCC 12701) before and after removal of soluble microbial product, loosely-bound EPS, and tightly-bound EPS. **, $p < 0.01$.

LB- and TB-EPS were removed from cells of *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7, and the membrane filtration test was performed using NANOSEP MF GHP 0.45 μm filter in a spin column to analyze the effect of the localization of bacterial HBGAs on the removal property of noroviral particles. The mixture/filtrate ratio of ELISA signal was compared between bacterial cells before and after the EPS removal. As a result, the removal of EPS from *Enterobacter* sp. SENG-6 cells has been attributed to a significantly lower mixture/filtrate ratio ($p < 0.01$). The difference in the ratio values for *Enterobacter* sp. SENG-6 with EPS between **Figure 5.4(C)** and **Figure 5.5** was caused by experimental random errors in both values of numerator (A490 of mixture) and denominator (A490 of filtrate) in the ratio calculation. A490 values of mixture in **Figure 5.4(C)** and **Figure 5.5** were 1.50 ± 0.13 and 1.13 ± 0.01, respectively, and those of filtrate were 0.24 ± 0.04 and 0.84 ± 0.08. Although we used the same amount of NoVLP in these experiments, this level variation in ELISA signal can be observed. On the other hand, the EPS-removed *E. coli* O86:K61:B7 cells gave a significantly higher value of the mixture/filtrate ratio ($p < 0.01$) **(Figure 5.5)**. These results demonstrate that the localization of bacterial HBGAs is an important factor for the norovirus removal with the MF membrane, and the HBGAs in LPS of *E. coli* O86:K61:B7 have to be exposed for the association with GII.6 NoVLP.

5.3.4 Immuno-TEM confirmation of the localization of bacterial HBGAs

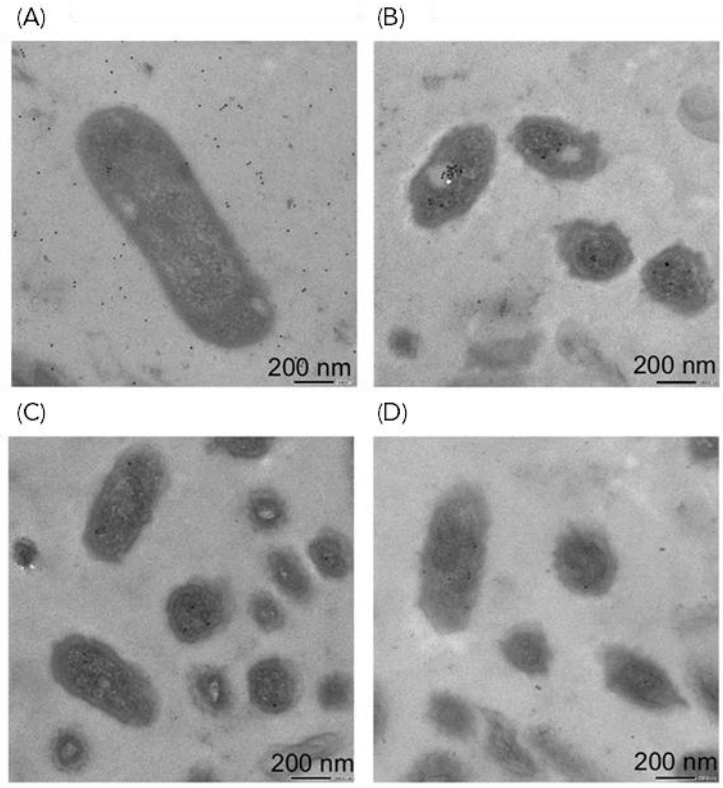

Figure 5.6: Localization of histo-blood group antigens in mixed liquor suspended solids from a membrane bioreactor pilot plant. Ultrathin section was labeled with immuno-gold after reacting with anti-blood group A, B, and O(H) antibodies (primary antibodies) and observed under transmission electron microscopy. Ultrathin sections without primary antibodies were used as controls. A) The A antigen, B) the B antigen, C) the O(H) antigen, and D) control

Ultrathin sections of MBR MLSS labeled with gold nanoparticles bearing anti-mouse IgM after reacting with anti-blood group A, B, or O(H) antibody (used as the primary antibody) were examined by immuno-TEM to study the localization of HBGAs. A number of gold nanoparticles were observed in the EPS region of MLSS (**Figure 5.6** (A)) that had reacted with anti-A antibody. In contrast, the B and O(H) antigens were not detected in the EPS region, but a few gold nanoparticles carrying anti-B and O(H) antibodies were observed inside bacterial cells (**Figure 5.6** (B) and (C)). These immuno-TEM observations indicate that at least the A antigens can be present in the EPS region of MBR MLSS.

5.4 DISCUSSION

We demonstrated the role of bacterial HBGAs on the MF membrane separation of HuNoVs. Two HBGA-positive bacteria, *Enterobacter* sp. SENG-6 and *E. coli* O86:K61:B7, or one HBGA-negative bacteria, *S. epidermidis*, were mixed with each genotype of NoVLP GII.3, GII.4, or GII.6, and the reduction of NoVLPs in the filtrate with an MF membrane (nominal pore size of 0.45 μm) installed in a spin column was compared. All results indicated that bacterial HBGAs in EPS and

LPS could capture noroviral particles and contribute to the MF membrane removal, but the attachment of NoVLPs to HBGA-positive LPS is physically hindered by EPS covering bacterial cells.

