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The detection of the norovirus in a wastewater treatment plant as a potential source of environmental contamination

Dorothee Goettert

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by

Dorothee Goettert

Undergraduate honors thesis under the direction of

Dr. Marlene Janes

Department of Nutrition and Food Sciences

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Louisiana State University
& Agricultural and Mechanical College
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ABSTRACT

The Norovirus, especially strains from the genogroups GI and GII are the number one cause of acute gastroenteritis worldwide. It is primarily transmitted through fecal contaminated food and water, but also by interpersonal contact, aerosolized vomit particles, and exposure to fomites. The Norovirus is very environmentally stable and relatively resistant to common disinfectants. Studies have shown that the Norovirus remains active after being treated with chlorine concentrations normally used in the treatment of wastewater.

In this research, both influent and effluent wastewater was analyzed for bacterial and viral indicators. *Escherichia coli, Enterococci*, and fecal coliforms were cultured on modified mTEC, mEI, and m-FC agar respectively after subjecting different wastewater concentrations to membrane filtration. Coliphage concentrations were also enumerated through the use of Triptic Soy Agar. Lastly, Norovirus GI, Norovirus GII, and other enteric viruses were extracted from the wastewater. These viral extractions were subjected to RNA extraction, which were then analyzed using RT-PCR (real time – polymerase chain reaction).

Wastewater samples were collected from July 2013 until January 2014. The results showed that ambient temperature slightly affects the efficiency of the chlorination treatment in the reduction of viruses while bacterial reductions remained relatively uniform, regardless of the ambient temperature. Overall, with a decreasing temperature, there was a slight increase in the virus concentration in the effluent wastewater. This and further studies need to take into account factors such as chlorine concentration and weather conditions in order to ensure that the cleanest water is released into the environment.

CHAPTER 1: INTRODUCTION

While drinking water is monitored by the US Environmental Protection Agency (EPA), nondrinking water, which includes irrigation water used in the production of fresh produce, is not regulated (13). In September 2012, during a routine inspection, the US Food and Drug Administration (FDA) collected several samples of alfalfa sprouts and spent irrigation water from Arizona Hydroponic Farming LLC. During testing, the FDA discovered that the alfalfa sprouts and the spent irrigation water both tested positive for *Salmonella cubana*. There are many sources that can contaminate water, including naturally occurring chemicals and minerals, land use practices (pesticides, fertilizers), manufacturing processes, sewer overflows, and wastewater releases (10). With the presence of contaminants in water, adverse health effects due to illness outbreaks begin to get noticed. In fact, according to the United States Centers for Disease Control and Prevention (CDC) the second highest cause of outbreaks in public water systems is the Norovirus (10).

In February 2013, the National Park Service created the “State of the River Report” to inform the public on the health of the Mississippi River. The Mississippi River, which flows into the Gulf of Mexico, a major provider for seafood, is responsible for draining all or part of 31 states in the US, and two Canadian provinces (15). Due to this, Mississippi River water can be a major source of contamination (15). Bacterial analyses were performed in Minnesota, with *Escherichia coli* (*E. coli*) being used as the indicator bacterium, because it can indicate fecal contamination. The study showed that most portions of the river had bacterial concentrations that were higher than the state standard (15). Many wastewater treatment facilities deposit their cleaned water into the Mississippi River after it has been sanitized. According to the Clean Water Act, wastewater needs to be tested for *Enterococci* and *E. coli*. As of yet, there are no regulations for the Norovirus, which can be transmitted to food sources through fecal contaminated water. It

can be found in raw oysters, harvested from contaminated water, or from fruit and vegetables which were contaminated in the field (11). Norovirus is the number one cause of nonbacterial epidemic gastroenteritis (2). According to the National Outbreak Reporting System, between 2009 and 2010, there were 2,259 epidemic gastroenteritis outbreaks through person-to-person contact, and 1,270 (89%) of these were suspected or confirmed to have been caused by the Norovirus (7).

The Norovirus also called the Norwalk-Virus, is one of four genera in the family *Caliciviridae*, which is comprised of small, circular-looking (icosahedral), nonenveloped viruses (2). It contains a linear, positive-sense, single-stranded RNA genome (3). It was first discovered by Kapikian et. al. (1972) when they received stool samples from a gastroenteritis outbreak at an elementary school in Norwalk, Ohio (5). It wasn't until 1990 however, nearly two decades later, when Jiang et al. (1990) was able to clone and do the initial characterization of Norwalk virus RNA (4). Currently, the Norovirus is divided into five genogroups; GI, GII, GIII, GIV, and GV. (1). Infections in humans have only been linked to GI, GII, and GIV, with GI and GII causing the majority of the infections (1).

According to the Louisiana Department of Health and Hospitals, the infectious dose of the Norovirus is very low, as little as 10 to 100 viral particles can cause an infection (8). Everyone is susceptible to a Norovirus infection, but it's usually acute and self-limiting, however in immunocompromised people, infants, and elderly people, it can become more severe and prolonged (6). It is primarily transmitted through fecal contaminated food or water, but also by person-to-person contact, aerosolized vomit particles, and exposure to fomites (6). A major problem is that the Norovirus is very stable in the environment and resistant to common

disinfectants, which leads to outbreaks happening in semi-closed communities, such as nursing homes, schools, hospitals, and cruise ships (6).

The Norovirus is the second cause of outbreaks in public water systems. Advancements have been made in developing tests for early identification of the Norovirus, such as the "Ridascreen Norovirus 3rd Generation EIA" developed by R-Biopharm AG (14). This test though can only be used for preliminary identification. Currently, the main diagnostic method to detect viral RNA or antigens is by using real-time reverse transcription-polymerase chain reaction (RT-qPCR) (12). The assays are extremely sensitive, being able to detect as little as 10 to 100 Norovirus copies in a reaction (12). Additionally, through the use of different primers, Norovirus genogroups GI and GII can be differentiated (12).

The goal of my thesis is examine the efficiency of the chlorination of wastewater in the reduction of the Norovirus (GI and GII) concentration through the use of RT-qPCR. This is then compared to the efficiency of chlorination in the reduction of bacteria and viruses normally found in wastewater (*E. coli*, *Enterococci*, Fecal Coliforms, and Coliphages).

