

WASTEWATER-BASED EPIDEMIOLOGY AND QUANTITATIVE MICROBIAL RISK ASSESSMENT
ASSOCIATED WITH WASTEWATER CONTACT OF NOROVIRUS IN BANGKOK, THAILAND



A Thesis Submitted in Partial Fulfillment of the Requirements
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ระบดวทยาทางน้ำเสยและการประเมยความเสยงทางจุลยทรยเจยงปรยมาณที่เจยวข้องกบการสมผส
น้ำเสยของนรไวรัสในกรงเทพมหานคร ประเทศไทย



วทยาพนนทน์รฐเป็นส่วหนยงของการศยษาตามหลกสูตรปรยญาวทยาศาสตรมหาบัณทต
สาขาวิชาพชวทยาอุตสาหกรรณและการประเมยความเสยง ภาควชวทยาศาสตรลยงแวดลลอม
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Thesis Title	WASTEWATER-BASED EPIDEMIOLOGY AND QUANTITATIVE MICROBIAL RISK ASSESSMENT ASSOCIATED WITH WASTEWATER CONTACT OF NOROVIRUS IN BANGKOK, THAILAND
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นันทน์รัฐ ศรีโพธิ์รัตนะ : ระบาดวิทยาทางน้ำเสียและการประเมินความเสี่ยงทางจุลินทรีย์เชิงปริมาณที่เกี่ยวข้องกับการ
สัมผัสน้ำเสียของโนโรไวรัสในกรุงเทพมหานคร ประเทศไทย. (WASTEWATER-BASED EPIDEMIOLOGY AND
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โนโรไวรัสเป็นสาเหตุหนึ่งที่สำคัญของโรคกระเพาะลำไส้อักเสบเฉียบพลันทั่วโลก การเฝ้าระวังโดยใช้น้ำเสียนั้นมี
ประโยชน์อย่างมาก เช่น ช่วยเพิ่มความเข้าใจเกี่ยวกับการเกิดโรคในประชากร ประเมินประสิทธิภาพของการลดจำนวนเชื้อโรคใน
กระบวนการบำบัด และติดตามการแพร่กระจายของเชื้อโรคในสิ่งแวดล้อมที่ก่อให้เกิดความเสี่ยงต่อสุขภาพของมนุษย์ ดังนั้นการเฝ้า
ระวังด้วยน้ำเสียจึงเป็นสิ่งสำคัญ ในการศึกษาครั้งนี้ทำการเก็บตัวอย่างน้ำเข้าระบบและน้ำออกจากระบบบำบัดสองครั้งต่อเดือนเริ่มจาก
เดือนมีนาคม 2563 ถึงเดือนมีนาคม 2564 จากโรงบำบัดน้ำเสียทั้งสามแห่งในกรุงเทพฯ น้ำเสียตัวอย่างถูกทำให้มีความเข้มข้น
เพิ่มขึ้นและวิเคราะห์ปริมาณโนโรไวรัสด้วยเทคนิคปฏิกิริยาลูกโซ่โพลีเมอเรสเชิงปริมาณ(qPCR) เพื่อประเมินความเข้มข้นของโนโร
ไวรัส นอกจากนี้ยังมีการประเมินตัวบ่งชี้ทางจุลินทรีย์ โคลิฟอร์มแบคทีเรีย และโคลิฟาจโดยใช้วิธีเพาะเลี้ยงเซลล์ สำหรับความ
เข้มข้นของอีโคไลและโคลิฟอร์มแบคทีเรียในน้ำเข้าและน้ำออกจากระบบมีค่าตั้งแต่ 4.04 – 5.03 log CFU/ml และ 2.64 – 3.76
log CFU/ml ตามลำดับ ความเข้มข้นของโคลิฟาจในน้ำเข้าและน้ำออกจากระบบมีค่าตั้งแต่ 1.76 log PFU/ml และ 0.42 log
PFU/ml ตามลำดับ ความเข้มข้นเฉลี่ยของโนโรไวรัส GI และ GII คือ (1.90 และ 1.35 copies/ml) และ (0.91 และ 0.37
copies/ml) ในน้ำเข้าและน้ำออกจากระบบตามลำดับ ในขณะที่เดียวกันสามารถกำจัดโนโรไวรัส GI และ GII อยู่ที่ 0.75 และ 0.54
log copies/ml. เมื่อสังเกตความผันแปรของฤดูกาลโดยจุลินทรีย์มีความเข้มข้นสูงในช่วงฤดูฝนและความเข้มข้นของโนโรไวรัสสูงสุด
ในช่วงฤดูหนาว เมื่อทำการเปรียบเทียบทางระบาดวิทยาระหว่างความเข้มข้นของโนโรไวรัสจากโรงบำบัดน้ำเสียกับรายงานผู้ป่วยที่ติด
เชื้อโนโรไวรัสพบว่ารูปแบบที่คล้ายคลึงกันซึ่งชี้ให้เห็นว่าน้ำเสียสามารถใช้เป็นเครื่องมือที่มีประโยชน์ในการตรวจหาการติดเชื้อ
ในประชากรโดยเฉพาะในสถานการณ์ที่มีข้อมูลทางคลินิกจำกัด ตัวอย่างน้ำออกจากระบบถูกนำมาหาค่าสหสัมพันธ์แบบสเปียร์แมน
ระหว่างจุลินทรีย์ตัวบ่งชี้และสายพันธุ์ของโนโรไวรัส จากการวิเคราะห์พบว่าตัวบ่งชี้จุลินทรีย์เหล่านี้ไม่สามารถเป็นตัวแทนของโนโร
ไวรัสได้เนื่องจากมีค่าสหสัมพันธ์ที่ต่ำ ($r_s < 0.3$) อีกทั้งน้ำออกจากระบบยังถูกนำมาประเมินความน่าจะเป็นของการเจ็บป่วยด้วยโรค
กระเพาะลำไส้อักเสบเฉียบพลันจากการรับสัมผัสน้ำเสียด้วยการประเมินความเสี่ยงทางจุลินทรีย์เชิงปริมาณของกิจกรรมตกปลาและ
การโดยสารด้วยเรือขนาดเล็ก การสัมผัสน้ำออกจากระบบมีความน่าจะเป็นของทั้งสองกิจกรรมมีค่ามัธยฐานของโอกาสในการ
เจ็บป่วยต่ำกว่า 0.036 แม้ว่าโรงบำบัดน้ำเสียจะสามารถลดจำนวนจุลินทรีย์ลงได้ แต่น้ำเสียที่ผ่านการบำบัดยังคงมีจุลินทรีย์ตกค้าง
ซึ่งก่อให้เกิดความเสี่ยงต่อสุขภาพหากปล่อยลงสู่แหล่งน้ำสาธารณะหรือใช้ในกิจกรรมต่างๆ โดยแนะนำให้พัฒนาระบบบำบัดน้ำเสีย
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KEYWORD: Norovirus, Microbial indicator, Wastewater-based epidemiology (WBE), Quantitative microbial risk assessment (QMRA)

Nonnarit Sriporatana : WASTEWATER-BASED EPIDEMIOLOGY AND QUANTITATIVE MICROBIAL RISK ASSESSMENT ASSOCIATED WITH WASTEWATER CONTACT OF NOROVIRUS IN BANGKOK, THAILAND. Advisor: JATUWAT SANGSANONT, Ph.D.

Norovirus is one of the leading causes of acute gastroenteritis worldwide. Wastewater surveillance offer significant benefits such as enhancing our understanding of the disease occurrence in the population, evaluating the effectiveness of pathogen reduction in the treatment process, and tracking the spread of pathogens in the environment which pose a health risk to humans. Hence, wastewater surveillance is crucial. In this study, the influent and effluent samples were collected bi-monthly from March 2020 to March 2021 from three distinct wastewater treatment plants in Bangkok. Wastewater samples were concentrated and subjected to quantitative polymerase chain reaction (qPCR) to assess norovirus concentrations. Additionally, microbial indicators, coliform bacteria and coliphage, were evaluated using cell culture methods. Concentration of *E. coli* and total coliform in the influent and effluent ranged from 4.04 – 5.03 log CFU/mL and 2.64 – 3.76 log CFU/ml, respectively. Concentration of coliphage in the influent and effluent ranged from 1.76 log PFU/ml and 0.42 log PFU/ml, respectively. Average concentrations of norovirus GI and GII were (1.90 and 1.35 log copies/ml) and (0.91 and 0.37 log copies/ml) in the influent and effluent, respectively. Removal efficiency for microbial indicators was 1.26 – 1.40 log CFU, PFU/ml while the removal of norovirus GI and GII were 0.75 and 0.54 log copies/ml. Seasonal variations were observed, with the highest concentrations of microorganisms during the rainy season and the highest norovirus concentration in winter. An epidemiological comparison between norovirus concentrations from wastewater treatment plants and reported norovirus cases showed similar patterns, suggesting that wastewater could serve as a useful tool for detecting infections within a population, particularly when clinical data are limited. Effluent samples were used to determine the Spearman's correlation between the microbial indicators and both norovirus strains. Our analysis found that these microbial indicators could not represent norovirus due to low correlation ($r_s < 0.3$). Additionally, the effluent was also used to assess the probability of illness with acute gastroenteritis from exposure to wastewater by quantitative microbial risk assessment from fishing activities and small craft boating. Exposure to effluent from the system, the probability of both activities had a median probability of illness below 0.036. Despite the ability of wastewater treatment plants to reduce the number of microorganisms, the treated wastewater still harbored these microorganisms presenting a potential health risk if discharged into public water sources or used in activities. Efficient wastewater treatment systems are recommended to be developed, including disinfection methods, to mitigate the risks from waterborne pathogens.

Field of Study:	Industrial Toxicology and Risk Assessment	Student's Signature
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CHAPTER 1

INTRODUCTION

1.1 Background

Diarrhea diseases have been associated to be major causes of mortality and morbidity with significance in developing countries in children less than the age of 5 years (Ugboko et al., 2020). There are 716,513 diarrhea illness cases and 3 deaths, reported by department of Disease Control, ministry of public health, Thailand in 2019. The majority of outbreaks is widespread, leading to high numbers of people and their symptoms can lead to serious complications such as kidney failure or death especially in young children and elderly. Diarrhea disease is transmitted predominantly through the fecal-oral route by ingesting water and food contaminated Up to 70% of acute diarrhea in children were also due to the pathogenic viruses. (Fewtrell & Kay, 2015). Among the viral infection causing diarrhea disease, norovirus (NoV) is the one most important causes of acute gastroenteritis worldwide (Van Trang et al., 2014) which incidence of diarrhea from both viruses are obtain from clinical testing in infected people.

For gastrointestinal epidemiology study, it can be done through the examination of patient's feces. However, only patients who have already been diagnosed with symptoms have been subjected to cause of disease analysis. Diarrhea clinical examination was therefore unable to reflect infections in the overall population. There are several reports on asymptomatic infection of gastroenteritis disease. For example, asymptomatic excretion of NoV was detected in stool samples of 31 of 63 (49.2%) asymptomatic children in Periurban, Mexico (Garcia et al., 2006). Hence, asymptomatic people in community are a clinical epidemiology limitation. Therefore, the epidemiology surveillance was developed strategy to help illustrate the overall epidemic.

Wastewater can be one of the channels for studying the epidemiology of viral infection among the population. There are several studies using wastewater-based epidemiology (WBE) to detect infectious people in the interested area (Aguiar-Oliveira et al., 2020). The viral concentrations found in wastewater can reflect the correspondingly variable number of infected patients. Viruses were excreted from infected people both symptomatic and asymptomatic may represent the amount of virus circulating in the community (Hellmer et al., 2014). WBE is particularly useful for early warning of public health virus outbreaks (Asghar et al., 2014). In Thailand, there is still little research on the WBE, while these tools an effective aid in public health surveillance (Campos et al., 2016). In addition, the process of handling wastewater contaminated with the virus is an important environmental and health risk.