The membrane used in this study is an MF membrane with a nominal pore size of 0.45 μm. MF membranes with a nominal pore size of 0.1 to 0.45 μm are very frequently employed in MBR systems for wastewater treatment (193). A membrane pore size larger than 0.1 μm is commonly preferable because the membrane fouling associated with pore plugging is one of the most crucial problems in MBR operation (212). Noroviral particles have a diameter of around 40 nm (213) and mixture/filtration ratios around 1.0 is obtained in the absence of bacterial cells with all 3 types of NoVLPs (**Figure 5.4**, VLP only), which explains the sieve mechanism does not appear to work in the removal of NoVLPs, as observed in the membrane filtration test of noroviral particles indigenous in wastewater (214). However, the significant reduction of NoVLPs of all genotypes was observed in the presence of *Enterobacter* sp. SENG-6 **(Figure 5.4)**. *Enterobacter* sp. SENG-6 produced HBGAs in EPS **(Table 5.2)**, which contributed to the efficient removal of all norovirus genotypes of GII.3, GII.4, and GII.6 with the MF membrane. For the efficient removal of HuNoV in the MBR, the association of viral particles to mixed liquor suspended solids (MLSS) and/or the cake layer formed on the membrane surface is of primary importance (7, 138). MLSS and the cake layer are mainly composed of bacterial cells and EPS (215), and the immuno-TEM images show that the A antigen was present in the EPS region of MBR MLSS **(Figure 5.6(A))**. A thorough understanding about the prevalence and spatial/temporal (seasonal) variation of the expression level of these bacterial HBGAs and the binding property of noroviral particles to bacterial components including EPS is thus critical for discussing the contribution of bacterial HBGAs in MLSS on the removal of HuNoVs in MBR.

The removal efficiency of NoVLPs in the presence of *E. coli* O86:K61:B7 was genotype-dependent, in which GII.4 NoVLP was well removed but GII.3 and GII.6 were not **(Figure 5.4)**. The NoV genotype GII.4 can recognize broad types of epitope compared to the other genotypes (216–218). It is likely that the interaction between the GII.4 NoVLPs and *E. coli* O86:K61:B7 **(Figure 5.4(B))** is caused by the broad spectrum of HBGA-epitope recognition of this genotype. Several phenomena have been proposed as the determination factors of virus removal efficiency with membrane filtration, including the attachment of viruses to biosolids (7, 65), the virus retention by cake layer on membrane surface (165), the virus retention on membrane surface (65), the initial concentration level of virus in feed water (96), and the virus types (12). This study verified for the first time that the epitope-recognizing capability of viral particle is one of the factors determining virus removal efficiency with membrane filtration.

All results from the present study provide evidence that the removal efficiency of NoV particles with the MF membrane is dependent on the location of bacterial HBGAs and the norovirus genotype. Different norovirus genotypes have different HBGA-binding profiles (106, 219, 220),

which means that norovirus genotypes other than those tested in the present study have different removal properties by the MF membrane and HBGA-positive bacteria. Since a recent report describes that the HBGA-binding profile can also vary within a genotype (221), a strain-dependent removal property can be observed when multiple strains from an identical genotype are used to test the removal efficiency with the MF membrane. Particularly, the rapid evolution of NoV GII.4 should be paid attention, because new variants have appear every 2-3 years and increased relative affinity towards the A, B, O(H) and Lewis antigens over the evolution has been observed (216). The GII.4 NoVLP used in this study is a variant strain of Den Haag 2006b (200), which can recognize all the A, B, O(H), and Lewis antigens (222) as well as the other GII.4 strains (106). This means that the Den Haag 2006b strain can be representative among GII.4 strains in terms of the MF membrane removal with HBGA-positive bacteria. Recent studies have shown the emergence of NoV GII.17 strains and the replacement of the currently predominant GII.4 Sydney strain (116, 223). It is expected that these GII.17 strains can be effectively removed by MF membrane because of the broad HBGA binding spectrum (116), but it is necessary to conduct the filtration test as in this study for coming to a conclusion about the removal efficiency of the emerging NoV strains with MF membarne.