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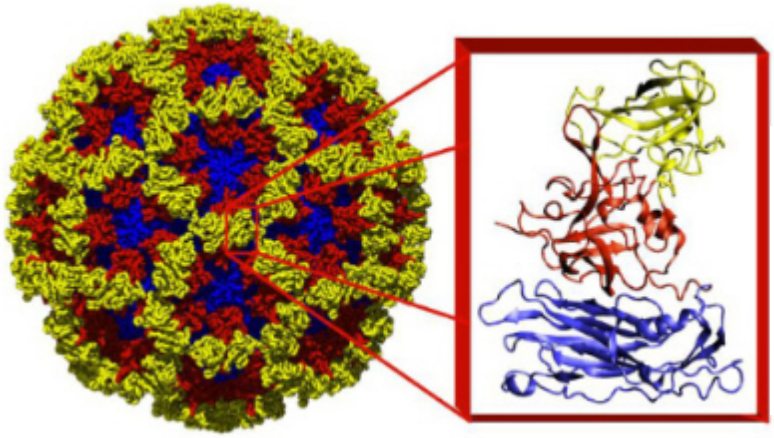
CHAPTER 2: LITERATURE REVIEW

2.1: History and General Information of the Norovirus

The genus Norovirus is one of four genera in the *Caliciviridae* family; the other three being *Sapovirus*, *Vesuvius*, and *Lagovirus* (5). The members of the *Calciviride* family are small (27 to 40 nm), nonenveloped, icosahedral viruses, with a genome that is composed of a linear, positive-sense, single-stranded piece of RNA (5). Figure 1 below shows the structure of the Norovirus (17).

Figure 1: X-ray structure of the Norwalk virus capsid



X-ray structure of the Norwalk virus capsid, with the inset showing details of the structure of the subunits. The different colors represent different regions of the capsid protein.
Courtesy: Dr. B.V.V. Prasad

The Norwalk virus, which is the only species in the genus Norovirus, was first discovered by Kapikian and others in 1972 when a filtered stool sample was studied using immune electron microscopy, due to a gastroenteritis outbreak in Norwalk, Ohio (9). Through this discovery, the Norwalk virus became the prototype for the "Norwalk-like virus group," which was composed of similar "small round structured viruses," and which is now known as Noroviruses (5).

An important advancement came with the initial characterization of the Norwalk virus RNA genome and its cloning in 1990 by Jiang and others (8). This firmly placed the Norwalk virus into the *Caliciviridae* family. This step led to the ability of characterizing the genomes of other Norovirus types, including those found in swine, cattle, and mice (5).

Understanding the basic replication technique and pathogenic mechanisms of the Norovirus have not been advancing rapidly because of the inability to grow the human Norovirus in a cell culture (6). There was a breakthrough however when the murine Norovirus was isolated and then successfully grown in a culture of macrophages and dendritic cells (6). The murine Norovirus is genetically similar to the human Norovirus, which will aide in the study of the human Norovirus until it can be grown in a culture (6). The replication technique of the Norovirus is believed to be similar to that of other ssRNA viruses (6). To infect a human, specific strains of Noroviruses recognize different HBGAs in human intestinal epithelial cells (7). Once the virus attaches, it releases its genome into the host cells' cytoplasm, where the RNA is then translated.

The Norovirus RNA genome is composed of three major open reading frames (ORFs), where the nonstructural proteins are encoded at the ORF1, which is the 5'-end of the genome (5). The structural proteins are encoded in the ORF2 (encodes VP1) and ORF3 (encodes VP2) regions, which are located more towards the 3'-end of the RNA genome (5). The VP1 protein is the major structural protein in the viral capsid where both the key antigens of the virus are located and it's determined how the virus will interact with its host cell (5, 12). The VP2 is only a minor structural protein, with only one or two copies present in each virion (5). Its presence is necessary for both the production of the infectious particles and later the change in the host cell membrane that facilitates apoptosis (13, 14). VPg is the third type of protein; there are usually only one or two copies present per virion (5). It is covalently linked to the RNA, where its function is probably acting as a nonstructural protein during replication (5).

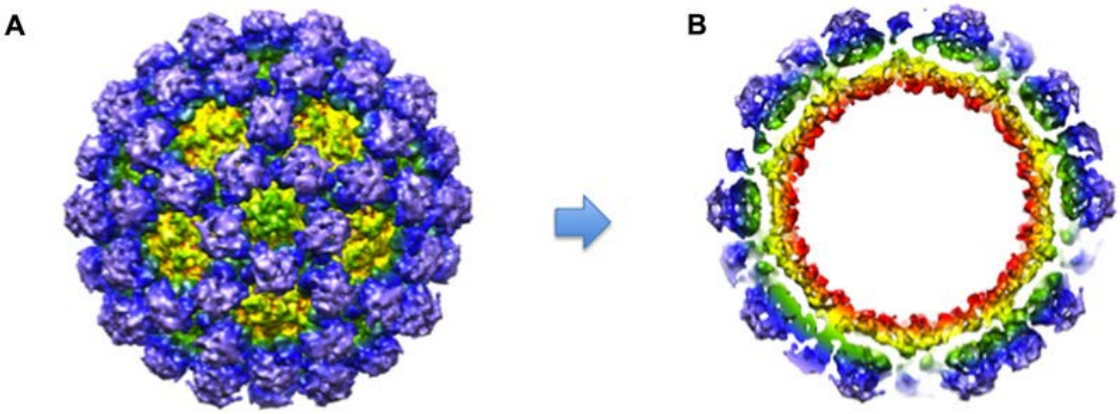
2.2: Taxonomy

The genus Norovirus is in the family *Caliciviridae*. Currently the Norovirus has five genogroups: GI, GII, GIII, GIV, and GV. Of these five groups, GI, GII, and GIV have been

linked to infections in humans, with GI and GII affecting humans the most (2). Worldwide studies have shown that the GII Norovirus causes more outbreaks than the GI. Within the genogroups, there also exist a variety of genotypes. The Norovirus is classified as a Category B biodefense agent, which means it is "moderately easy to disseminate, results in moderate morbidity rates and low mortality rates, and requires specific enhancements for diagnostic capacity and enhanced disease surveillance (24)."