Wastewater treatment plants are used to reduce wastewater contaminated by pathogenic microorganisms. Quality of the effluent is therefore important to consider as it is relevant to health. As well as people and workers in the area may be exposed to treated wastewater that is still contaminated with microbes. The assessment of probability of gastroenteritis illness of effluent contact was assessed by Quantitative Microbial Risk Assessment (QMRA) for making decision the control strategy of pathogenic microorganisms releasing from wastewater treatment plant.

The effluent quality must be up to standard of WHO by examining the amount of fecal indicator bacteria (FIB) found in treated effluent. Total coliform is presently being used to assess contamination in water quality management. In addition, some viruses as bacteriophage that must be considered. There is few research has associated the relationship between microbial indicators and virus in wastewater in tropical region. Therefore, this research is part of the study to find relationship of microbial in effluent to compare and reduce the microbial load in the discharge to an acceptable level before it is released into the water catchment. Therefore, WBE and QMRA are efficiency tools for aid in surveillance and assess the health risks of wastewater to assist in better management of quality in wastewater treatment plants in the future.

1.2 Objectives

1. To compare the data between the norovirus concentration in influent from wastewater treatment plant and clinical cases.
2. To assess the probability of infection and illness from gastroenteritis due to norovirus exposure in wastewater effluent.
3. To determine the correlation between norovirus and microbial indicators in effluent.

1.3 Scope and limitation of the study

1. The experiment will be conducted using samples from three wastewater treatment plants in Bangkok: Din Daeng WWTP, Chong Nonsi WWTP and Nong Khaem WWTP.
2. For our study, influent samples will be used for epidemiological studies, while effluent samples will be used for quantitative microbial risk assessment (QMRA) of norovirus
3. Norovirus levels in wastewater will be measured using quantitative PCR. However, the data does not differentiate between infectious and non-infectious viruses. Despite this limitation, the information will still be used for quantitative microbial risk assessment (QMRA).

4. In QMRA model, we calculated the probability of illness under the assumption that the population had no acquired immunity, there was no secondary transmission, and no presence of sensitive groups.

1.4 conceptual and framework

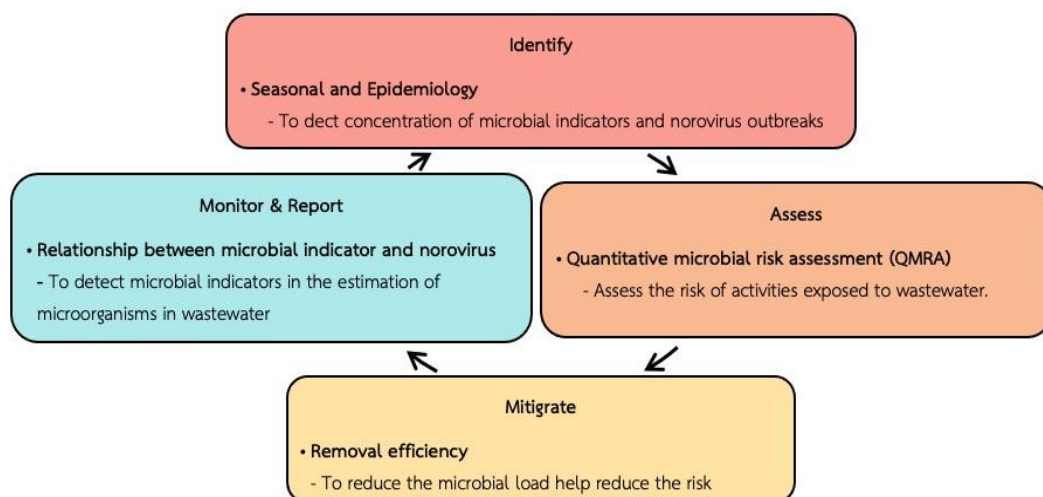


Figure 1 Conceptual and framework



CHAPTER 2 THEORETICAL BACKGROUNDS

2.1 Viral gastroenteritis

Acute gastroenteritis is a disease that occurs all over the world. Most of the causes of disease are viral infections. Acute diarrheal disease often occurs in developed countries with significant implications for illness among children and the elderly. In underdeveloped countries, viral diarrhea is the leading cause of death, especially in infants (Shane et al., 2017). According to the Centers for Disease Control, viral gastroenteritis, defined as diarrhea or watery diarrhea occurring or more times in a day, contributes to the deaths of more than 200,000 people worldwide each year. Viral gastroenteritis is a known cause of nausea, vomiting, diarrhea, anorexia, weight loss, and dehydration. Symptoms usually last less than a week, and often get better after 1 to 3 days but diarrhea can last for a couple of weeks. Acute diarrhea is an immediate symptom within 1-2 hours of exposure to the virus. Most of this transmission occurs through consumption of water contaminated with food and people to people by oral feces, contact with contaminated surfaces or airborne transmission. Viral gastroenteritis often occurs in outbreaks in neighboring communities such as daycare centers, hospitals and schools. There are many types of viruses that can cause this disease. NoV and RoV are the two most important causes of acute gastroenteritis in worldwide (Ugboko et al., 2020).

2.2 Norovirus

2.2.1 Structure and Genotypes

Norovirus, previously known as the Norwalk virus, was first found in a stool sample collected during a gastroenteritis outbreak in Norwalk, Ohio, and was the first virus to cause gastroenteritis (Kapikian et al., 1972). Its immunoelectron microscopy image was shown in Figure 1. Norovirus is classified as a family *Caliciviridae* and the genus *Norovirus*. They have linear RNA genome approximately 7.6 kb nucleotides in length, positive-sense and single-stranded (Capece & Gignac, 2023). Norovirus are classified into five genogroups by using techniques phylogenetic analysis of the viral capsid (VP1). The most outbreaks of acute gastroenteritis in worldwide are caused by norovirus GI.1 and GII.4 (Bull et al., 2006).

The genome is organized into open reading frames consist of ORF1 - ORF3. ORF1 can transcribe for a structural protein VP and nonstructural proteins such as a 3C-like-proteolytic enzyme and 3D-like RNA polymerase. ORF2, encodes for structural capsid protein called viral protein 1 (VP1). ORF3, encodes for the VP2 is minor capsid which serves to stabilize the capsid

(Donaldson et al., 2008). Transfection are complete when RNA genome of NoV can build intact virus particles, suggesting that the genome itself is infectious (Garcia et al., 2006).

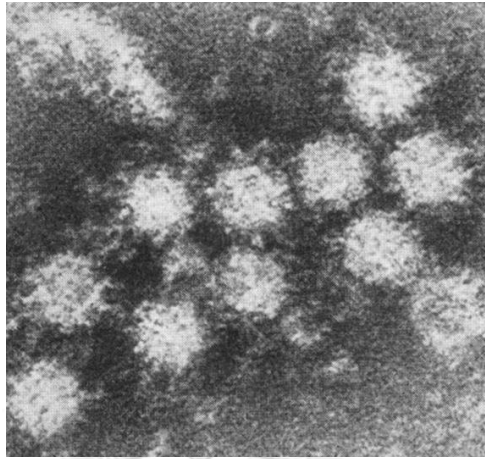


Figure 2 Immunoelectron microscopy of norovirus (Kapikian et al., 1972b)

Norovirus, previously known as the Norwalk virus, was first found in a stool sample collected during a gastroenteritis outbreak in Norwalk, Ohio, and was the first virus to cause gastroenteritis (Kapikian et al., 1972). Norovirus is classified as a family *Caliciviridae* and the genus *Norovirus*. They have linear RNA genome approximately 7.6 kb nucleotides in length, positive-sense and single-stranded (Jiang et al., 1996). Norovirus are classified into five genogroups by using techniques phylogenetic analysis of the viral capsid (VP1). Norovirus genogroups I (GI) and II (GII) are most associated with human infections. Most outbreaks worldwide are caused by GI.1 NoV and GII.4 NoVs (Bull et al., 2006).

The genome is organized into open reading frames consist of ORF1 - ORF3. ORF1 can transcribe for a structural protein VP and nonstructural proteins such as a 3C-like-proteolytic enzyme and 3D-like RNA polymerase. ORF2, encodes for structural capsid protein called viral protein 1 (VP1). ORF3, encodes for the VP2 is minor capsid which serves to stabilize the capsid (Domain, 2013). Transfection are complete when RNA genome of NoV can build intact virus particles, suggesting that the genome itself is infectious (García et al., 2006)

2.2.2 Epidemic and seasonality

Several studies have been conducted on seasonal norovirus outbreaks. Concentrations of norovirus in wastewater and patient outbreaks during winter were higher than in other seasons (Haramoto et al., 2006; Victoria et al., 2010). Pathogen seasonality is the occurrence of repeated

epidemics at predetermined times of the year (Dowell, 2001). The characteristics and timing of norovirus epidemics are extremely similar from year to year, peaking in winter from October to April (Bull et al., 2006). Zeng et al. (2012) analyzed outbreaks of norovirus in southwestern China by utilizing one step real-time RT-PCR technique on patient fecal samples. They found that the incidents was higher in Shanghai and Hangzhou during the late summer and fall, and lower during the winter. This is proven by the norovirus' continued presence in gastroenteritis throughout the off-season (May-September). In other studies, although outbreak rates drop in the summer, the data prompt us to wonder why norovirus infections peak in the winter and show a decreased occurrence in the summer, without going away. Pathogen seasonality is linked to changes in host behavior, environmental changes, and pathogens presence and disappearance (Dowell, 2001).

Norovirus outbreaks appear sporadic towards the end of the summer. and more epidemic in the winter, which could show that norovirus survives long-term in the host and another seasonal outbreak pattern. There are three reasons that contribute to the ability of norovirus to survive for a long time and can be spread effectively and silently from asymptomatic to non-infected people.

1. Norovirus is shed in very high loads.
2. It caused a self-limiting disease that lasts from 24 to 48 hours in otherwise healthy individuals, which facilitates unnoticed transmission.
3. Asymptomatic individuals can shed the virus over time and at high loads, enabling efficient chain-to-chain transmission.

This kind of silent transmission is comparable to the spread of influenza virus. In fact, noroviruses exhibit evolutionary patterns resembling those of influenza (i.e., antigenic drift punctuated by antigenic shift every two to three years) (Lindesmith et al., 2008). This pattern of evolution allows noroviruses to persist in human populations by evading host immunological pressure through antigenic drift and/or invading naive populations (Donaldson et al., 2008).

Changes in the natural environment can influence the seasonal impact on the waterborne pathogen in various ways. Seasonality of infectious diseases is correlated with changes in the environment, including humidity (Chew et al., 1998), temperature (Checkley et al., 2000), rainfall patterns, and winds. Changes in humidity have been shown to facilitate viral persistence in gastrointestinal infectious agents like rotaviruses, raising the likelihood of transmission through contaminated surfaces. For influenza viruses, comparable findings were made (Anastasi et al., 2012). Increased humidity may likely help the noroviruses transmit more effectively, as these viruses can spread not only through the fecal-oral pathway but also by aerosols (vomit). It is tempting to hypothesize that variations in temperature and humidity may affect norovirus virulence,

transmission, and/or resistance. The frequency of norovirus in sewage water during the floods caused by the Elbe River spilling its banks was previously covered by us (Rohayem et al., 2006). The previous study's findings show that norovirus prevalence increased when water temperatures were low, suggesting that the virions may have a favorable physical habitat. However, more research is necessary to explore the potential impact of temperature and humidity on the frequency and seasonality of noroviruses. In addition, there are other studies of Ahmed et al., (2013) studied the data showed that 52.7% of the cases occurred and 41.2% of the outbreaks occurred during the winter. Both cases and outbreaks the severity of the norovirus outbreak is positive with the average rainfall in the wettest months. In the same way the studies of Lopman et al., (2009) was found Lower temperatures, decreased relative humidity, decreased population immunity, and the emergence of new norovirus strains were independently associated with increases in norovirus. The temperature and humidity have affected a reduction in the number of viruses.