Jones et al. reported that *Enterobacter cloacae* (ATCC 13047), a relative of *Enterobacter* sp. SENG-6 (99.9% identity in the 16s rRNA gene sequence), bound norovirus GII.4 Sydney strain through the bacterial O(H) antigen, which resulted in the infection of the Sydney strain to cultivated B cells (224). Jones et al. (2014) used whole cell components of *Enterobacter cloacae* and detected the H antigen by western blotting. From the bacteriological viewpoint, even the 100% identity of 16S rRNA gene sequence is not enough to identify bacterial species, and multiple phenotypes including substrate availability must be tested for the species identification. Since enteric bacteria are so diverse, it is highly likely that *Enterobacter* sp. SENG-6 is identified as a new species in genus *Enterobacter*, which is different from *Enterobacter cloacae*. The comparison of the HBGA expression profile among *Enterobacter* spp. and other enteric bacteria is necessary to discuss the ubiquity of HBGA-positive bacteria in nature. Further understanding of the prevalence of HBGA-positive bacteria in environments may lead to better insights about norovirus survival and persistence in environmental waters and removability in wastewater treatment processes, including MBR. A small-scale (500µl) test for the membrane filtration was performed in this study to investigate the effect of specific interaction between bacterial HBGAs and noroviral particles on the removal efficiency, and the observations at a larger scale must be validated in the further study.

5.5 CONCLUSIONS

- The interaction with HBGA-positive bacteria contributes to the removal of HuNoV particles with MF membrane.
- Cell-associated and HBGA-positive EPS is important for removing HuNoV particles with MF membrane.
- The removal efficiency of HuNoV particles with MF membrane and bacterial HBGA is genotype-dependent.
- HBGAs are expressed in mixed liquor suspended solids from MBR, which can be available for the removal of NoVs in wastewater.

Chapter 6 : HISTO-BLOOD GROUP ANTIGEN (HBGA) POSITIVE BACTERIA IN THE MIXED LIQUOR CONTRIBUTES TO THE HUMAN ROTAVIRUS REMOVAL BY CROSS – FLOW MEMBRANE FILTRATION

6.1 INTRODUCTION

Virus removal efficiency obtainable in wastewater treatment process chains are based on the mechanisms of virus removal in different unit processes. For MBR systems, pore size, attachment to the gel cake layer and attachment to mixed liquor suspended solids and inactivation by mixed liquor suspended solids are considered as crucial mechanisms for virus removal (64–66). In wastewater treatment pond systems adsorption to solids and sedimentation, predation by organisms of higher trophic levels, and sunlight mediated inactivation are the major mechanisms of virus removal (62). In the case of activated sludge, it is reported that the influent viruses are rapidly adsorbed to the MLSS (7, 63, 64).

By observing above mechanisms, it is clear that the adsorption to wastewater solids plays a major role in virus removal from all types of treatment unit processes. Gerba et al. (1978) reported that the percentage of animal enteric viruses associated with solids in the treated sewage effluents varied from 3-100% (17). Norovirus GI and GII were attached to large settleable particles (>180µm), smaller suspended particles (>0.45µm) and colloidal particles in a waste stabilization pond (18). Rotavirus was detected from suspended solids and estuarine sediments and the attached quantities were different depending on the solids (19). The study by Miura et al. (2015) described enteroviruses are less associated with activated sludge than calicivirus (7). In contrast, interactions between wastewater solids and enteric viruses are reported to be responsible for the survival of human enteric viruses from inactivation. According to the studies by Winward et al. (2008), microorganisms associated with wastewater solids are more resistant to chlorine than the free microorganisms (20). Based on the observations by Hejkal et al. (1979), compared to free or secondarily adsorbed poliovirus, fourfold increase of combined chlorine was necessary to achieve the same degree of inactivation in fecal particle associated or occluded poliovirus (21). The recent review by Chahal et al. (2016) described that wastewater solids can shield microorganisms in different ways by providing shading or partial absorption of UV energy to reduce the effective dose, or by scattering the light (121). Templeton et al. (2005) study on the inactivation of viruses by UV light compliments the fact as humic acid and activated sludge flocs shielded MS2 and T4 by UV inactivation to a statistically significant degree (with >99%CI) relative to the particle free conditions (125).

All the studies on virus adsorption to wastewater solids are attributed to the Derjaguin, Landau, Verwey and Overbeek theory (DLVO theory) where interactions between two particles in a dispersion are thought to result from a balance between repulsive double-layer interactions and

attractive van der Waals forces (2). Based on that, attachment of viral particles to solids such as settleable, suspended, colloidal particles and MLSS has been already studied for different enteric viruses (7, 18, 135, 200, 225). Da Silva et al. (2011) has confirmed the presence of Ca^{2+} and Mg^{2+} has dramatically improved the attachment of norovirus GI and GII.4 into silica because the addition of cationic salts decreases the layer thickness as less volume is needed to contain enough counter-ions and facilitates the approach of the two surfaces allowing van der Waals forces to have an effect (2, 196).

However, most of the studies have focused on the non-specific interactions between wastewater solids-human enteric virus and therefore the contribution of specific wastewater solids-human enteric virus interactions on the virus removal and survival in wastewater has not been extensively analyzed. The first study regarding this topic was published by Miura et al. (2013) and it reported that *Enterobacter cloacae* SENG-6 isolated from a fecal sample of a healthy individual interacts with human norovirus-like particles (NoVLPs) through extracellular polymeric substances (EPS) where HBGA-like substances were localized (128). Therefore, HBGA – human norovirus interaction provides an excellent platform to analyze specific enteric virus – wastewater solids interactions. Following the idea, many studies have been conducted and much evidence on the importance of HBGA-human norovirus interaction on norovirus life cycle has been reported. HBGA like substances excreted in the EPS of *Enterobacter cloacae* SENG-6 displayed the strain dependent recognition and removal of human norovirus like particles (NoVLPs) (176). HBGA-expressing *Escherichia coli* maintained the antigen integrity and mucin-binding ability of NoVLPs after heat treatment (129). A set of bacterial species representative of human gut microbiota isolated from a norovirus GI.6 stool sample bound to GII.4 New Orleans 2009, GII.4 Sydney 2012 and GI.6 strains (226).