Figure 2 (15) shows the different parts of the Norovirus, in this case it is a Norovirus from the genogroup GII. The red part indicates the N-terminal regions, the yellow part represents the Shell (S) domain, the green part is the Protruding arm 1 (P1) domain, and the blue, purple color is the P2 domain.

Figure 2: Depiction of Norovirus (GII) Capsid and Inner Layers



A. Depicts a Norovirus particle. piece

B. Depicts a cross sectional of the Norovirus.

The N-terminal and S domain are relatively conserved in their sequence when compared to other Norovirus strains (5). The P domains however, which are attached to the S domain with a flexible hinge, vary more in their amino acid sequences, with P2 varying the most (5). The function of the S domain is thought to be that it acts as an icosahedral scaffold, where the N-

terminal provides a switch that allows for the correct curvature (5). The P domains are thought to be replaceable modules which provide strain differentiation and antigenic specificity (5).

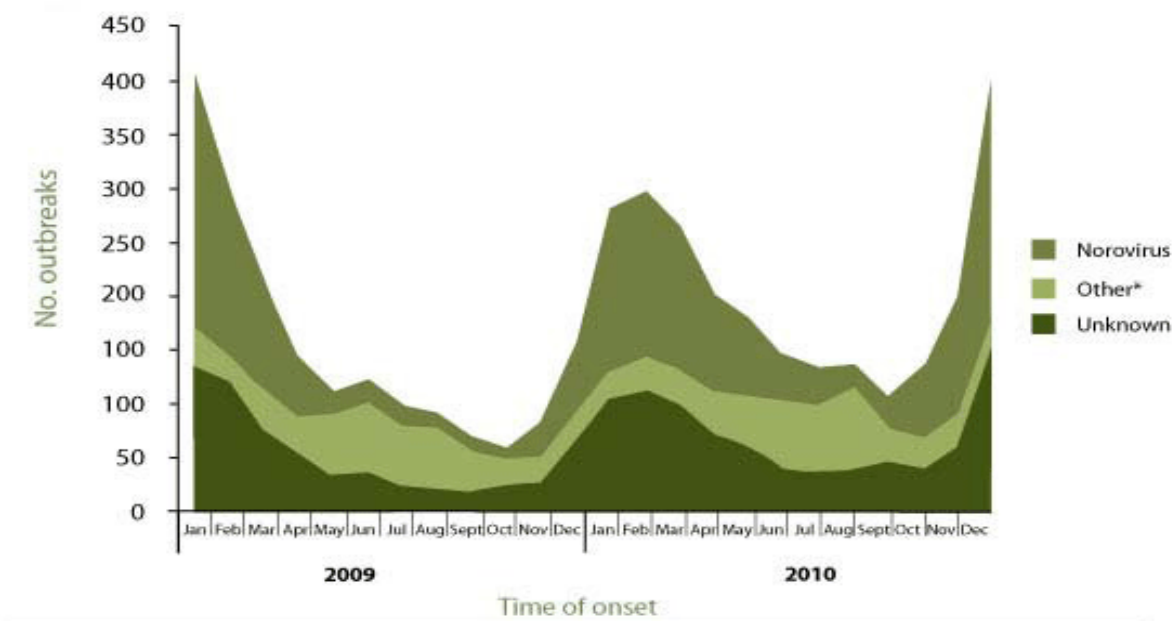
2.3: Infectious Dose

The infectious dose of the Norovirus is very low, inoculums as low as ten viral particles can be sufficient to infect someone (16). The incubation period, or the time elapsed between infection and showing symptoms, is about 24 to 48 hours, with the illness lasting on average anywhere from 12 to 60 hours (20).

2.4: Target Population

Outbreaks often occur in semi-closed communities, including nursing homes, schools, hospitals, and cruise ships (10). Surveillance of Norovirus outbreaks has shown that even though outbreaks occur throughout the year, 80% of them occur between November to April (18). See Figure 3 (19).

Figure 3: Graph of the Prevalence of Norovirus Outbreaks over a Year



2.5: Clinical Illnesses Caused by the Norovirus

The human Norovirus is responsible for at least 95% of viral outbreaks and over 50% of all outbreaks worldwide; it is a major cause of epidemic gastroenteritis (10). The illness is usually acute and self-limiting, but it can become more severe and prolonged in immunocompromised, infants, and elderly people (10). According to the CDC (Centers for Disease Control and Prevention) the Norovirus causes 19-21 million cases of acute gastroenteritis each year in the United States (22). This leads to an average of about 56,000-71,000 hospitalizations and 570-800 deaths (mostly young children and the elderly) yearly (22).

It is believed that the primary replication site of the Norovirus is in the upper intestinal tract (small intestines) (5). This theory is supported by a study performed by Agus et al. (1973), where patients were infected with the Norwalk virus. Histological signs showed blunted villi, shortened microvilli, dilated endoplasmic reticula, and an increase in the number of intracellular multivesicular bodies in the small intestine (1).

2.6: Detection Methods of the Norovirus

Since the Norovirus has been identified, efforts have been made to grow it in a cell culture system. This has been unsuccessful to this day for the Noroviruses associated with gastroenteritis in humans (4). Currently, the main diagnostic method to detect viral RNA or antigens is by using real-time reverse transcription-polymerase chain reaction (RT-qPCR) (21). The assays are extremely sensitive, being able to detect as little as 10 to 100 Norovirus copies in a reaction (21). Additionally, through the use of different primers, Norovirus genogroups GI and GII can be differentiated (21).

Recent advancements have been made in developing tests for early identification of the Norovirus. One such test, the "Ridascreen Norovirus 3rd Generation EIA" developed by R-Biopharm AG (23), has been approved by the FDA to detect Norovirus outbreaks. This test though can only be used for preliminary identification of sporadic cases because it is not sensitive enough to diagnose individual patients (23). As a result, RT-qPCR would still have to be performed.