In order to explain increased transmission and the subsequent occurrence of infectious diseases, population density is usually invoked (Dowell, 2001). Highlighting the importance of host behavior in disease transmission, the transmission models for infectious diseases among children indicate that patterns of transmission are linked to the frequency of contact. Clinical cases tended to rise during school terms and decline during holiday periods. Thus, it is possible to human behavior for facilitating the spread of the norovirus throughout the winter.

The epidemiological pattern of norovirus strains showed that norovirus GII strains were more widespread. This conclusion was drawn from the data found in wastewater and the number of cases reported by hospitals (Kazama et al., 2017). In Thailand, statistics of norovirus outbreaks are primarily collected in infected patients, which may lack data on other forms of outbreaks. However, other channels such as wastewater may be able to predict the infection of the population.

2.3 Wastewater treatment Plant

For the preservation of natural ecosystems and human health, it is crucial to remove the pollutants causing by households and agriculture. Wastewater treatment plant (WWTP) use a variety of mechanical and biological procedures to remove various organic and inorganic pollutants, transforming polluted water into a state that is suitably clean (Ahkola et al., 2021). Large-scale plants typically treat wastewater in three phases. Physical methods of water purification are used in the first stage, and chemical and/or biological treatment in bioreactors with suspended or

attached activated sludge (AS) is used in the second stage. The third stage is the final treatment disinfection. In Bangkok there are 8 large wastewater treatment plants covering 21 districts with a total area of 212.74 square kilometers (Figure2). The effluent from WWTP in Bangkok has treatment requirements shown in Table 1. The WWTP in Bangkok only have first and second treatment systems without disinfection systems. Treated wastewater may contain amounts of microorganisms. When the wastewater treatment meets the specified standards, the wastewater will be discharged into public water sources canal or used in various activities. The amount of microorganisms remaining in the treated wastewater is not measured. This oversight poses a risk with exposure to treated wastewater.

Table 1 Effluent standards from the municipal wastewater treatment system

Parameter	unit	standard
1.pH	-	5.-5 – 9.0
2.biochemical oxygen demand (BOO)	mg/ml	20
3. suspended solid (SS)	mg/ml	30
In case that the final treatment pond is stabilization or oxidation pond	mg/ml	50
4.Oil and grease	mg/ml	5
5. total nitrogen (TN)	mg/ml	20
6. total phosphorus (TP)	mg/ml	2

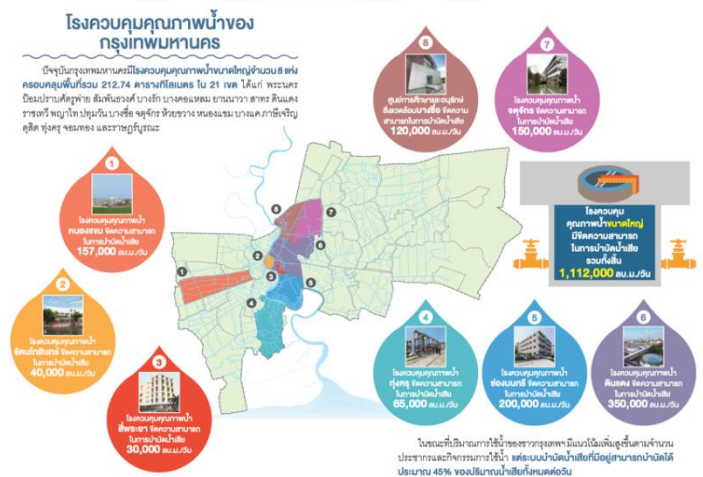


Figure 3 The area covered by eight large wastewater treatment plants in Bangkok.

Previous research has found that various microorganisms remain in the treated wastewater, such as *E. coli* bacteriophage and norovirus. Previous studies have found that various

microorganisms remain. The study by Campos et al., (2016) found norovirus GI and GII in treated wastewater. Secondary treatment can reduce GI and GII by only 3.11 and 2.34 respectively. Improving the efficiency of the activated sludge process may lead to a better reduction in the amount of norovirus. Meanwhile, Wastewater treatment plant in Bangkok found norovirus in 44.4% of samples of reclaimed water and 73.9% of samples of sewage sludge. In recycled water samples, norovirus GI.2 and GII.4 were found at concentrations of 2.19×10^1 and 3.26×10^4 copies/L, respectively. In sewage sludge samples, six different GI genotypes and GII.17 were found at concentrations ranging from 1.99×10^1 - 1.43×10^5 copies/g. Sewage sludge samples contained the four recombinant norovirus GII strains (Kittigul et al., 2019). Noroviruses can be found in treated water and waste sludge, causing a health risk from environmental exposure.

In addition to norovirus, treated wastewater contains other viruses, such as rotavirus, that also cause acute diarrheal d. A study of effluent from a WWTP in Thailand by Kittigul & Pombubpa (2021), was found there is rotavirus in 30.2 % of treated water samples and 50.0 % of sewage sludge samples, respectively. G1 was consistently present in treated wastewater. The rotavirus strains found in this study have a strong nucleotide identity to human rotavirus strains, indicating that they are derived from humans. As different viruses have been found in treated wastewater. WWTP need to improve the microbial load reduction in the effluent to reduce the risk of exposure to microorganisms.

2.4 Relation between virus and microbial indicator

Microorganisms found in water bodies, such as bacteria and viruses, are referred to as indicator organisms and are used as a substitute for pathogens in pathogen detection. It is preferable that these bacteria have no or minimal growth in water, are nonpathogenic, and can be consistently detected at low concentrations. The indicator organisms must be more prevalent than the linked pathogen and, ideally, have comparable rates of survival to the infection. As was covered in this part, several indicator organisms can be employed to monitor water quality, and the accuracy of pathogen prediction depends on the organisms detection limit, susceptibility to environmental stresses, and other contaminations (Motlagh & Yang, 2019).

Fecal indicator bacteria (FIB) are currently being used to identify waterborne fecal contamination and to screen for bacteria that are susceptible to other pathogens, as well as to determine the distribution of bacteria in this community as a microbiological water quality indicator. Fecal Streptococcus, *E. coli*, and fecal coliforms. To determine pollution in the control of water quality, there are still several viruses to member. The distribution in the environment is different

due to the different topography. Few research has been done on the relationship between FIB and enteric virus environmental viruses in terms of microbial marker distribution.

Campos et al., (2016) was found sample size and the number of samples positive for pathogens were the most significant factors in assessing associations between indicator–pathogen pairs. Correlations between markers and pathogens are unaffected by pathogen origins, detection processes, or other variables. The findings indicate that much of the debate about indicator–pathogen associations stems from research with inadequate data to determine correlations.

2.4.1 Coliform bacteria

Coliform bacteria are a type of microbial indicator that is generally used to assess how much feces is polluted in water. Fecal markers such as *E. coli* are thought to be a good microbial agent for studying waterborne pathogen activity, and they were created to replicate environmental conditions. *E. coli* is a valuable method for analyzing the behavior of waterborne pathogens, and it was developed to imitate natural conditions (Garcia-Aljaro et al., 2019). However, *E. coli* does not seem to be successful in all tests. *E. coli* is a weak predictor for predicting the risk of fecal pathogen exposure in shellfish, according (Flannery et al., 2012).

2.4.2 Bacteriophages

Bacteriophages, also known as phages, are viruses that specifically infect bacteria. They are composed of a nucleic acid molecule, called the genome, which is surrounded by a protein coat called a capsid. In addition to the capsid, many phages possess other structures like tails and spikes. Although it is less common, some phages may also contain lipids. Phages vary in size and typically fall within the range of viral particles. This similarity in size suggests that phages share several characteristics, including composition, structure, morphology, and capsid size, with animal viruses and, consequently, human viruses. It is widely acknowledged that the persistence of bacteriophages in the environment and their resistance to treatment bear resemblances to the behavior and characteristics of viruses. Bacteriophages can replicate only within host bacteria that are susceptible and metabolically active. They exhibit host specificity, meaning that a particular bacteriophage can only infect certain types of bacteria. The extent of this host specificity varies depending on the specific bacteriophage. The host specificity of phages is primarily determined by receptor molecules present on the surface of bacteria. These receptor molecules can be found in various parts of the bacteria, including the capsule, cell wall, flagella, and pili. Among these, phages that attach to receptors located in the cell wall, known as somatic phages, are the most common type (Jofre et al., 2016).

Two groups of bacteriophages that infect *E. coli*, namely somatic coliphages and F-specific coliphages, have been widely employed as indicators for both fecal contamination and viral presence. These coliphages are found in high concentrations in raw wastewater and other matrices that are contaminated with fecal matter (Jofre, 2007). Their detection and enumeration methods are relatively simple, fast, and cost-effective. Moreover, they exhibit persistence in water environments and are resistant to treatment methods commonly used for viruses. These make indicator bacteriophages suitable surrogate indicators for various applications.

Coliphages are a specific group of bacteriophages that infect *E. coli*. However, the term "coliphage" is quite broad, as it can encompass phages that infect various strains of *E. coli*, and different strains of host bacteria may have varying susceptibilities to different sets of coliphages. In the context of using phages as indicators in the water environment, there is an unofficial consensus that the term "coliphage" specifically refers to phages that infect specific host strains (Muniesa et al., 1999). This distinction will be further explained in the Methods section below. In the field of water quality, the term "coliphage" typically refers to two main groups: somatic coliphages and F-specific coliphages.

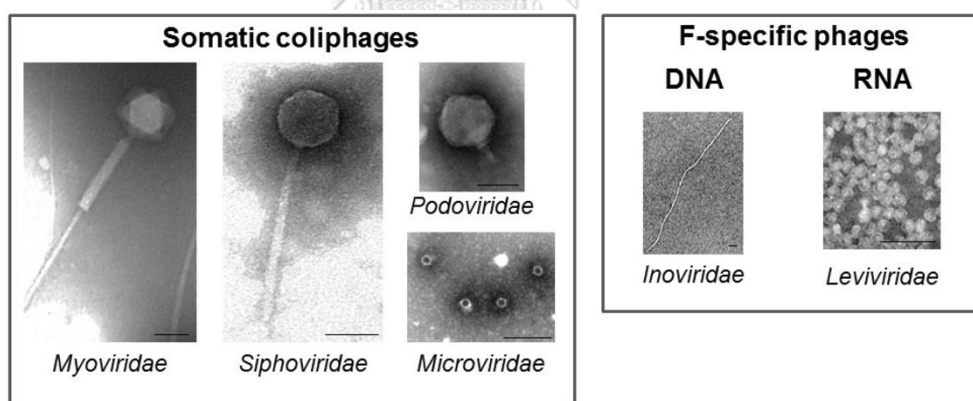


Figure 4 The morphological types in somatic coliphages and F-specific phages size of 50 nm.

Somatic coliphages are a diverse group of bacteriophages that infect *E. coli* by attaching to receptors on the bacterial cell wall. Among the somatic coliphages detected using the host strains recommended in standardized methods and commonly found in municipal wastewater, the majority belong to several families, including *Myoviridae*, *Siphoviridae*, *Podoviridae*, and *Microviridae*. These families encompass a wide range of phage morphologies and characteristics. By targeting specific host strains and using standardized methods, researchers and practitioners can identify and classify somatic coliphages present in wastewater samples, providing valuable

information about the composition and diversity of this group of bacteriophages (Muniesa et al., 1999).

F-specific bacteriophages, also known as sexual coliphages or male-specific bacteriophages, are a type of bacteriophage that infect bacteria by binding to the sex pili. These pili are encoded by the F plasmid, which was initially identified in *E. coli* K-12. Among F-specific bacteriophages, there is a subgroup called F-specific RNA bacteriophages. These phages have a simple capsid with cubic symmetry and a diameter ranging from 21 nm to 30 nm. Their genome consists of a single-stranded RNA molecule. The F-specific RNA bacteriophage group belongs to the *Leviviridae* family and includes two genera: *Levivirus* and *Allolevirus*. Additionally, there are three minor unclassified groups within this phage group. The *Levivirus* genus consists of subgroups I and II, while the *Allolevirus* genus comprises subgroups III and IV. These classifications help categorize and distinguish different strains and variants of F-specific RNA bacteriophages (Fauquet & Fargette, 2005).