Up to now, all the conclusions made with regard to specific interactions between HBGA positive bacteria and enteric viruses are based on the experiments performed in microliter scale using HBGA-positive bacteria and human norovirus like particles (NoVLPs). The prevalence of HBGA positive bacteria-human enteric virus interactions in large scale wastewater treatment plants and their contribution on the human enteric virus removal and survival has not been evaluated yet. Meanwhile, current protocols of cultivating human noroviruses under laboratory conditions involve large costs, time and labour (227). Therefore, in evaluating the interactions between HBGA positive bacteria-human enteric virus interactions in large scale, it is necessary to find a surrogate to replace human norovirus which can be cultivated in large scale.

Proteolysis of spike protein VP4 in human rotavirus generates VP5* and VP8* proteins. VP8* of some human rotavirus strains specifically interacts with A-type histo-blood group antigens (99). In addition, human rotavirus can be cultivated in-vitro using MA104 cells (228) which makes an

ideal candidate as a surrogate for human norovirus. Therefore, in this study, we used HBGA – human rotavirus interaction as a model to analyze the specific interactions between the human enteric bacteria and human enteric viruses in wastewater and the potential effects of specific interactions on virus removal from wastewater.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial strains and human rotavirus strain used

Enterobacter cloacae SENG-6 is used as the HBGA-positive strain and the HBGA-activity is reported elsewhere (128, 176). Human rotavirus HAL1166 (G8P[14]) strain which is reported to recognize A-type HBGAs (117) is used throughout the study.

6.2.2 Specific interactions between *Enterobacter cloacae* SENG-6 and human rotavirus HAL1166

First, 200µl of *Enterobacter cloacae* SENG-6 (optical density at 600nm=1.0) is inoculated in triplicate to the relevant wells of a Microlon 96 well flat bottom high binding ELISA plate (greiner bio-one, Germany) and kept overnight at 4^0C until the bacteria are attached to the wells properly. The following day bacteria were removed and the wells were dried properly. Then the wells were washed thoroughly 2 times using 200µl of PBS (pH=6.5) per well. Blocking the sites that bacteria were not attached was performed using a 5% Bovine Serum Albumin (BSA) (Wako chemicals, Japan) in PBS solution. Three hundred and thirty five microliters of the solution was filled to each well and incubated at room temperature (RT) for 2 hours. Then the solution was removed and the drying and washing step was performed as mentioned earlier. Human rotavirus HAL1166 treated with or without trypsin is added to the relevant wells. Remaining wells were filled with PBS and incubated at RT for 1 hour. For the next step, mouse monoclonal antibody to rotavirus (GroupA) (0.1 mg/ml, AMO1341PU-N, Acris Antibodies Inc, USA) was used. Antibody was serially diluted up to 10 times and 50 µl was added to each well after washing wells with PBS as mentioned above and kept for 1 hour. Goat anti-mouse IgG H&L HRP-conjugated antibody (ab6789, abcam) was used as the secondary antibody for the experiment. Antibody was diluted 100 times with BSA and 50 µl was added to each well after washing with PBS. The plate was incubated at room temperature for 1 hour. O-phenylenediamine (OPD) solution was prepared for the final step by dissolving 0.52 g of Citric acid, 0.71 g of Disodium Hydrogen Phosphate (Na_2HPO_4), one OPD tablet and 30 µl of Hydrogen Peroxide (H_2O_2) dissolved in 100 ml of MilliQ water. One hundred microliters of the OPD solution was added and the plate was incubated at room temperature for 30 mins. Then 50 µl of 2M H_2SO_4 was added to neutralize the reaction before absorbance measurement. Absorbance at 490 nm (0.1s) was measured using a multi label counter (ARVO MX; Perkin Elmer Inc., Japan).

6.2.3 Analysis of the effect of bacterial HBGAs on virus removal in a lab scale cross-flow membrane system

A laboratory scale cross-flow membrane system is developed as depicted in **Figure 6.1**. Cross flow membrane device had a surface area of 2000mm^2 and equipped with a 0.2µm nominal pore size PTFE membrane (Advantec, Japan). *Enterobacter cloacae* SENG-6 and *E. coli* K12ΔwcaF was cultivated overnight in LB –medium and the OD_{600} value is adjusted to around 1.0. Rotavirus HAL1166 was treated with 1.0mg/ml trypsin from porcine pancreas (Type IX-S, Sigma Aldrich, USA) for 30mins at 37^{0}C or used without trypsin treatment. One hundred and eighty ml of *Enterobacter cloacae* SENG-6 or *E. coli* K12ΔwcaF is mixed with 1.8 ml of trypsin treated or non-trypsin treated rotavirus HAL1166. The suspension is then mixed for 50 mins at 4^{0}C using a magnetic stirrer (229). The container including the mixture is then connected to the system and the recirculation and permeate pumps were started. Permeate pump had an initial flow rate of 0.8ml/min. Paired samples were collected from permeate and the reactor at different time intervals and the genome copies of rotavirus were quantified by RT-qPCR. Reactor bacteria concentrations were quantified at 600nm using a spectrophotometer at every sampling event (Bio-Rad Laboratories, USA).