2.7: Methods of Contamination

The virus is transmitted primarily through contaminated food or water, but also from person-to-person contact, aerosolized vomitus particles, and exposure to fomites (10). Common foods involved in Norovirus outbreaks are leafy greens such as lettuce, fresh fruits, and shellfish such as oysters, which are often eaten raw (22). The Norovirus is highly contagious, very stable in the environment, it is resistant to common disinfectants, and it is the leading cause of gastroenteritis (vomiting, diarrhea, and stomach cramping), a debilitating illness (10, 19). Additionally, they can remain active after freezing and heating of up to 60°C (20). According to the CDC, infection from the Norovirus is reported year round, but that it is most common during the winter months (22).

2.8: Prevention

Through experimentation, it was found that the Norovirus is able to infect humans even after being placed under the following conditions: exposure to a pH of 2.7 for 3 hours at room temperature, treatment with 20% ether for 18 hours at 4°C, and incubation for 30 minutes at 60°C (3). Additionally, it was found that the Norovirus remains active after being treated with 3.75 to 6.25 mg/L of chlorine, which had a free residual chlorine content of 0.5 to 1.0 mg/L (11).

This amount of chlorine is the concentration used in drinking water distribution systems (11). However, when the Norovirus is treated with 10 mg/L of chlorine, it was found to be inactivated (11). Ten mg/L is the chlorine concentration used in water supply systems when contamination has been detected (11).

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CHAPTER 3: EXPERIMENTS

3.1: Wastewater Treatment as performed by a Wastewater Treatment Plant in New Orleans, Louisiana

The process employed by a wastewater treatment plant in New Orleans, Louisiana is depicted in Figure 4 on page 18.

The incoming wastewater, called the influent wastewater is brought to the first step in a long series of steps called the Headworks. In the Headworks, large solids such as toilet paper, trash, and grit, are removed from the wastewater using bar screens. The trash that is removed gets sent to the dumpster, whose contents are then transported to a landfill. The next step is the raw sewage or grit channel, where inorganic substances, such as sand and coffee grounds settle at the bottom. These inorganic substances are then collected and also sent to the dumpster, and later on to a landfill. After this, the wastewater is sent to the oxygen or sequencing batch reactors. During this part of the process, oxygen is pumped into the wastewater to remove biological nutrients. The wastewater undergoes nitrification, denitrification, and even some phosphorus removal (4).

The next step is sending the wastewater to the clarifiers. Clarifiers have a cone-shaped bottom, which allows them to remove the final solids when they gather at the bottom. The idea behind this step is known as a weir. This means that the clarifier is acting as a barrier, allowing the cleaner water at the top to steadily spill over the top and gather for the final step. The last step in the treatment of the wastewater is chlorination. When looking at the diagram below, the liquid chlorine is indicated with the yellow arrows. The chlorine is transported using railway carts to the beginning of the effluent channel where it is added to the water that spilled over the clarifier's edge. This channel feeds directly into the river into which the cleaned wastewater is released.

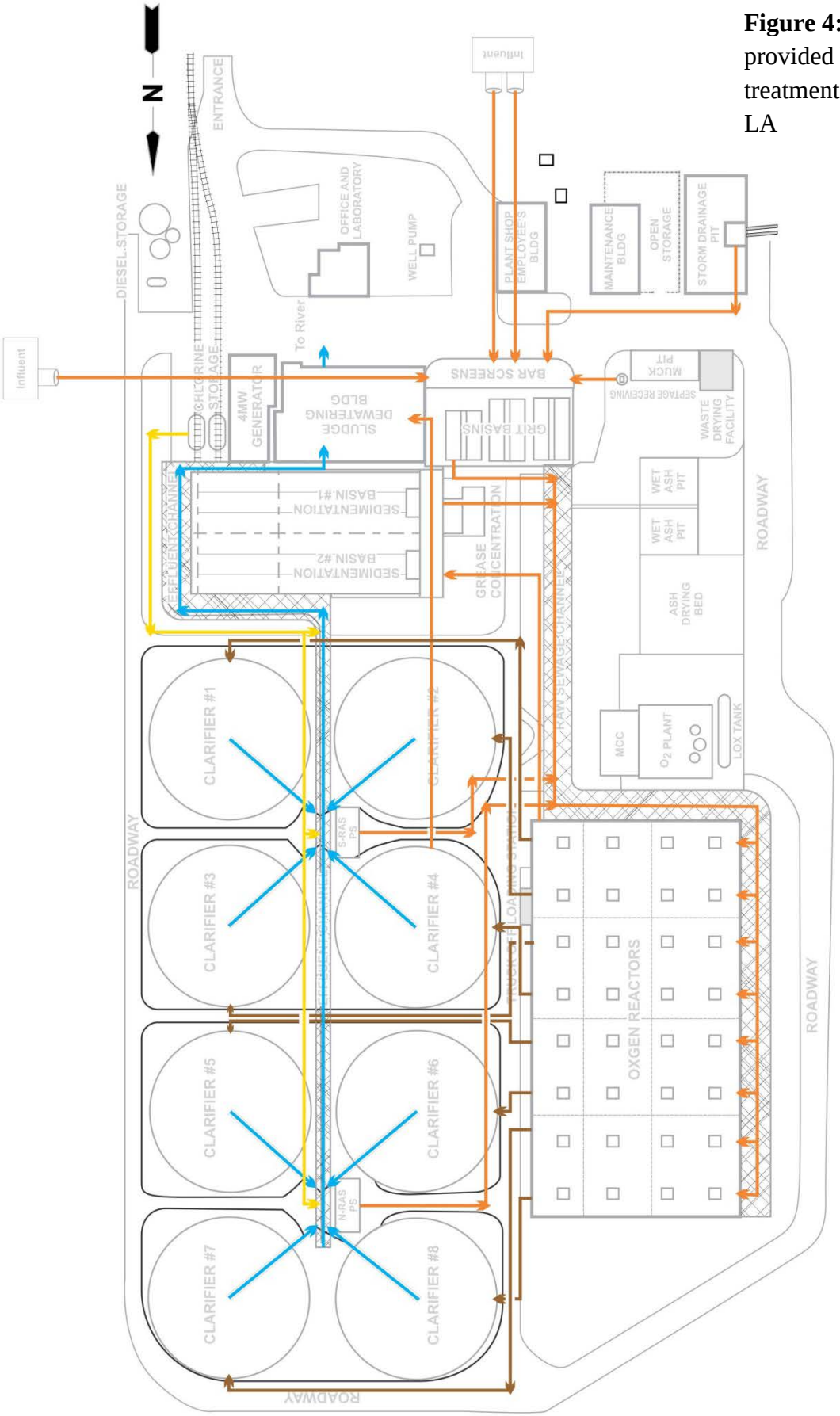


Figure 4: Process Flow Diagram, provided by the wastewater treatment plant in New Orleans, LA

In addition to wastewater, sewage sludge is also produced during the wastewater treatment. At the wastewater treatment facility in New Orleans, the sewage sludge is collected and thickened in clarifier #4. During that time, water is squeezed out, and what remains is sent to burn in the furnace. The ash from the furnace is removed and then brought to a landfill.