As described in further detail above, both somatic coliphages and F-specific coliphages are present in substantial quantities in raw municipal wastewater, animal wastewater, animal slurries, and manure worldwide. It is noteworthy that neither of these coliphage groups exhibits seasonal variation in pollution sources or surface waters. This indicates that their presence and abundance remain relatively consistent throughout the year, regardless of environmental factors or specific sources of contamination. The consistent occurrence of these coliphages underscores their potential as reliable indicators of fecal contamination and viral presence in various water sources. The study of Antony et al., (2014) identified that, due to their structure, morphology, scale, environmental persistence, and resistance to treatment methods, bacteriophage (phages) has been suggested as a possible candidate for indicator for waterborne pathogen. It is nonpathogenic to humans and more similar to enteric viruses than indicator bacteria. In addition, another study on bacteriophage by Cole et al., (2003) explored the possibility study of using bacteriophages as indicators for viral pathogens in shellfish. Their results indicated that they could be used as predictive measure for the contamination of pathogens in water. In some cases, bacteriophages were detected even when *E. coli* was not, which suggests that bacteriophages may serve as a more sensitive marker for viral risk assessment.

2.5 Wastewater-based Epidemiology (WBE)

Wastewater-based epidemiology (WBE) is a tool used for studying infectious disease or public health data by analyzing wastewater. WBE analyzes the presence or amount of a chemical or biological signals in a pooled sample of sewage collected from the sewer network or wastewater treatment plant (WWTP). Measurements of chemical or biological signal concentrations can reveal patterns of drug or substance consumption in a community or reveal signs of chemical exposure or disease. The degree of sickness or the spread of diseases within a community can be determined by other human indicators or biological signals like viral RNA. The processes involved in WBE programs include sampling, sample preparation, analysis, data processing and interpretation, and reporting. Each of these steps has the potential to incorporate uncertainty into the data's ultimate interpretation (O'Keeffe, 2021).

There are many different uses for WBE, and some of them are more developed technologically and in use than others. One of the earliest uses of WBE that was suggested was the analysis of wastewater for signs of illicit drug usage (de Oliveira et al., 2023). Since then, WBE has been used to analyze a wide range of indicators, including markers of antimicrobial resistance (AMR), alcohol, tobacco, infections, treatments, and more (Donaldson et al., 2008). The upcoming discussion content will focus on estimating the prevalence of infectious diseases.

Direct measurements of microbial DNA/RNA for infectious diseases brought on by viruses and bacteria can show the presence of infection in a population, and the strength of the signal can show the prevalence. One of the first examples of this was for poliovirus surveillance. Following a poliovirus outbreak in Israel in 2013, the virus was identified in sewage, and a dose-dependent relationship between the viral content in sewage and the quantity of positive cases (active shedders) was discovered (Berchenko et al., 2017). The molecular properties of the virus that was found made it possible to identify the origin and location of patients (Contreras et al., 2017). In order to supplement clinical data, WBE is a component of the Global Polio Eradication Initiative's surveillance plan in a number of nations (Global Polio Eradication Initiative, 2021). A number of additional human viruses, such as enteroviruses and norovirus (Hellmér et al., 2014; Lu et al., 2021) have been quantified using WBE at the population level. Some of these research have utilized WBE to show patterns in the prevalence of certain viruses (Brinkman et al., 2017) or as an early indication of outbreaks (Hellmér et al., 2014).

In the event of the COVID-19 pandemic, a study of Aguiar-Oliveira et al., 2020 was found the high frequency of SARS-CoV-2 RNA detection in influent and effluents as hospital sewage samples collected from May to June 2020 in untreated wastewater samples. The amount of SARS-CoV-2 The RNA detected in wastewater can vary greatly from day to day. High concentrations in wastewater may be detected 10-14 days before the outbreak in the population. The future of the WBE may be used for public notice, health policy and practice and decision-making in public health. WBE can be applied as a tool to predict the outbreak of various viruses from wastewater.

2.6 Quantitative microbial risk assessment

Quantitative Microbial Risk Assessment (QMRA) is assessing human health risks associated with exposure to pathogens which makes it possible to get infected or illness. It was calculated from exposure assessment and dose-response relationships. QMRA is used to assess water-related risk issues and identify important uncertainties and knowledge gaps. QMRA is used as THE US EPA tool to inform the criteria for the quality of recreational water source (EPA, 2012).

2.6.1 Hazard identification

One of the first steps in a quantitative microbiological risk assessment (QMRA) is hazard identification. The list of dangers related to these problems must be described after the problem formulation, which may include discussion of the locations, circumstances, and issues that need to be addressed. For QMRA, the hazard identification includes a wide range of details on the infectious agents as well as general information about the microbial agent (pathogens) and the negative effects on the host from infection. An endpoint of infection relates to a pathogen that can infect host organisms, proliferate, and possibly generate asymptomatic infections without disease. A disease's latency, incubation periods, infectiousness and disease duration, percentage of cases with various symptoms, excretion rates, and immunity (e.g., the length of time immunity lasts), can all be quantitatively defined.

2.6.2 Exposure assessment

The simplest definition of exposure is the amount of the pathogen that a person consumes, inhales, or comes into contact with. The dose-response models use this quantity to forecast the likelihood of infection. However, determining the date of the exposure as well as the methods used to quantify the microorganisms and their concentrations, for instance in water or the air, makes up exposure assessment, which is a very complex process. Most of the time, exposure can

be thought of as a conduit leading from the pathogen's source (such as the discharge of pathogens by infected people or concentrations in sewage) to the exposure site (such as swimming at the beach). Understanding the microbe's survival and transportation is also necessary (Haas, 2014).

2.6.3 Dose-response model

Dose-response models are that forecast the association between the degree of microbial exposure and the likelihood of unfavorable health effects. In the broadest sense, a dose-response model is a mathematical function that accepts a dose measure as an argument, which can be any non-negative integer, and produces the likelihood of a specific adverse impact, which is bounded by zero (no effect). As for the models in the dose, there are many variations. Two models are shown as examples. The single-parameter exponential function (Eq. 1) and the two-parameter beta-Poisson (Eq. 2) are two dose-response models that have been shown to be highly applicable to the majority of microorganisms and exposure pathways (Haas, 2014). In selecting the model depending on the spread of the infection, such as *Cryptosporidium spp.*, the study found that the probability of infection is suitable for the model of exponential. At the same time, the viral infectivity model follows beta-Poisson.

$$P(d) = 1 - e^{-kd} \quad \text{Eq.1}$$

the exponential function Eq. (1), $P(d)$ represents the probability of infection and d is a single dose at exposure. The base of the natural logarithm (e) and the probability that one organism survives to initiate the health outcome (k) are pathogen infectivity constants (Haas, 2014).

$$P(d) = 1 - \left[1 + \left(\frac{d}{N_{50}} \right) \times \left(2^{\frac{1}{\alpha}} - 1 \right) \right]^{-\alpha} \quad \text{Eq.2}$$

the beta-Poisson function shown in Eq. (2), $P(d)$ represents the probability of infection and d a single dose at exposure, with model slope parameter α and median effective dose N_{50} . The data analyses used to develop the functions originates primarily from clinical trials (Haas, 2014).

2.6.4 Risk characterization

The fourth and last phase involves incorporating the results of the first three into a mathematical model that determines the risk by calculating the likelihood of contracting an infection, becoming ill, or passing away. This stage is a little challenging because the previous three only produced a range of values for the danger, dose, and exposure. As a result, it becomes

necessary to assess risk for all values within the range of these variables. A complete list of potential risks, including best, mean, and worst case scenarios, is produced and examined by decision-makers to better design policies and by health officials to determine whether further research to analyze the risk is necessary (Gusain, 1994).



CHAPTER 3 METHODOLOGY

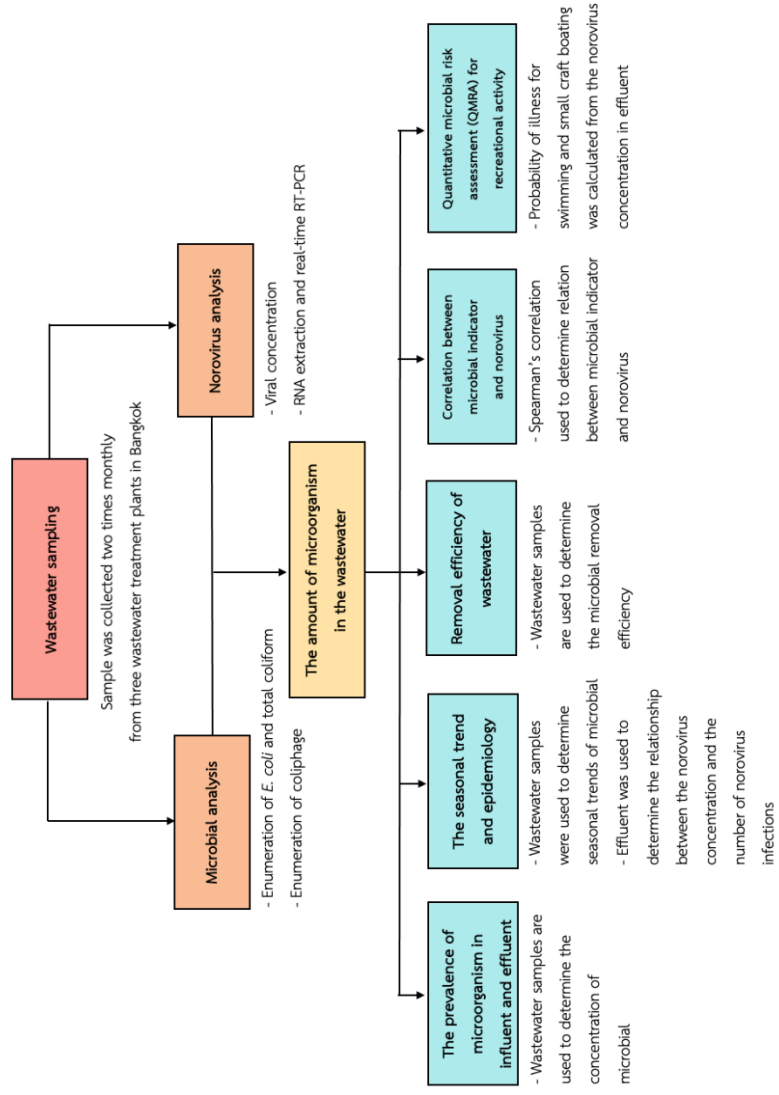


Figure 5 Method of experiment

3.1 Sampling sites and approach

Between March 2020 to March 2021, wastewater sampling was collected two times monthly from three wastewater treatment plants located in Bangkok (Figure3). Plant A, Dindaeng wastewater treatment plant utilized a Biological activated sludge process with nutrients removal. Plant B, Chong Nonsi wastewater treatment plant utilized a Cyclic activated sludge process and Plant C, Nong khaem wastewater treatment plant utilized a Vertical loop reactor with nutrients removal. The characteristics of each plant are described in Table 1. Effluent and Influent were collected into 1,000 ml High Density Polyethylene (HDPE) containers using a grab sampling technique. The sample containers were labelled and stored in the dark inside cool boxes containing freezer packs and transported to Laboratory for testing within 24 hours.



Figure 6 Wastewater treatment plants in Bangkok

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Table 2 Characteristics of sewage treatment works monitored during the study.