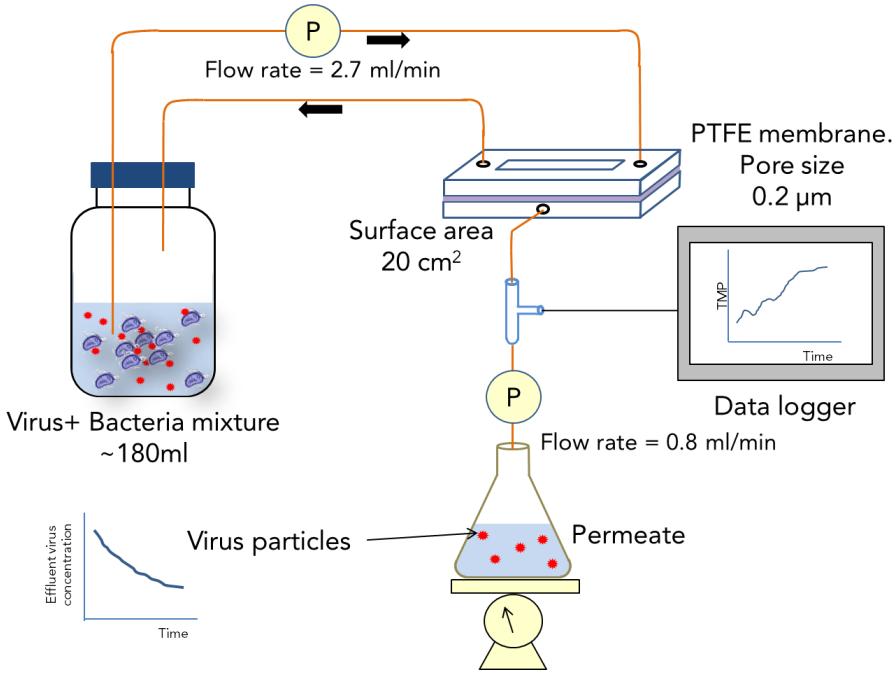

Figure 6.1: Experimental set up for cross-flow membrane experiment

6.2.4 RNA-extraction, cDNA synthesis and RT-qPCR for the quantification of human rotavirus

RNA extraction was performed using the spin protocol of QIAamp Viral RNA mini kit (QIAGEN Sciences, Maryland, USA) according to the manufacturer's instructions. Briefly 140 µl of the sample is lysed with 560 µl of lysis buffer under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Next 560 µl Ethanol is added to adjust buffering conditions to provide optimum binding of RNA to the membrane. Then sample is loaded and

centrifuged in the mini spin column. RNA is bounded to the membrane and contaminants are washed away with two wash buffers. After the extraction of RNA, samples were heated at 95^0C for 5 mins followed by cooling on ice to relax the secondary structures. RNA is then eluted in a special RNase free buffer and stored at -80^0C until further use.

Synthesis of cDNA from extracted RNA was performed using PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara Bio Inc., Japan) as per the instruction manual. Briefly, 4µl of 5×Prime script buffer, 1µl of RT enzyme mix, 1µl Oligo dT primer, 4µl of random 6 mers and 6µl of RNase free water were mixed with 4µl of the extracted RNA in a PCR tube. Mixture was incubated in a 96 well Veriti thermal cycler (Applied Biosystems, USA) at 37^0C for 15 mins, 42^0C for 15 mins and 85^0C for 5 sec. Prepared cDNA was stored at -20^0C until further processing.

RT-qPCR was performed using the primers NVP3-F (ACCATCTACACATGACCCTC), NVP3-R (GGTCACATAACGCCCC) and Taqman probe (5'-/56-FAM/ATG AGC ACA /ZEN/ATA GTT AAA AGC TAA CAC TGT CAA/3IABkFQ/-3') as recommended by Pang et al. (2004) (230). In a 96-well PCR plate (µltraAmp, Sorenson BioScience, Inc. Utah 84107, USA), 25µl of the reaction mixture was prepared by mixing 12.5 µl Premix Ex Taq, 0.5µl of 10µM TaqMan probe, 0.5µl of 10µM each primer, 0.5µl of Rox reference dye II (Takara Bio Inc, Japan), 5.5µl of RNase-free water and 4µl of cDNA. Using MilliQ water, 10 times serial dilution series from 4.78×10^7 copies/µl to 4.78×10^1 copies/µl was prepared from the standard plasmid. All the reactions were performed in duplicate. Incubation was performed using a 7500-Fast Real Time PCR system (Applied Biosystems Inc, CA, USA). Reaction conditions were set as follows: initial denaturation at 95^0C for 5 mins followed by 45 cycles of amplification with denaturation at 94^0C for 20 seconds and annealing and extension at 60^0C for 1 min and final extension at 72^0C for 5 mins. Amplification data were collected and analyzed with 7500 software (version 2.0.4) (Life Technologies Corp, USA).