3.2 Wastewater Sample Collection

A composite sample of influent and effluent wastewater was taken once a month. Due to numerous analyses 3 Liters of effluent wastewater and 1 Liter of influent wastewater were taken. Immediately after taking the samples, 0.5 mL of 10% sodium thiosulfate were added to each liter of effluent wastewater. The samples were then placed in a cooler with ice until they were used.

3.3: RT-PCR

3.3.1: Reagents Used During Virus and RNA extraction

Before performing the concentration and extraction of viruses, reagents had to be prepared ahead of time. First, 0.25N/0.05M glycine buffer was prepared by dissolving 3.75 grams of glycine (Sigma G-7126) in 1 Liter of water. The pH was adjusted to 9.5, after which the reagent was autoclaved at 121°C (15 PSI) for 15 minutes and then stored at 4°C until it was used. Next, both 1xPBS and 2xPBS tissue culture grade (tc) were prepared, the 2xPBS (tc) contained twice the amount of reagents as the 1xPBS (tc). For the 1xPBS (tc), 8.0 grams of NaCl, 0.2 grams of KCl, 0.12 grams of KH_2PO_4, and 0.91 grams of Na_2HPO_4 were added to 1 Liter of distilled water. The pH was adjusted to 7.5, and then the samples were autoclaved at 121°C (15 PSI) for 15 minutes and then stored at 4°C until they were used.

For the RNA extraction, 3 reagents had to be prepared. 6M guanidine isothiocyanate (GITC) was prepared by dissolving 7.1 grams of GITC in 4.5 mL of sterile distilled water. The

dissolved solution was then filtered through a 0.45µ filter, and stored in the dark for up to 3 weeks. Next 50% ethanol (EtOH) was prepared by adding 5 mL of ethanol to 5 mL of sterile distilled water.

3.3.2: Concentration and Extraction of Viruses in Sewage

All samples were run in duplicate. First 60 mL of both influent and effluent wastewater were pipetted aseptically from shaken sample bottles. The 60 mL were placed into appropriate glass tubes and centrifuged at 37,000 rpm for 1 hour. Due to the sensitivity of the ultra-centrifuge, the weight of the tubes had to be within 0.05 grams. After one hour, the tubes were removed, and the supernatant was discarded. The pellet was suspended in 6 mL of 0.25N (0.05M) glycine buffer (pH 9.5). This mixture was pipetted into 50 mL plastic tubes. While in the tubes, the samples were incubated on ice for 30 minutes, and shaken every 5-10 minutes using a shaker. After the 30 minutes, 6 mL of 2X PBS (tc) were added and the sample was shaken again. These tubes were then weighed to ±0.5 grams and then centrifuged at 5000 rpm for 20 minutes at 4°C.

While the samples were centrifuging for 20 minutes, the glass tubes used for the first centrifugation were cleaned using a 10% bleach solution and UV light, whose peak UVC emission is 253.7 nanometers (2). After 20 minutes in the centrifuge, the tubes were removed, aspirated, and the supernatant was then transferred into the clean 60 mL glass tubes and 50 mL of 1xPBS (tc) were then added to each glass tube. These were then centrifuged for 1 hour at 37,000 rpm. After 1 hour, the tubes were removed, and the supernatant was discarded. The pellets were suspended using 600µL 1xPBS (tc) and then mixed well. The samples were finally evenly distributed into three 2.0 mL micro-centrifuge tubes, with each tube containing 200µL of sample. The tubes were then stored at -70°C until RNA extraction was performed.

3.3.3: RNA Extraction

A tube of each of the four virus extracts from the previous extraction was removed from its -70°C incubation and allowed to thaw. Then 500 µL of 6M GITC was added to each pellet, after waiting for one minute, the tubes were vortexed in order to lyse the virus and dissolve the pellet. Next, 700 µL of 50% EtOH was added to each tube, after which the tubes were inverted twice to precipitate the RNA. Seven hundred µL of each mixture was then pipetted to a mini spin column that contained a silica gel filter membrane. The mini spin column was then centrifuged at 10,000 rpm for one minute. The eluent was discarded and the remaining sample was pipetted into each spin column, which was then centrifuged again at 10,000 rpm for one minute.

Next, 700 µL of washing buffer RW1 was added to each spin column, which then sat at room temperature for 15 minutes. The spin columns were then centrifuged at 10,000 rpm for 45 seconds, and the eluent was discarded. Five hundred µL of the RPE buffer were then added to the spin columns as a second washing. The spin columns sat at room temperature again for 15 minutes, after which they were centrifuged at 10,000 rpm for 45 seconds. During this time the frozen primer TE buffer (THE RNA Storage Solution, *Sodium Citrate* pH 6.4) was placed on a heating plate, so that it would reach 70°C by the time it was needed.

After centrifugation, the spin columns were removed, the eluent was discarded, and 500 µL of RPE buffer were added again. This time the spin columns were centrifuged immediately after adding the RPE buffer for 2 minutes at 10,000 rpm. The collecting tube was then changed, and the spin column was dried by spinning it for one minute at full speed 13,200 rpm. The collecting tubes were then discarded, and the spin column was placed in 1.5 mL DNase/RNase free tubes. Twenty µL of the primer TE buffer (THE RNA Storage Solution, *Sodium Citrate* pH 6.4) were added to the column and allowed to sit for one minute in order to elute the RNA. The

spin columns were then centrifuged at 10,000 rpm for one minute. Twenty µL of primer TE buffer (THE RNA Storage Solution, *Sodium Citrate* pH 6.4) were then added to the spin column again and allowed to sit for one minute to elute the rest of the RNA. The spin columns were then centrifuged again for one minute at 10,000 rpm. The RNA extract was then evenly divided into two 1.5 mL DNase/RNase free tubes, which were then stored at -70°C until RT-PCR was performed on them.