WWTP	WWTP A	WWTP B	WWTP C
Wastewater volume (m ³ /day)	350,000	200,00	157,00
Population equivalent	1,080,000	580,000	520,000
Treatment type	Biological activated sludge process with nutrients removal	Cyclic Biological activated sludge process	Vertical loop Biological activated sludge process
Length of sewer (km)	66	55	46
Area of sewer coverage (km ²)	37	28.5	44

3.2 Virus concentration

The concentration of viruses in the wastewater samples was based on the method mentioned (Katayama et al., 2002). This concentrate method can recovery norovirus in tap water, river water and pond water with value 80%, 39% and 15% respectively (Haramoto et al., 2009). Cellulose nitrate HA negatively charged membrane filters (Nihon Millipore) with a 0.45 um pore size and a 90 mm diameter was used with a vacuum pump system. One liters of sample was added 5.3 g of MgCl₂ 500 to 800 mL of sample was filtered to adsorb the viruses to the membrane, 200 ml of 0.5 mM H₂SO₄ (pH 3.0) was passed through the membrane to rinse out the cation, and then 10 ml of 1 mM NaOH (pH 10.5 to 10.8) was poured on the membrane for virus elution. The eluted filtrate was recovered in a tube containing 0.1 ml of 50 mM H₂SO₄ and 0.1 of 100 Tris-EDTA buffer (pH 8.0) for neutralization. The samples were stored at -80°C until further processing. The eluates were further concentrated using a secondary concentrated by Amicon® Ultra-15 centrifugal filter (Merck Millipore) to obtain a final volume of approximately 300 ul, according to the manufacturer's protocol.

3.3 RNA extraction

Norovirus RNA in the concentrated samples 300 ul were extracted using the QIAamp Viral RNA Mini Kit (Qiagen) by spin protocol. When the RNA extraction is complete Viral RNA is stored at -30 to -15°C or at -90 to -65°C.

3.4 Quantification of viruses by qPCR

The quantitative PCR (qPCR) will be performed using 4X Taqman Fast Virus 1-Step Master Mix (ThermoFisher). The qPCR thermal cycler of novorius GI, GII are 10 min at 50°C, followed by 45 cycles of 20 second at 95°C, 3 sec at 95°C, and 30s at 60°C. The sequences of the primers and TaqMan probes used are given in Table 3. The CT values obtained for sewage samples in the qPCR were used to calculate the number of viral genomes of norovirus. This quantity was corrected using the percentage extraction efficiency and converted into a concentration in copies/ml wastewater.

Table 3 Primers and TaqMan probes for qPCR assay.

Viruses	Function	Sequence (5' – 3')	References
Norovirus GI	Forward primer	5'-CGCTGGATGCGNTTCCATGA-3'	(Kageyama et al., 2003)
	Reverse primer	5'-CTTAGACGCCATCATCATTTAC-3'	
	Taqman probe	5'-/5HEX/AGATYGCGATCTCCTGTCCA/3BHQ_1/-3	
Norovirus GII	Forward primer	5'-AGRTGGATGAGRTTYTCWGA-3'	(Kageyama et al., 2003)
	Reverse primer	5'-TCGACGCCATCTTCATTCACA-3'	
	Taqman probe	5'-/56-FAM/TGGGAGGGCGATCGCAATCT/3BHQ_1/-3'	

3.5 Enumeration of *E. coli* and Total coliform bacteria

To prepare the agar, 26.5 g of Chromocult coliform agar (Sigma-Aldrich) was dissolved in 1 liter of deionized water. The mixture was heated on a hot plate until it starts boiling, stirring frequently until the agar is completely dissolved. The solution is then poured into a Petri dish and allowed to solidify at room temperature. Next, a dilution series is created from the sample. One milliliter of diluted or non-diluted sample was added to the Petri dish. The sample was then evenly spread over the surface of agar using the sterile glass spreader. The plate is incubated the plate at 37 °C for 18-24 hours. Finally, *E.coli* and total coliforms were enumerated and reported as CFU/ml.

3.6 Host cell preparation and enumeration of coliphage using *E. coli* K12 A/λ (F+) as host strain

Agar preparation involved dissolving 2 g of Luria-Bertani (LB) Broth and 1 g of Bacto agar (Sigma-Aldrich) with 0.8 g CaCl₂ in 100 ml of deionized water. The mixture was sterilized by autoclaving at 121.5 °C for 15 minutes. A dilution series was prepared from the sample, and 1 ml of each dilution was plated on the prepared agar plate. The plates were incubated at 37 °C for

18 - 24 hours. Following incubation, microbial concentrations were determined and reported as plaque-forming units (PFU) per ml.

3.6.1 Bacteriophage cell culture

Bacteriophage was assayed using agar layer method. *E. coli* K12 A/λ was used as host strain at a concentration of amount 10^8 CFU/ml. Briefly, *E. coli* K12 A/λ host strain was prepared by incubated in LB broth for 4-6 hours at 37°C with shaking at 120 rpm. *E. coli* K12 A/λ host culture were mixed with autoclaved agar and one milliliter of samples was poured into the petri dish with agar. The overlays were incubated overnight at 37°C. The concentration of microbe was reported as PFU/ml.

3.6.2 *E. coli* K12 A/λ (F+) cell culture

The *E. coli* K12 A/λ (F+) stock was thawed and 1 ml was added into 9 ml of Luria-Bertani (LB) broth. The mixture was then incubated at 37 °C with continuous shaking for 18 to 24 hours. Glycerol was prepared to a final concentration of 50% (by dissolving 63 g of 95% Glycerol in deionized water to a final volume of 100 ml). The glycerol was then added to the incubated mixture to achieve a final concentration of 20%. This solution was aliquoted into 1.5 ml tubes with 1 ml in each tube and store at -20 °C. For working stock solution, the stock was thawed, added to 10 ml of LB broth, and incubated at 37°C while shaking for 5-6 hours. To prepare 100 ml of LB agar, 2 ml of the inoculum culture was used.

3.7 Clinical data

Clinical data of gastroenteritis cases of all ages was obtained from the Center of Excellence in Clinical Virology at the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The hospitals mentioned above are public hospitals where lots of patients came to receive treatment. The data was used as a representative for diarrhea cases in Bangkok. No patient information was used, and all data were maintained anonymously and securely.

3.8 Quantitative microbial and risk assessment

Quantitative microbial risk assessment of norovirus contaminated in effluent from wastewater treatment plant will be evaluated after obtaining concentrations of norovirus GI and GII, the Probability of infection was calculated based on the Eq. 4

3.8.1 Exposure assessment

The exposure assessment quantifies the mean dose copies/ml/event of norovirus used to predict microbial exposure applied on the Eq.3 (Van Abel et al., 2017) and use the data in table 4.

$$D_{NoV} = C_{NoV} \times Df \times 10^{-Inact} \times Inf \times V_{cons} \quad Eq.3$$

where D_{NoV} is dose of norovirus intake

C_{NoV} is concentration of norovirus in wastewater

Df is dilution factor

Inf is proportion of viruses that are infectious

$Inact$ is inactivation (degradation) factor

V_{cons} is volume of water consumed

3.8.2 Dose-response model

Dose-response model are used to predict the relationship between among of viral exposure and probability of adverse health effect. beta-Poisson is widely used to predict microbial exposure based on the Eq.4 (Haas et al.,2014) with disaggregated models.

Probability of infection $P(d)$

$P(d)$ is represent the probability of infection and d is a single dose at exposure for recreational (Table4), with model slope parameter α and median effective dose N_{50} (Table4).

$$P(d) = 1 - \left[1 + \left(\frac{dNoV}{N_{50}} \right) \times \left(2^{\frac{1}{\alpha}} - 1 \right) \right]^{-\alpha} \quad Eq.4$$

Where $P(d)$ is probability of infection

d_{NoV} is a single dose at exposure

N_{50} is median effective dose

α is slope parameter

Table 4 Exposure assessment and dose-response models and parameters for use in the quantitative microbial risk assessment (QMRA) estimating acute gastroenteritis of norovirus.

Variable	Definition	units	distribution	value	references
C_{NoV}	concentration of norovirus	Copies/ml	Log normal	Collected & analyzed data from WWTPs	
Inf	proportion of viruses that are infectious	%	-	100	(Van Abel et al., 2017)
Inact	Inactivation by sunlight	Log reduction	Uniform	Min = 1 Max = 3	(Flannery et al., 2013)
Df	Dilution factor	Log reduction	Uniform	Min = 1 Max = 4	Best estimate
α	slope parameter	-	-	0.04	(Ahmed et al., 2018)
N_{50}	median effective dose	-	-	1,845,494	(Ahmed et al., 2018)
$V_{cons, Swimming}$	volume of water consumed	ml/event	Triangular	Min = 0 Max = 53 Mode = 16	(McBride et al., 2013)
$V_{cons, Small craft boating}$	volume of water consumed	ml/event	Triangular	Min = 3 Max = 4 Mode = 3.8	(McBride et al., 2013)

3.8.2 Probability of illness (P_{ill})

The probability of illness was estimated using Monte Carlo simulations with 10,000 simulations was conducted in R-studio software. The risk estimates were calculated at a 95% confidence level. The equation utilized to estimate these outcomes was adapted from Haas et al. (2014), Howard et al. (2006) and WHO (2016). The estimated probabilities of gastroenteritis (P_{ill}) per person per single exposure event were calculated for each water contact activity. P_{ill} was used to determine the morbidity ratio as presented in Table 5 and calculated using Eq.5.

$$P_{ill,path} = P_{inf,path} \times P_{ill|inf} \quad \text{Eq.5}$$

Where $P_{ill,path}$ is probability of illness per pathogen, per exposure pathway

$P_{inf,path}$ is probability of infection per pathogen per single exposure

$P_{ill|inf}$ is morbidity ratio

Table 5 Morbidity ratios estimating probability of illness conditional upon infection for selected pathogens ($P_{ill|inf}$) for viral gastrointestinal attributable to wastewater treatment systems.

Pathogen	$P_{ill inf}$	References
Norovirus	0.6	(Teunis et al., 2008)

3.9 Statistical analysis

3.9.1 One-way ANOVA analysis

The concentration of norovirus in wastewater at each sampling site was analyzed for significant differences. The level of significance for all analyses was set at $p < 0.05$. Any p-value less than this was considered statistically significant.

3.9.2 Correlation analysis

The concentrations of microbes, specifically *E. coli*, total coliform, and coliphage, were analyzed to observe their Spearman's correlation with norovirus data from each treatment plant. The level of significant was set at $p < 0.05$, any p-value less than this was considered statistically significant.

CHAPTER 4

RESULT AND DISCUSSION

The results of the study were divided into 5 parts: 1) the prevalence of microbial indicators and norovirus in influent and effluent wastewater, 2) removal efficiency of microorganisms in wastewater treatment plants, 3) seasonal epidemiology, 4) correlation of microbial indicator and 5) quantitative microbial risk assessment (QMRA) of norovirus in surface water contaminated by treated wastewater

4.1 The prevalence of microbial indicators and norovirus in influent and effluent wastewater.

4.1.1 The prevalence of microbial indicators in influent and effluent wastewater.

E. coli and total coliforms were detected in all influents (25/25) and effluent (25/25), while bacteriophages were detected all influent samples. For effluent samples, bacteriophages were 13 out of 24 samples from DD, 19 out of 24 samples from CH, and 11 out of 24 samples from NK.

The NK plant had higher concentrations both influent and effluent of *E. coli* and total coliform than the other two treatment plants. While, bacteriophage concentration of DD was highest, and effluent of CH was highest. Different microbial indicator concentrations in each other WWTP can be due to many reasons such as wastewater characteristics, population density, sanitation practices, sewerage system, geographic location, and environmental factors (Cydzik-Kwiatkowska & Zielinska, 2016; Milledge et al., 2018; Numberger et al., 2019; Saleem et al., 2001).

The average influent and effluent concentrations (Table.6) of *E. coli* were 4.04 ± 0.36 and 2.64 ± 0.39 , respectively, samples concentration of total coliform was 5.03 ± 0.39 and 3.79 ± 0.41 , respectively. The average influent and effluent concentrations of coliphage were 1.76 ± 0.52 and 0.42 ± 0.51 , respectively. Influent and effluent were compared concentrations between WWTPs using ANOVA analysis. *E. coli* and total coliform concentration in influent and effluent didn't show the significantly difference, while the concentration of coliphage in influent and effluent showed significantly different concentrations ($p < 0.05$) among WWTPs. The difference in coliphage concentrations in the influent may be the result of different wastewater profiles and wastewater collection systems in each WWTP. While the different concentrations of coliphage in the effluent may be a result of the initial concentration in the influent and the efficiency of wastewater treatment, it differs between WWTP.