6.3 RESULTS

6.3.1 Specific interactions between *Enterobacter cloacae* SENG-6 and human rotavirus HAL1166

ELISA resulted in a significant difference ($p<0.001$, t-test) in the S/N ratios obtained for the interaction between *Enterobacter cloacae* SENG-6 and trypsin-treated rotavirus HAL1166 compared to non-trypsin treated rotavirus HAL1166 **(Figure 6.2)**. The results confirm the specificity of interaction between the A-type HBGA-like substances in the *Enterobacter cloacae* SENG-6 and VP8* protein of rotavirus HAL1166.

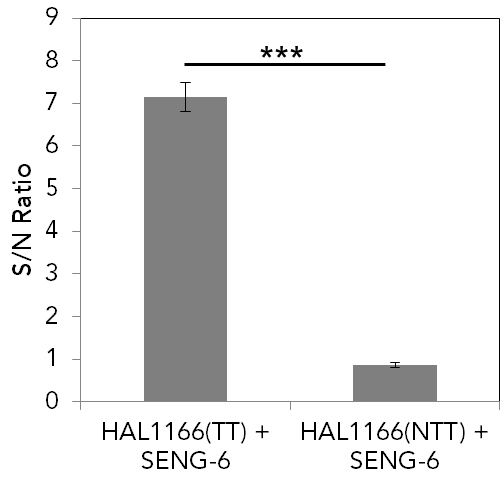

Figure 6.2: Specificity of interaction between *Enterobacter cloacae* SENG-6 and rotavirus HAL1166

6.3.2 Variation of rotavirus HAL1166 concentration in reactor effluent

Variation of trypsin treated and non-trypsin treated rotavirus HAL1166 in the membrane filtration effluent in the presence of *Enterobacter cloacae* SENG-6 is shown in **Figure 6.3**. Trypsin-treated rotavirus HAL1166 concentration in the effluent displayed a decreasing trend with larger coefficients of determinations (R^2 >0.75) **(Figure 6.3(a))**. Non-trypsin treated rotavirus HAL1166 concentration in the effluent displayed reduction of copy numbers or remain unchanged depending on the trial (R^2 <0.5) **(Figure 6.3(b))**.

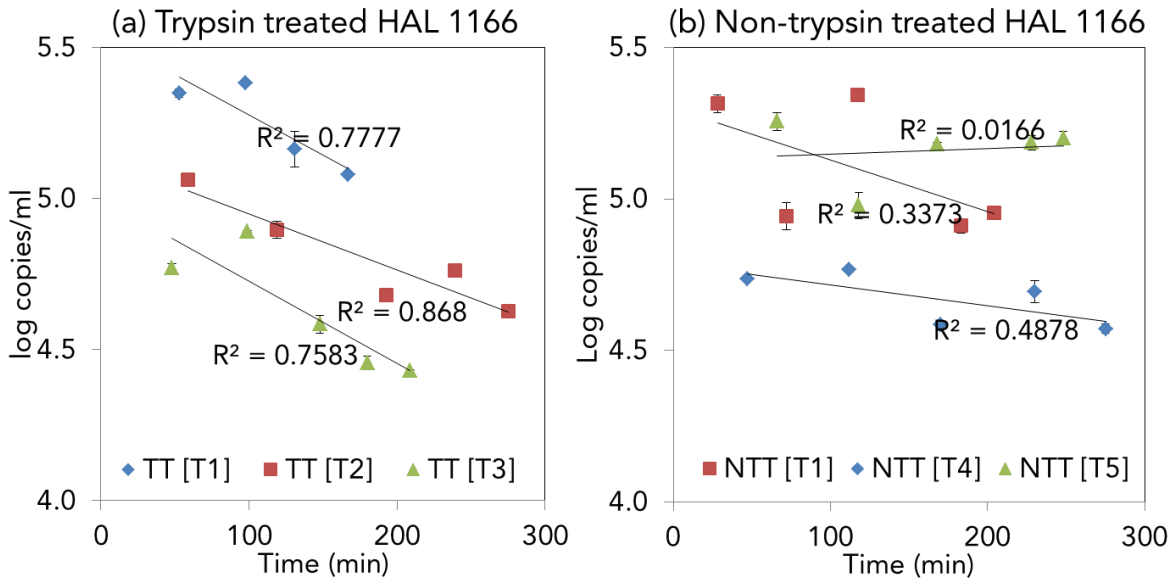

Figure 6.3: Variation of effluent rotavirus HAL1166 concentration in (a) Trypsin treated rotavirus HAL1166 and (b) Non-trypsin treated rotavirus HAL1166