3.3.4: RT-PCR Standard Curve and Sample Testing

Before performing RT-PCR, the appropriate concentration of an Internal Amplification Control (IAC) must be prepared. After this, to perform RT-PCR, the first step was to prepare the mixture of the reagents, primers, and probes. An example of this mixture can be seen in Table 1 below.

Table 1: Example Amounts of the Reagents, Primers, and Probes used in RT-PCR

	Reagents, Primers, and Probes	**Volume (µL)**
1	Water	60.00
2	5 X OneStep RTPCR-PCR buffer	60.00
3	$MgCl_2$ (25 mM)	18.00
4	dNTP Mix	12.00
5	COGI-Forward[A]	9.00
6	COGI-Reverse[A]	9.00
7	COGII-Forward[A]	9.00
8	COGII-Reverse[A]	9.00
9	Enteroviruses Forward[B]	12.00
10	Enteroviruses Reverse[B]	12.00
11	IC 46Forward[A]	2.26
12	IC 194Reverse[A]	2.26
13	COGI-Cy5[A]	3.00
14	COGIb-Cy5[A]	3.00
15	COGII-Cy3[A]	3.00
16	EV-FAM[B]	9.00
17	IC-TxR[A]	4.50
18	OneStep RT-PCR Enzyme Mix	12.00
19	Superase In	3.00

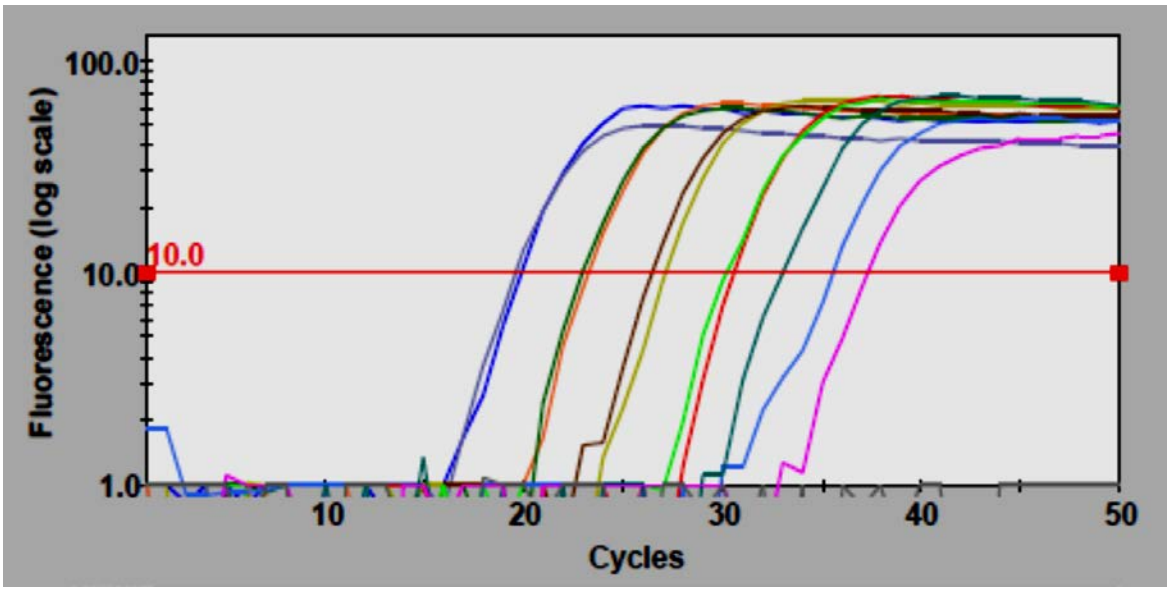
20	IAC[A]	12.00
21	RNA	3.00

[A] These probes, primers, and IAC are from reference 1.
[B] The enterovirus primers and probes are from an unpublished paper.

For reagents 1-12, both the reagents and the mixture were vortexed and then centrifuged to make sure there was even distribution. Additionally, reagents 18 and 20 were only added to the mixture immediately prior to adding the master mix to the RT-PCR test tubes. The RNA was not added to the mixture, the 3.00 µL shown in the table above is the amount that was added to each RT-PCR test tube. From the table above, enough of the mixture was made to run RT-PCR on 12 test tubes, two of which were used for a positive and negative control. The rest of the test tubes were filled with different dilutions of the sample.

After the tubes had been filled with mixture from the Table 1 and sample, they were vortexed for 10 seconds and then checked to see if there were any bubbles in the test tube. If there were no bubbles, the test tubes were placed in the RT-PCR machine and the appropriate program was started. In this experiment, the RT-PCR analysis went through 50 cycles. An example of a calibration curve for Norovirus GII can be seen in Figure 5.

Figure 5: Calibration Curve for Norovirus GII



3.4: Microbial Tests

3.4.1: Media Preparation for Membrane Filtration

Three types of media were used during the process of membrane filtration. Modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC) was used for the detection and enumeration of thermotolerant *Escherichia coli.* M-FC agar was used for the detection and enumeration of fecal coliforms. Lastly, mEI agar was used for the detection and enumeration of enterococci.

Modified mTEC plates were prepared by adding 45.6 grams of the dry ingredients mixture to 1 Liter of reagent-grade water. This was then mixed thoroughly on a heated hot plate until everything was completely dissolved. The mixture was then autoclaved at 121°C (15 PSI) for 15 minutes, after which it cooled in a 50°C water bath. After it had cooled to the temperature of the water bath, the pH was adjusted to 7.3± 0.2 using 1.0 N hydrochloric acid or 1.0 N sodium hydroxide. Once the ideal pH was reached, 6 mL of agar were pipetted aseptically into sterile 60 mm plates.