The microbial concentrations of WWTPs in Bangkok were in the same range as concentrations from WWTPs in other countries such as Montreal city in Canada (Frigon et al., 2013) and Milan city in Italy (Raboni et al., 2016). Previous studies have shown that the average concentrations in influent of *E. coli*, total coliform and coliphage range from 2.4 – 6.6 log₁₀ CFU/ml, 3.8 – 5.8 log₁₀ CFU/ml and 0.8 – 4.3 log₁₀ PFU/ml, respectively.

Table 6 The average Log concentration of microbial indicators from WWTPs.

WWTP	Average Log concentration (CFU/ml)					
	<i>E. coli</i>		Total coliform		coliphage	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
DD	4.05 ± 0.36	2.54 ± 0.38	5.06 ± 0.43	3.78 ± 0.40	2.01 ± 0.54	0.33 ± 0.40
CH	3.95 ± 0.37	2.76 ± 0.42	4.94 ± 0.41	3.75 ± 0.34	2.01 ± 0.33	0.78 ± 0.60
NK	4.13 ± 0.34	2.62 ± 0.39	5.08 ± 0.34	3.84 ± 0.49	1.23 ± 0.37	0.17 ± 0.25
All WWTP	4.04 ± 0.36	2.64 ± 0.39	5.03 ± 0.39	3.79 ± 0.41	1.76 ± 0.52	0.42 ± 0.51

The concentration levels of microbial indicators in the samples showed the following order: total coliform > *E. coli* > coliphage in all WWTPs. Coliphage are classified as viruses. Usually, the prevalence of viruses is less than that of bacteria already in the environment (Bréchet et al., 2014). Previous studies have also shown that coliphage tend to have lower concentrations compared to coliform indicators (Muniesa & Jofre, 1998). Coliform bacteria may serve as better indicators of wastewater contamination than coliphage due to their higher prevalence. However, other factors such as persistence need to be considered when selecting appropriate indicators for assessing wastewater contamination.

4.1.2 The prevalence of norovirus in influent and effluent wastewater.

Table 7. show the influent of the DD had the highest mean concentration at 2.14 ± 0.44. The CH had the highest average influent concentration at 1.41 ± 0.36, as compared to the average concentration among the three WWTPs was not significant difference ($p > 0.05$). Log average concentrations of norovirus GI in influent and effluent samples were 1.90 ± 0.48 and 1.35 ± 0.32, respectively.

The influent and effluent concentrations of norovirus GII (Table7.) in DD were the highest when compared to other WWTPs. The influent concentration in DD was 1.04 ± 0.33, and the effluent concentration was 0.40 ± 0.19. When comparing the average concentration among the

three WWTPs were statistically significant differences ($p < 0.05$). The log average influent and effluent concentrations of norovirus GII were $*0.91 \pm 0.39$ and $*0.37 \pm 0.17$, respectively. However, norovirus GII was not detected in sample of NK.

Table 7 The average Log concentration of norovirus GI and GII from WWTPs.

WWTPs	Average Log concentration (copies/ml)			
	Norovirus GI		Norovirus GII	
	Influent	Effluent	Influent	Effluent
DD	2.14 ± 0.44	1.40 ± 0.33	1.04 ± 0.33	0.40 ± 0.19
CH	1.99 ± 0.48	1.41 ± 0.36	0.75 ± 0.41	0.28 ± 0.08
NK	1.52 ± 0.38	1.14 ± 0.16	-	-
All WWTPs	1.90 ± 0.48	1.35 ± 0.32	$*0.91 \pm 0.39$	$*0.37 \pm 0.17$

The detection of norovirus in wastewater may indicate infection in that area. A high concentration of norovirus may reflect many infections. WWTP have different collection areas and population numbers, resulting in different norovirus concentrations as well. Concentrations of norovirus GI greater than GII may also represent a greater number of GI infections than GII. Our research is in line with previous studies in detecting noroviruses in wastewater where GI was greater than GII in Thailand (Kittigul et al., 2012). On the other hand, the concentration of norovirus in wastewater in Thailand may differ from other countries where more GII were found in wastewater than GI. the study conducted in Brazil. GII concentrations were higher than GI in both influent and effluent, with GI values of 1.12 - 1.94 log copies/ml and 0.32 - 1.06 log copies/ml. while, GII with value 1.2 - 2.61 log copies/ml and 0.27 - 1.53 log copies/ml for GII, respectively (Victoria et al., 2010).

The reason of higher prevalence of norovirus GI than GII might be that norovirus GI are more resistant to decomposition in waste water than GII (da Silva et al., 2007). Although few data are available about the resistance of norovirus strains to environmental conditions, this may be due to differences in the capsid constituents of GI and GII (Tan & Jiang, 2005). However, it's important to note that the detection efficiency varies between GI and GII. The qPCR efficiency of norovirus GII was relatively lower than GI. The qPCR detection efficiency for GII and GI is 87.55% and 99.68%, respectively.

4.2 Removal efficiency of microorganisms in wastewater treatment plants.

The three WWTPs have different secondary treatment systems. DD: activated sludge process with nutrients removal, CH: cyclic activated sludge and NK: vertical loop activated sludge. The log removal of microbial indicators and norovirus are summarized in Table 8. DD can remove microorganisms better than two WWTPs. DD can reduce *E. coli* was 1.51 ± 0.44 , total coliform was 1.33 ± 0.46 , coliphage was 1.74 ± 0.58 , and norovirus GI and GII were 1.10 ± 0.43 and 0.63 ± 0.43 , respectively. The three WWTPs had an average log removal of *E. coli* was 1.40 ± 0.50 , total coliform was 1.26 ± 0.46 , coliphage was 1.33 ± 0.66 respectively. For norovirus, the average log removal of GI was 0.75 ± 0.57 for all WWTPs, while norovirus GII was 0.54 ± 0.41 . The log removal of norovirus GII was only shown for two WWTPs due to the undetectable amount of GII in the NK effluent and the relatively low PCR efficiency for GII. When comparing the log removal of *E. coli*, coliphage, and norovirus GI by ANOVA test among the three WWTPs, the difference was found to be significant ($p < 0.05$). while norovirus GII was found to be significantly (t -test, $p < 0.05$).

Significant differences in treatment efficiency may represent varying levels of efficacy in secondary treatment of each WWTP. The activated sludge process with nutrients removal used in the secondary treatment system of DD was able to reduce the microbial load more effectively than the system at other treatment plants. There are currently few studies on microbial removal by the cyclic activated sludge and vertical loop activated sludge processes, making these areas of interest for future research. The information can be valuable for decision-making and the design of wastewater treatment systems.

Table 8 The log removal of microbial indicators and norovirus from three WWTPs

WWTP	Log removal (CFU/ml, PFU/ml, copies/ml)				
	<i>E. coli</i>	Total coliform	coliphage	Nov GI	Nov GII
DD	1.51 ± 0.43	1.34 ± 0.45	1.75 ± 0.57	1.10 ± 0.43	0.63 ± 0.43
CH	1.19 ± 0.44	1.19 ± 0.41	1.19 ± 0.70	0.35 ± 0.45	0.46 ± 0.05
NK	1.51 ± 0.53	1.23 ± 0.48	1.05 ± 0.49	0.56 ± 0.22	-
All WWTPs	1.40 ± 0.50	1.26 ± 0.46	1.33 ± 0.66	0.75 ± 0.57	* 0.54 ± 0.41

E. coli and total coliform removal efficiencies of the three WWTPs in this study were found to be within the same range as that of the conventional activated sludge process in wastewater treatment system, as reported in a previous study, ranging from 1.1 to 1.9 log (Barrios-Hernandez

et al., 2020). However, the log removal of coliphages for the all WWTPs was comparatively lower when compared to other studies, ranging from 1.3 to 2.4 log (Amarasiri et al., 2017; Barrios-Hernandez et al., 2020).

For norovirus GI and GII, the three WWTPs in Bangkok exhibited lower removal efficiency for norovirus compared to the wastewater treatment plants from the previous study in Japan, a reduction of 1.82 log reduction was achieved for GI, and 2.72 log reduction was achieved for GII. The previous study also exhibited that the implementation of a chlorination process can further reduce the norovirus levels by an additional 0.45 to 0.95 log (Haramoto et al., 2006).

Since the removal efficiency of microbial indicators from each WWTP was higher than that of norovirus, estimating norovirus removal by using microbial indicators may lead to an overestimation of the actual reduction of norovirus. Therefore, conducting the direct investigation of norovirus removal efficiency will provide accurate results for assessing norovirus reduction.

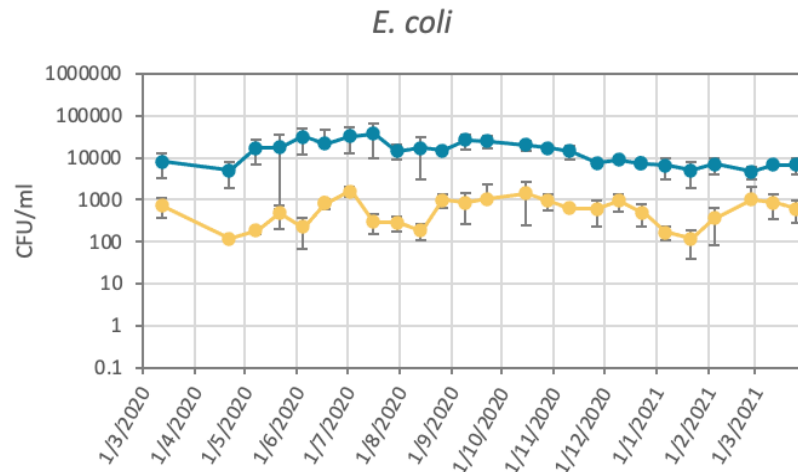
Although the wastewater treatment system can reduce the number of microorganisms in the influent, the treated wastewater may still contain microbial residues. This becomes a concern when the effluent is released into the public water supply or used in activities that may expose individuals to the risk of microbial infection. As Thailand does not establish criteria for microbial counts in effluent after treatment, wastewater treatment plants should consider installing disinfection systems and conducting additional microbial testing to minimize health risks for downstream residents.

4.3 The seasonal trend and epidemiology

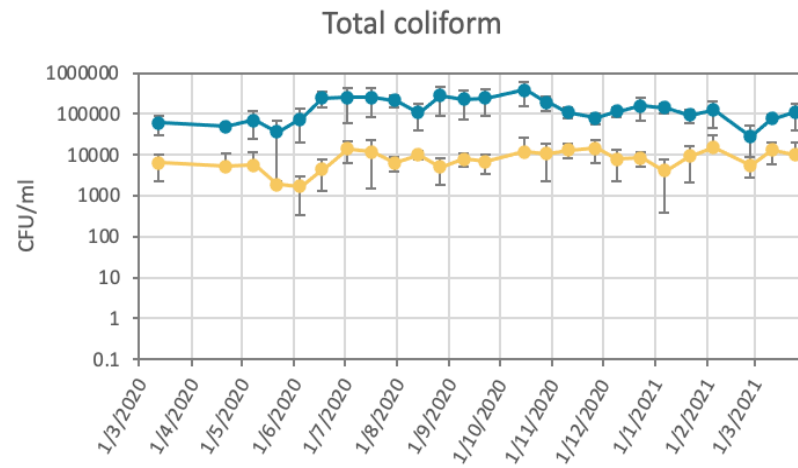
4.3.1 The seasonal trend of microbial indicator

Wastewater samples were collected over a period of 1 year during the wet season from mid-May 2020 to mid-October 2020 (n=9), and the dry season from mid-October 2020 to mid-March 2021 with March 2020 to mid-May 2020 (n=16). Figure4 (a) shows the average influent concentrations of *E. coli* during the wet and dry seasons as 2.37×10^4 and 8.68×10^3 CFU/ml, respectively. The highest concentration of 3.80×10^4 CFU/ml in July 2020 and the lowest concentration of 5.00×10^3 CFU/ml in January 2021. Statistical analysis using an independent sample *t*-test indicated a significant difference between the wet and dry seasons ($p < 0.05$).