6.3.3 Correlation between the log reduction of rotavirus HAL1166 and the presence of *Enterobacter cloacae* SENG-6

To confirm the contribution of *Enterobacter cloacae* SENG-6 on the rotavirus HAL1166 removal, we evaluated the relationship between the *Enterobacter cloacae* SENG-6 deposition in the membrane and the LRV. Considering the mass balance for bacteria and assuming no losses, equation 1 was used to calculate the quantity of bacteria deposited in the membrane.

$$\begin{aligned}\text{Bacteria deposited by time (T)} = &[\text{Initial bacteria concentraton} \times \text{Initial volume}] - \\ &[\text{Bacteria concentraton at time (T)} \times (\text{Initial volume} - \text{Permeate volume} - \text{Sample volume})]\end{aligned} \quad \textbf{(1)}$$

Deposited bacteria quantity is plotted with the LRV of rotavirus HAL1166 quantified and the Pearson correlation coefficient is calculated to evaluate the how bacterial deposition contributed to the LR of rotavirus HAL1166 **(Figure 6.4)**. For trypsin-treated rotavirus HAL1166 **(Figure 6.4(a))**, Pearson's r was 0.60 which indicated a strong positive relationship between the bacterial deposition and virus removal. In addition, test for correlation coefficient yielded a linear relationship (α=0.05) at the range of values tested. In contrast, non-trypsin treated rotavirus HAL1166 **(Figure 6.4(b))** yielded a Pearson's r of -0.06 which is indicative of no or negligible relationship between the bacterial deposition and virus removal.

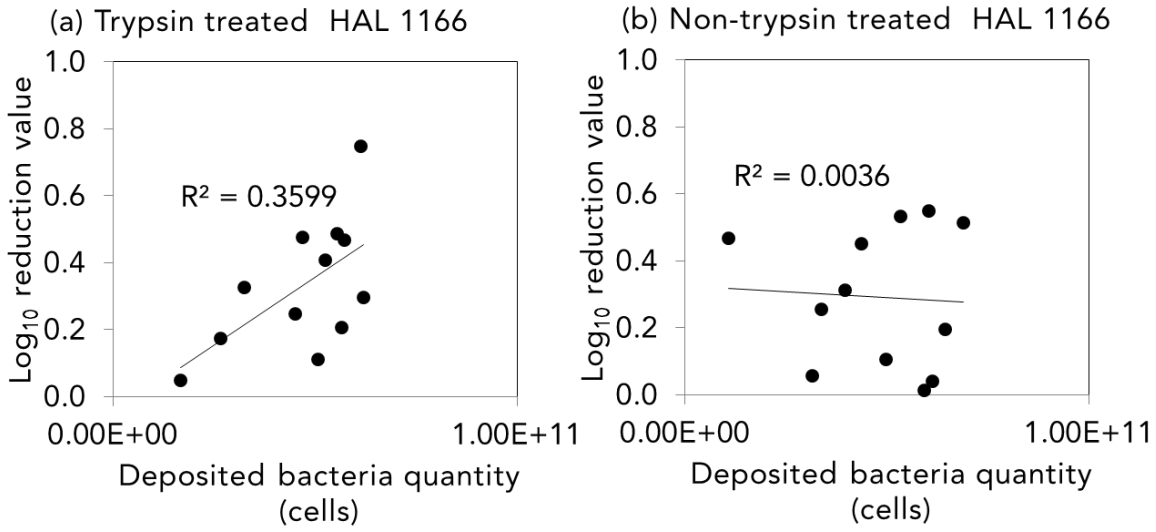

Figure 6.4: Correlation between the quantity of deposited bacteria and the log reduction value of rotavirus HAL1166

6.4 DISCUSSION

This study evaluated the specific virus–wastewater solids interactions using the *Enterobacter cloacae* SENG-6 – rotavirus HAL 1166 interaction as the model. A cross – flow membrane filtration test is performed and the effluent virus concentration reduces with time when HBGA – HAL1166 interactions are prevalent. There is a correlation between the deposition of HBGA-positive bacteria in the membrane and the virus log reduction.

Carbohydrate – protein interactions are reversible and the HBGA – norovirus/ rotavirus interaction is a typical carbohydrate – protein interaction (231, 232). Trypsin-treatment of HAL 1166 cleaves the VP4 protein and produces VP8* which specifically interacts with the receptors (60). In the current study, HBGA-like substances of *Enterobacter cloacae* SENG-6 acts as the receptor and the trypsin treatment activates the A-type HBGA binding of rotavirus HAL1166 and facilitates the interaction with *Enterobacter cloacae* SENG-6. Since trypsin treatment is the only difference between the two experimental conditions, specific interactions between the HBGA-like substances and trypsin treated rotavirus HAL1166 can be considered as the contributing factor for the decreasing trend of trypsin treated rotavirus HAL1166 concentration in the effluent with time compared to the non-trypsin treated HAL1166 concentration. Bacterial cell quantity deposited in the membrane increases with time and in the presence of both trypsin treated and non-trypsin treated rotavirus HAL1166 conditions bacterial cell quantities deposited on the membrane are similar. However, considering the LRV of HAL1166, only the log reduction of trypsin treated rotavirus HAL1166 displayed a strong positive correlation ($r = 0.60$) with the deposited bacteria quantity. It is expected that after the receptor binding, ligand also displays the motional properties similar to the receptor (231). Therefore, we can consider that trypsin treated rotavirus HAL1166 which are attached to the *Enterobacter cloacae* SENG-6 during the mixing phase is deposited in the membrane with bacteria (176). There are no specific interactions between *Enterobacter cloacae* SENG-6 and non-trypsin treated rotavirus HAL1166 and the rotavirus particles (~100nm diameter) (56) are free to pass through the 0.2µm pore size membrane.