M-FC plates were prepared by suspending 52 grams of the medium to 1 Liter of purified water. Then 1% rosolic acid was prepared by dissolving 1 gram of rosolic acid into 100 mL of 0.2 N NaOH. Ten mL of 1% rosolic acid in 0.2 N NaOH was then added to the m-FC. The mixture was then heated and agitated on a hot plate. After boiling for one minute to completely dissolve the medium, the mixture was removed and if necessary, the pH was adjusted to 7.4 using 1N HCl. Once the ideal pH had been reached, the agar was placed in a 45-50°C water bath to cool. After reaching 50°C, 6 ml of agar were aseptically pipetted into sterile 60 mm plates.

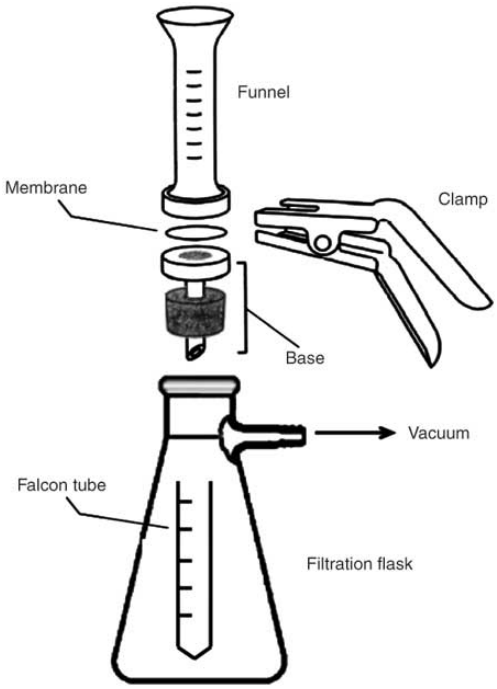
The mEI plates were prepared by first suspending 72 grams of medium powder in 1 Liter of purified water. This was thoroughly heated with frequent agitation and allowed to boil for 1

minute, so that the powder was completely dissolved. The mixture was then autoclaved at 121°C for 15 minutes, after which it was cooled in a 50°C water bath. Next a nalidixic acid solution was prepared by adding 0.24 grams of nalidixic acid to 5 mL of purified water. A few drops of 0.1 N NaOH were added to the nalidixic acid solution to dissolve everything. This entire solution was then added to the 1 Liter of mEI medium. Next, 0.02 grams of triphenyltetrazolium chloride were added to the mEI medium. The entire mixture was thoroughly mixed, after which 6 ml of agar were aseptically pipetted into sterile 60 mm plates.

3.4.2: The Process of Membrane Filtration

First all 60 mm plates were labeled, and then the membrane filtration apparatus was set up under the hood. A sterile membrane filter, grid side up, was added, and the funnel was attached to it. The apparatus set up is depicted in Figure 6 (3).

Figure 6: Set Up of the Filtering Apparatus



The effluent (Ef) wastewater samples were run first so as to reduce the risk of possible contamination from the Influent wastewater sample. Two concentrations of wastewater were run in duplicate per media. For modified mTEC, 50 mL of wastewater were used for Ef-A and 100 mL of wastewater were used for Ef-B. For m-FC, 100 mL of wastewater were used for Ef-A and 150 mL of wastewater were used for Ef-B. Lastly for mEI, for both Ef-A and Ef-B, 150 mL of wastewater were filtered. The appropriate amounts of wastewater samples were removed from a vigorously shaken bottle. The vacuum was turned on until all the water had filtered through, after which the vacuum was turned off and the funnel was removed. Sterile forceps were then used to aseptically remove the membrane filter, which was then rolled grid face down onto the appropriate agar to avoid bubble formation. To make sure that the filter was sealed onto the agar, the forceps were run around the edge of the filter.

The procedure for filtering the influent wastewater was the same as above. The only exception was that because less than 20 mL of wastewater were tested, 20-30 mL of PBS were used to rinse the side of the funnel and to make sure the sample was evenly distributed on the membrane. For modified mTEC, In-A used 1 mL of 10^{-2} concentrated wastewater sample and In-B used 1 mL of 10^{-3} concentrated wastewater sample. For m-FC, I In-A used 1 mL of 10^{-2} concentrated wastewater sample and In-B used 1 mL of 10^{-3} concentrated wastewater sample. Lastly for mEI, in In-A, 1 mL of 10^{-1} concentrated sample was used and in In-B, 1 mL of 10^{-2} concentrated sample was used.

3.4.3: Membrane Filtration Media Incubation

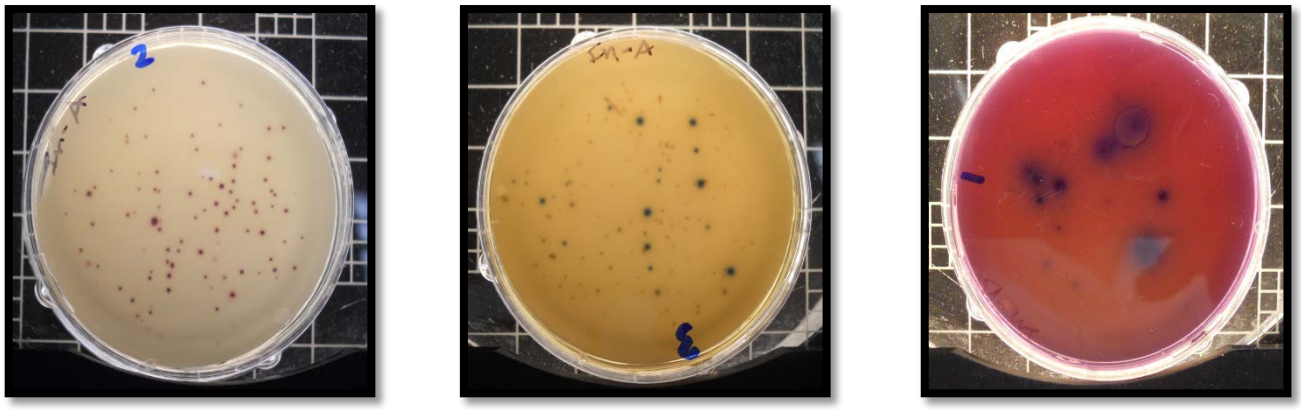
The modified mTEC agar plates were first placed in a 35°C incubator for 2 hours so that injured or stressed bacteria could be resuscitated. After the 2 hours, the plates were placed in a

44.5°C incubator for 22 hours. The m-FC agar plates were placed into a 44.5°C incubator for 24 hours. Lastly, the mEI plates were placed into a 41°C incubator for 24 hours.