(a)



(b)



(c)

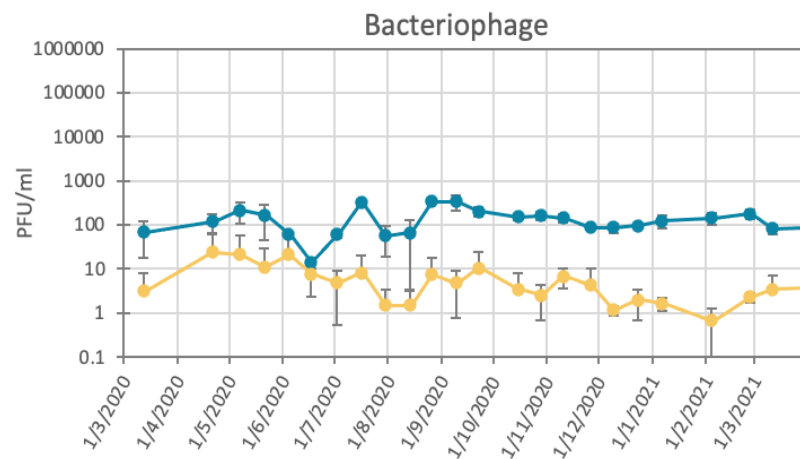


Figure 7 Seasonal trend of (a) *E. coli*, (b) total coliform and (c) coliphage from WWTPs

●, Influent; ●, Effluent

Figure (b) shows the average concentrations of total coliform in influent during the wet and dry seasons were found to be 2.20×10^5 and 9.57×10^4 CFU/ml, respectively. The highest concentration of total coliform was observed in September 2020 at 3.90×10^5 CFU/ml, while the lowest concentration was observed at 7.10×10^4 CFU/ml in May 2020. Statistical analysis using an independent sample *t*-test indicated a significant difference between the wet and dry seasons ($p < 0.05$). The higher concentration of *E. coli* and total coliform concentrations during the wet season, compared to the dry season, can be due to temperature fluctuations and the influence of rainfall, resulting in increased surface runoff. These factors can contribute to the elevated microbial detections observed in the wet season (Grøndahl-Rosado et al., 2014). A run-off in the wet season causes more leaching of the microbial contamination source than in the dry season, which causes higher microbial concentrations (Monteiro et al., 2021). The experimental results of the research are in line with research conducted in other continents, which showed that microbial concentrations in wastewater tend to be higher during the wet season than the dry season.

Figure (c) shows the average concentrations of coliphage. The influent showed the log concentration of 1.35×10^2 PFU/ml, while the effluent had an average log concentration of 6.38 PFU/ml. The sample in May 2020 showed the highest concentration with a log concentration of 2.15×10^2 PFU/ml. On the other hand, the lowest concentration was obtained in June 2020 with a log concentration of 1.42×10^2 PFU/ml. Comparisons were made between the mean concentrations during the wet season with the concentration of 1.62×10^2 PFU/ml, and dry seasons, with a concentration of 1.14×10^2 PFU/ml. The result shows that the average concentration of coliphage in wet and dry seasons was similar, when statistically tested by *t*-test, there was no significant difference in concentration ($p > 0.05$).

Based on the data, it was observed that the concentration of coliphage did not differ between season (wet and dry season). While it was observed from the previous studies that the concentration of coliphage in surface water was higher in winter than in summer. As the water temperature decreases, the rate of coliphage is greatly reduced in summer because higher water temperatures and sunlight intensity affect the survival rate of the coliphage (Hata et al., 2016). In addition, decreasing bacterial count was also positively correlated with the growth of the host. However, it should be noted that other studies utilized different criteria for seasonal stratification.

4.3.2 The seasonal trend and epidemiology of norovirus

The average concentration of norovirus GI and GII in influent wastewater from three WWTP from March 2020 – 2021. The data was divided into three seasons: summer, which extends from

March 2020 to mid-May 2020 with mid-February 2021 to mid-march 2021 (n=10), the rainy season, which spans from mid-May 2020 to mid-October 2020 (n=9), and winter, which spans from mid-October 2020 to mid-February 2021 (n=6). Influent concentrations of norovirus GI reached their peak in January 2021 (Figure a) with values of 5.65×10^2 log copy/ml.

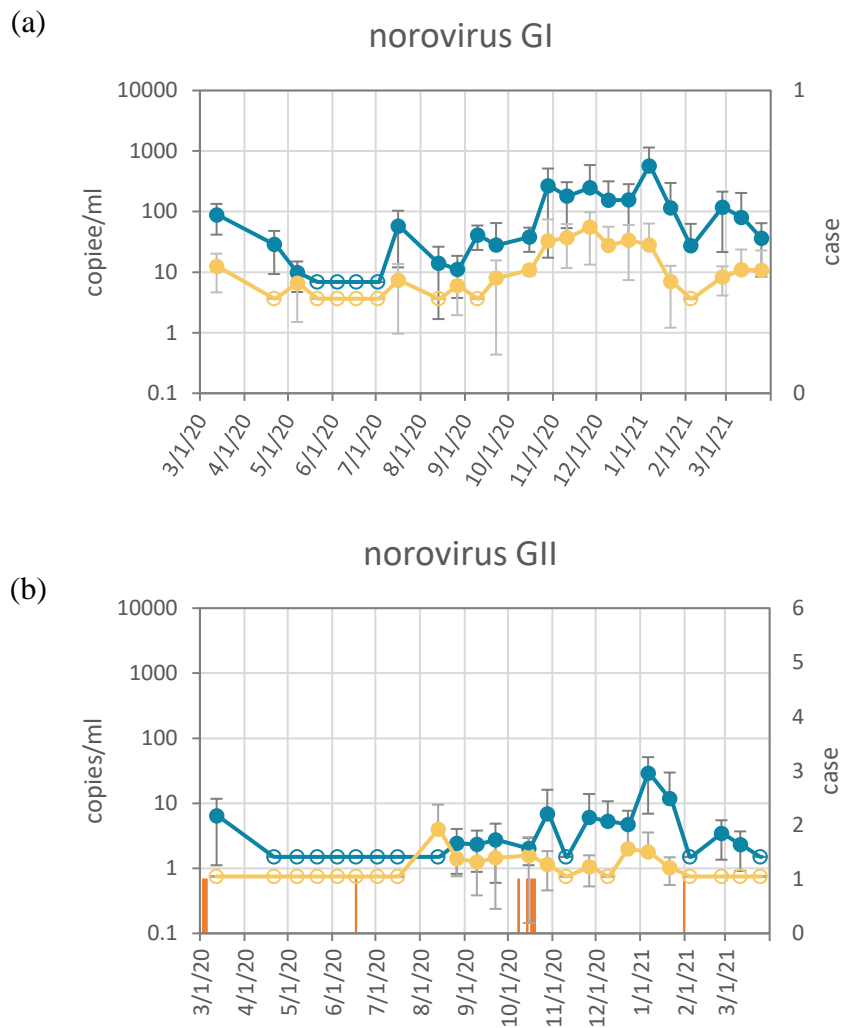


Figure 8 Seasonal trend of (a) norovirus GI (b) norovirus GII from WWTPs and case of gastroenteritis

●, Influent; ●, Effluent ○, Influent > LOQ; ○, effluent > LOQ and — case

Norovirus GI concentration tends to increase from the rainy season to winter and decrease during summer. ANOVA statistical analysis indicated a significant difference in concentration among the seasons for norovirus GI ($p < 0.05$). Norovirus GI concentrations were higher during winter than other seasons of the year. Trends between the association of the number of infected patients and the concentration of norovirus GI in wastewater. None of the infected cases were reported for the

total number of 217 cases, the effluent may contribute to a greater overview of the population with asymptomatic infection in the absence of clinical data.

Influent concentrations of GII reached their peak in January 2021 same the norovirus GI, with values 2.90×10^1 log copy/ml. The influent concentration of GII tends to increase in winter and decrease during summer. ANOVA statistical analysis indicated a significant difference in concentration among the seasons for GII ($p < 0.05$). Norovirus GII concentrations were higher during winter than other seasons of the year. The trend between the number of infected patients and the concentration of norovirus GII in wastewater was found. There is a small number of reported cases of infected patients, 8/217 cases. November 2020, at the start of the winter season, the highest number of reported 4 cases was. However, in January 2021, the highest concentration of norovirus GII was detected in wastewater, but no case count was reported. This suggests the presence of latent infections in the area that may not have been captured in medical treatment data. The concentration of norovirus GII in wastewater may show a positive correlation with the number of infected individuals in area.

From the data in this study, seasonality may affect the concentration and number of estimated infected people from wastewater. In winter, the concentration of norovirus is highest. There is evidence from previous research demonstrating similar outbreak patterns of norovirus during the winter in other countries (Dowell, 2001), where climate conditions such as temperature, humidity, and wind play a role in spread of infection (Rohayem, 2009). The persistence of viruses in wastewater may increase due to lower temperatures. For norovirus, increased humidity may affect the spread of viral particles. On the one hand, it has been concluded that lower water temperatures in winter can better detect norovirus concentrations compared to other seasons (Rohayem, 2009).

The above patient data for 2020-2021 represents a small number of reported cases. In the population, there may be many symptomatic and asymptomatic infections, which may vary from region to region and over time. The number of gastroenteritis cases in this data indicates a low detection rate of norovirus compared to historical data. It is important to note that due to the spread of COVID-19, individuals residing in areas with norovirus-infected wastewater treatment plants may not be admitted to hospitals, and the overview only represents symptomatic patients. If retrospective data from January 2015 and February 2017 (Thanusuwannasak et al., 2018) were compared with the period of norovirus epidemic with the concentration in 2020, it was found that the trend remained consistent. The number of cases increased in the winter and decreased in the

summer. In our study, while the concentration of norovirus GI was greater than GII in wastewater, the number of GI cases was less than that of GII based on clinical data from patients. The discrepancy could be due to GII infections being more severe than those caused by GI (Bhavanam et al., 2020). Reported outbreaks of GI cases are relatively low, likely due to their mild symptoms, which often prevent them from being brought to a physician's attention or treated (Da Silva et al., 2007). The concentration of norovirus in wastewater may reflect a more comprehensive picture of norovirus infection in the population, compare to the number of reported medical cases, because it reflected both symptomatic and asymptomatic infected patients. In addition, wastewater surveillance may serve as a valuable tool for monitoring and providing early warnings of viral outbreaks, yielding significant public health benefits.

4.4 Correlation of microbial indicator and norovirus

The concentrations of microbes, specifically *E. coli*, total coliform, and coliphage, were analyzed to observe their Spearman's correlation with norovirus data from each treatment plant as present in table9. All of Spearman's coefficients between any microbial indicators and norovirus were $r_s < 0.3$, which indicates a weak correlation between these two variables.

Table 9 Spearman's correlation coefficients between microbial indicator and norovirus in effluent

Microbial indicator /WWTP	DD		CH		NK	
	Nov GI	Nov GII	Nov GI	Nov GII	Nov GI	Nov GII
<i>E. coli</i>	-0.270	0.040	0.047	0.111	0.231	-0.060
Total coliform	0.209	0.377	0.170	0.088	0.092	-0.291
Coliphage	0.251	0.226	-0.337	0.162	0.056	0.285
Norovirus GI	-	0.140	-	-0.299		-0.069

This study revealed that all WWTP demonstrated a weak correlation between microbial indicator and norovirus. Specifically, *E. coli* and total coliform showed a weak correlation with norovirus across all WWTPs. In the present, fecal coliform is widely used as a microbial indicator for wastewater contamination. Using fecal indicators such as *E. coli* is believed to be a suitable microbial agent to study the behavior of aquatic pathogens, but *E. coli* is classified as a group of bacteria. But in wastewater there are other non-bacterial organisms such as viruses and protozoa.