Several studies have reported the presence of human enteric viruses in the wastewater effluents and it is a necessity to address this concern in order to provide microbiologically safe reclaimed water for any other beneficial purposes (67, 68, 71). In a previous study, genotype-dependent human norovirus removal in the presence of HBGA-positive *Enterobacter cloacae* SENG-6 is reported in microliter scale (176). Current study analyzed the importance of specific interactions between wastewater solids – human enteric viruses on the virus removal and reinforced the idea that specific virus – wastewater solids interactions can lead to increased removals of human enteric viruses.

Higher log reduction of human enteric viruses during the biological treatment can positively affect the subsequent disinfection processes; in particularly disinfection. Chemicals used in the disinfection process is reported to generate disinfection by products (233–235) which are reported to be associated with adverse health effects (236, 237). Therefore, improved biological treatment unit processes can reduce the usage of disinfection chemicals since the performance targets expected from disinfection can be lowered.

HBGA-positive bacteria are ubiquitously present in the environment (Amarasiri et al. unpublished data) and therefore the prevalence of specific enteric virus – wastewater solids interactions can be expected in wastewater. A further analysis on the environmental factors which can

lead to improved specific interactions can contribute to improved human enteric virus removal and provide a better understanding on the life cycle of human enteric virus associated with wastewater solids.

Chapter 7 : CONCLUSIONS AND RECOMMENDATIONS

Removal of human enteric viruses from wastewater before further beneficial reuse purposes is a must to avoid water-borne virus disease outbreaks. Many countries and states have published guidelines regarding the $\log_{10}$ removal of human enteric viruses necessary before reclaimed wastewater is suitable to be used in agricultural irrigation, landscape irrigation, urban non-irrigation uses, environmental and recreational, groundwater recharge and indirect and direct potable reuse. To achieve the performance targets, multiple-barrier systems are employed and the design of multiple-barrier systems needs reference log reduction values for each treatment unit process. In this study, we calculated the average log reduction of human norovirus and enteroviruses LRV by MBR process and human norovirus and rotavirus LRV by CAS. However, the number of studies which reports the LR of human enteric viruses by different treatment unit processes was limited and it was suggested to provide LR data of different treatment unit processes in future studies. In addition, it is found that many studies replaced zero or detection limit of the assay when addressing the left-censored data. Importance of using proper statistical methods to address left-censored data in future studies is emphasized.

Suitability of bacteriophages as the validation and operational monitoring indicators of human enteric virus removal is discussed using a meta-analysis approach. Meta-analysis results displayed intra- and inter-process variations in bacteriophage reduction efficiency in wastewater treatment unit processes. The intra-process LRV variation mainly depends on operational conditions, while bacteriophage diversity and surface characteristics of bacteriophages are additional factors for the LRV variation. The inter-process LRV variation is due to the difference in virus removal mechanisms involved in a particular unit process. MS2 coliphage shows lower LRVs compared to human viruses and is suggested as a validation and operational monitoring indicator in MBR.

Specific interactions between wastewater solids and human enteric viruses and their contribution to the human enteric virus removal in wastewater treatment plants is analysed using a microfiltration device. The interaction with HBGA-positive bacteria contributed to the removal of human norovirus particles with MF membrane. Cell-associated and HBGA-positive EPS was important for removing human norovirus particles with MF membrane. The removal efficiency of human norovirus particles with MF membrane and bacterial HBGA displayed genotype dependency. The study was scaled up using HBGA-positive bacteria and human rotavirus to evaluate the contribution of specific enteric virus – wastewater solids interactions on human enteric virus removal. In the presence of HBGA-positive bacteria, trypsin treated human rotavirus concentration in the effluent reduced with time. There was a strong correlation (Perason's $r = 0.6$) between the bacterial

deposition and trypsin treated human rotavirus removal indicating the contribution of specific human enteric viruses – wastewater solids interaction on the improved removal of human rotavirus.

We found specific human enteric virus – wastewater solids interactions can contribute to the improved removal of human enteric viruses by microfiltration. Evaluation of environmental factors which contribute to specific interactions between wastewater solids and human enteric viruses may provide hints to better understand the behaviour of human enteric viruses attached to specific wastewater solids in the environment. Moreover, evaluation of the seasonality of HBGA-positive bacteria may provide insights in to the seasonal variations in the virus log removal. Attachment to wastewater solids is reported to protect viruses from inactivation stresses. A study on the specific human enteric virus – wastewater solids interactions on the environmental persistency of human enteric viruses will be invaluable to understand the life cycle of human enteric virus in water environments.

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