Therefore, *E. coli* and total coliform may not be suitable representatives of noroviruses in wastewater due to their different life cycles. Meanwhile, coliphages may be more suitable representatives of norovirus than bacterial groups. So, there might be something else more suitable than *E. coli*. However, several studies have now suggested coliphage as a potential marker of aquatic virus because the structure, morphology, and size can be compared with other intestinal viruses (Antony et al., 2014). According to data from the three WWTP in Bangkok, coliphage have low correlation for norovirus. Therefore, coliphages may not be good candidates for norovirus at the WWTP in Bangkok. In previous study demonstrated the strong correlation between adenoviruses and coliphage in water sample (Arredondo-Hernandez et al., 2017). The results imply that coliphage can potentially serve as indicators of pathogenic virus without seasonality, such as adenovirus. However, coliphage may not be representative of noroviruses as seasons affect the spread of noroviruses.

4.5 Quantitative microbial risk assessment (QMRA) of recreational activity

4.5.1 Probability of illness of recreational activity

Treated wastewater from three WWTP in Bangkok without disinfection systems in the final process. The effluent may be released into public water sources or recycled, for example to watering plants or bringing treated wastewater back to the canal for recreational use for the Chong Nonsi Canal. In this study, the risk of effluent into the public with public activities was assessed. The activities are divided into two categories: fishing and small craft boating.

The table 10. shows the probability of GI and GII illness from recreational contact with wastewater from each WWTP. The morbidity risk assessment was performed using a equation 3. When exposed to effluent from WWTPs in public water source, the result was found that the median P_{ill} was lower than 0.036, which is the illness benchmark set by the US EPA, in all WWTPs and activities. According to US EPA (US EPA, 2012), the P_{ill} is high when it exceeds the illness benchmark (36 GI illnesses/1000 exposures, or 0.036), and it is low when it is less than 0.036. The activity with the highest dose of GI and GII is swimming, as it has the highest exposure to polluted water compared to small craft boating.

Table 10 Probability of caused by norovirus GI and GII in effluent from WWTPs.

WWTP	DD		CH		NK	
	Nov GI	Nov GII	Nov GI	Nov GII	Nov GI	Nov GII
Swimming	0.0162	0.0021	0.0162	0.0015	0.0090	-
Small craft boating	0.0036	0.0003	0.0039	0.0003	0.0019	-

In comparison, the likelihood of GI illness was higher than that of GII illness in all activities because the concentration of GI in the effluent was greater than that of GII. The P_{ill} GI of swimming DD, CH and NK was 0.0162 0.0162 and 0.009 respectively, for the P_{ill} GI of DD CH were 0.0021 and 0.0015 respectively. The results were unable to assess the probability of illness GII for NK because only one sample of GII was detected in the exudate, making it impossible to determine the mean concentration. For small craft boating, the mean exposure dose was 3.8 ml. When randomized with Monte Carlo, the P_{ill} were similar. The P_{ill} corresponded to the GI and GII concentrations found in the effluent because of the differences in the constituents in the influent and the different treatment systems in each WWTP.

All data showed that the risk of using treated wastewater from the three treatment plants was lower than the acceptable risk if there is dilution from effluent or decomposition of the infection. In contrast, if direct exposed to treated water, there may be an exceed acceptable risk. However, wastewater treatment plants should eliminate norovirus concentrations below the acceptable risk of Benchmark Because there are other activities besides water recreation. some activities that use directly treated water, such as watering plants or machine equipment cleaning, which may pose a risk.

When analyzing the sensitivity of the exposure parameters (Figure 9), it was found that the main morbidity factor may depend on the dilution of norovirus concentrations and the rate of degradation by sunlight in public water sources. Dilution factor (Df) and inactivation factor, both variables had – (40 - 50 %) sensitivity percentage from the data of the three treatment plants. As for the decay rate, it may be one way to reduce norovirus loads if public water supplies are exposed to sunlight. At the same time, dilution in a high-volume source may reduce the likelihood of exposure. The initial concentration of bacteria in public water supplies may also affect risk assessment.

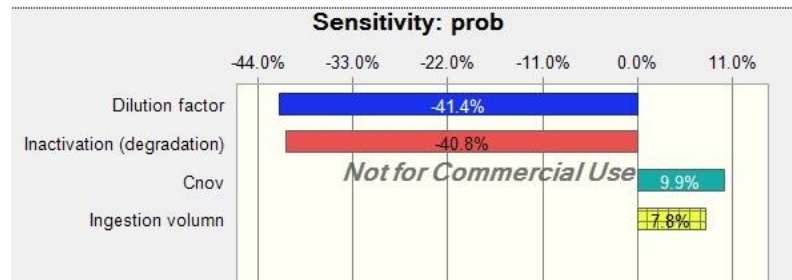


Figure 9 Sensitivity analysis for probability of illness

Other studies have shown the risks of using treated wastewater contaminated with microbial loads. The study of Schoen & Ashbolt, 2010 have shown the risk of GI illness in recreational. This indicates that the effluent from various WWTPs may not be able to remove the GI and GII content below the illness benchmark 0.036 may pose health risks. In addition, there are other studies of adenovirus concentration with the risk benchmark of second-treated wastewater samples. It was found that treated wastewater may need to reduce the amount adenovirus to be below 10^{-5} to make the value risk benchmark within the acceptable risk (Ahmed et al., 2018). It is recommended that a tertiary treatment be added to contain microbial loads to reduce the risk of exposure to wastewater activities.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Norovirus is one of the leading causes of acute gastroenteritis worldwide. The use of wastewater is one way to monitor the spread of pathogens in the environment that pose risks to human health. Therefore, monitoring of wastewater is important. Wastewater sample from three wastewater treatment plants in Bangkok was analyzed for indicator microbial and norovirus concentrations. The average log of *E. coli*, total coliform and coliphage in influent and effluent were $(4.04 \pm 0.36, 2.64 \pm 0.39 \text{ log CFU/ml})$, $(5.03 \pm 0.39, 3.79 \pm 0.41 \text{ log CFU/ml})$. and $(1.76 \pm 0.52, 0.42 \pm 0.51 \text{ log PFU/ml})$, respectively. The concentration levels of microbial indicators in the samples showed the following order: total coliform > *E. coli* > coliphages in all WWTP. When comparing the concentration between wastewater treatment plants, it was found that only coliphage with a concentration difference between the three wastewater treatment plants ($p < 0.05$). While the average log concentration of norovirus GI and GII in influent and effluent was $(1.90 \pm 0.48, 1.35 \pm 0.32 \text{ log copies/ml})$ and $(0.91 \pm 0.39, 0.37 \pm 0.17 \text{ log copies/ml})$, respectively. Concentration differences between norovirus GII wastewater treatment plants were found in both influent and effluent (t -test, $p < 0.05$).

From the results of the experiment, it was found that there was still microbial contamination in effluents. Therefore, the removal efficiency of wastewater treatment must be assessed. When assessing the average removal efficiency of the three wastewater treatment plants of *E. coli* ($1.40 \pm 0.50 \text{ log CFU/ml}$), total coliform ($1.26 \pm 0.46 \text{ log CFU/ml}$), coliphage ($1.33 \pm 0.66 \text{ log PFU/ml}$), norovirus GI ($0.75 + 0.57 \text{ log copies/ml}$) and GII ($0.54 + 0.41 \text{ log copies/ml}$). The difference in the secondary treatment process of the three wastewater treatment plants was found to be different in the removal efficiency of bacteriophage, norovirus GI and GII, the activated sludge process with nutrients removal is more effective in removing microorganisms than the cyclic activated sludge and vertical loop activated sludge.

The amount of microorganisms in the wastewater entering the system may vary depending on location and time period. Seasons can affect the amount and presence of microorganisms in wastewater. If comparing the intensity in different seasons, it was found that only *E. coli* and total coliform showed significant differences between the two seasons ($p < 0.05$), with the rainy season being more concentration than the summer season when compared. For trend and seasonal of

norovirus Influent concentrations of norovirus GI and GII reached their peak in January 2021 with values of 5.65×10^2 log copy/ml and 2.90×10^1 log copy/ml respectively. norovirus GI concentration tends to increase from the rainy season to winter and decrease during summer. While norovirus GI concentration tends to increase from the rainy season to winter and decrease during summer. Norovirus GI and GII concentrations were higher during winter than other seasons of the year ($p < 0.05$). In this study, although the concentration of norovirus GI was greater than that of GII in wastewater, the number of GI cases was less than that of GII according to clinical data from patients. The discrepancy could be due to GII infections being more severe than those caused by GI. Reported outbreaks of GI cases are relatively low, likely due to their mild symptoms, which often prevent them from being brought to a physician's attention or treated. The concentration of norovirus in wastewater may reflect norovirus infection in the population as reflected in both asymptomatic and asymptomatic infected patients. Surveillance and warning of virus outbreaks which brings great benefits to people's health.

Microbial indicators were used to correlate with norovirus as a representative for estimating microbial concentrations in the effluent. Spearman's coefficients between any microbial indicators and norovirus in all WWTP were $r_s < 0.3$, which indicates a weak correlation between these two variables. therefore, microbes may not be good candidates for the representation of noroviruses at WWTP in Bangkok. As noroviruses are characterized by their seasonal presence, the use of indicator microorganisms may lead to highly inaccurate assessments and therefore direct detection of noroviruses should be performed.

Treated wastewater contaminated with norovirus can cause health and environmental problems. When assessing the risk of exposure to treated wastewater in public water source from swimming activities and the use of small craft boating with quantitative microbial risk assessment. The result was that the median P_{ill} was below 0.036 of benchmark, the standard morbidity threshold set by the US EPA. The activity with the highest dose of norovirus GI and GII is swimming, as it has the highest exposure to polluted water compared to small craft boating. In comparison, the likelihood of norovirus GI illness was higher than that norovirus GII in all activities because the concentration of GI in the effluent was greater than that of GII. When treated wastewater is discharged into public water sources, it is diluted and decays norovirus particles when exposed to sun light, these two factors are important factors that reduce norovirus concentrations of effluent in water public source. However, if direct exposed to effluent, there may be an exceed acceptable risk. Wastewater treatment plants should remove norovirus concentrations below the acceptable risk threshold by installing a disinfection process because there are other activities besides water

recreation. some activities that use directly treated water, such as watering plants or machine equipment cleaning, which may pose a risk.

5.2 Limitations

1. When evaluating the correlation between norovirus concentrations in wastewater and the number of acute gastroenteritis cases, we encountered a limitation due to the COVID-19 pandemic in 2020. The pandemic may have discouraged norovirus-infected patients from visiting hospitals, leading to limited clinical data. Consequently, this paucity of data posed significant challenges for our statistical analysis.

2. In the quantitative microbial risk assessment related to various activities, we based our calculation on a worst-case scenario without considering pathogen dilution and degradation. As such, the resulting risk data can display considerable variability due to uncertainties associated with input variables. In order to reflect the actual situations where quantitative microbial risk assessment (QMRA) will be applied, efforts should be made to mitigate these uncertainties more accurately in future studies.

5.3 Recommendations

1. According to the results of the study, treated water still contains norovirus contaminants, contact with undiluted wastewater may pose a risk of illness. Wastewater treatment plants in Bangkok should measure microbial contaminants in their effluent and add disinfection systems to reduce the amount of microbial contamination in their effluent, thereby benefiting the environment and public health. At the same time, the risk assessment data may need to be added more concisely to allow more precise risk assessment to be developed as an effective tool to mitigate risks in treated effluent.

2. For future studies, it was found that between 2020 and 2021 there were only a few cases of norovirus-infected acute diarrhea. When comparing the concentration with the wastewater from the treatment plant Data comparison is difficult due to the COVID 2019 outbreak situation. On the other hand, few studies on the use of WBE are recommended in public health data research. Similarly, a higher number of rotavirus-associated acute diarrhea cases were found. If rotavirus data were included in the correlation findings, it may help to reflect that wastewater may have potential as a tool for monitoring outbreaks of acute diarrheal diseases.

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