3.4.4: Membrane Filtration After Incubation

After 24 hours, all plates were removed from their respective incubators, and the colonies were counted. For the modified mTEC, the red or magenta colored colonies were counted. For the m-FC, the fecal coliform colonies were various shades of blue, whereas the non-fecal coliforms had a gray to white color. Both types of colonies were recorded if both types were present, but only the fecal coliforms were used in the data analysis. Lastly, on the mEI plates, all colonies, regardless of their color were counted, as long as they had a blue halo. See Figure 7 below as examples of the plates.

Figure 7: Example plates of modified mTec, mEI, and m-FC



modified mTEC **mEI** **m-FC**

3.4.5: Prepartion of Overnight and Log Phase Cultures Used in the Enumeration of Coliphage in Wastewater

Overnight growth cultures were prepared the night before the test for the coliphage enumeration. The culture was prepared by inoculating 25 mL of 1x Triptic Soy Broth (TSB) with 250µL ampicillin-streptomycin antibiotic stock and a loopful of frozen *E.* coli coliphage culture. This mixture was then incubated on a shaker (100-150 RPM) at 36°C ± 1.0°C overnight. The next morning, the log-phase growth culture was prepared using 25 mL of 1xTSB, which was inoculated with 250 µL of ampicillin-streptomycin antibiotic stock and 1.0 mL of the overnight culture. Each tube was covered in parafilm and incubated on a shaker (100-150 RPM) at 36°C ± 1.0°C for 4-6 hours.

3.4.6: Media Preparation for the Enumeration of Coliphage in Wastewater

For the enumeration of coliphage, fresh, liquid 2xTriptic Soy Agar (TSA) was required. It was prepared by dissolving 66 grams of Triptic Soy Broth and 18 grams of Technical Agar in 1 Liter of purified water. 100 mL of this mixture was then aseptically pipetted into 6 250-500 mL Erlenmeyer flasks each and autoclaved at 121°C (15 PSI) for 15 minutes. After this the media was placed in a 45-48°C water bath for cooling. After the media has cooled to the temperature of the water bath, 2 mL of ampicillin-streptomycin antibiotic stock were added to each of the 6 Erlenmeyer flasks.

3.4.7: Procedure for the enumeration of coliphage in wastewater

While the Erlenmeyer flasks containing the 2x TSA were cooling in the water bath, wastewater samples, positive and negative controls were prepared. For the positive and negative controls, 100 mL of phosphate buffered saline broth (PBS) were aseptically pipetted into 250-500 mL Erlenmeyer flasks. Two concentrations of influent and effluent were tested. For the effluent, one Erlenmeyer flask contained 100 mL of wastewater, and the second flask contained 50 mL of wastewater and 50 mL of PBS. For the influent samples, one flask contained 5 mL of

wastewater and 95 mL of PBS, while the second flask contained 1 mL of wastewater and 99 mL of PBS. After all samples had been prepared, 100 µL of 10^{-7} concentrated coliphages was added to the positive control sample, so as to avoid contamination of the other flasks. After this, 0.5 mL of sterile magnesium chloride stock were added to each of the 6 sample flasks. These flasks were then placed in a 36°C water bath for 5 minutes, until they reached the temperature of the water bath. The temperature was checked using a temperature flask, which contained 100 mL of water.

After 5 minutes, the flasks were removed from the 36°C water bath, and 10 mL of the previously prepared log-phase stock containing *E. coli* solution was added to each flask. Ten mL of water were also added to the temperature flask. The flasks were then immediately transferred to a 45-48°C water bath. When the water in the temperature flask reached 43°C, all samples were removed.

The next step was plating. To avoid contamination, the negative control was plated first, followed by the effluent samples, then the influent samples, and lastly the positive control. Each mixture of PBS and sample was added to 100 mL of 2x TSA. The flask was then swirled around for 3 minutes. After this the contents of flask were poured into a series of ten 100 mm Petri dishes. The agar was allowed to harden, and the plates were then incubated for 16-24 hours at 36°C.

After the incubation time, the plaques were counted on each plate. An example of what a plaque looks like can be seen in Figure 8. The red circles indicate a few of the individual plaques.

Figure 8: Plate depicting coliphage plaques

3.5: References

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3. http://www.nature.com/nprot/journal/v1/n5/fig_tab/nprot.2006.372_F3.html
4. https://www.neiwpcc.org/neiwpcc_docs/sbr_manual.pdf

CHAPTER 4: RESULTS AND DISCUSSION

4.1: Bacterial Growth and the Effect of Temperature Over Time

According to the Environmental Protection Agency (EPA) the weather can have an effect on both influent and effluent wastewater. During the winter, for example, influent temperatures can cool rapidly, which in turn will affect the quality of the effluent wastewater (4). The EPA has performed studies, which showed that during winter and early spring the quality of the effluent wastewater was reduced, especially when ice was present on the water surface (4). In addition to this, the temperature can have an effect on chlorination. According to the EPA, increasing the temperature increases the rate of disinfection (1). The temperature range at which the plant will have optimum results is 20°C (72°F) to 35°C (95°F), with the majority of problems happening at the colder, wintery temperatures (5). It gets especially bad at below 5°C, which is when the biological treatment activity drops to almost zero (5).

The goal of this project was to examine the efficiency of chlorination in wastewater and the potential effect that temperature has on it. This was done by comparing bacteria (*E. coli*, *Enterococci*, Fecal Coliforms) and viruses (Coliphage, Norovirus GI and GII, enteric viruses) present in influent wastewater with what remained in the effluent wastewater. The average temperature of the testing dates was provided by the National Climatic Data Center, which is part of the National Oceanic Atmospheric Administration (8).

The first analysis involved growing cultures from wastewater filtrates on different media, where *Enterococci*, *E. coli*, and fecal coliforms were detected and enumerated. Figure 9 shows the log reduction of enterococci bacteria remained relatively unchanged when compared to the changing temperature.

Figure 9: Log reduction of *Enterococci* compared to the changing temperature over time

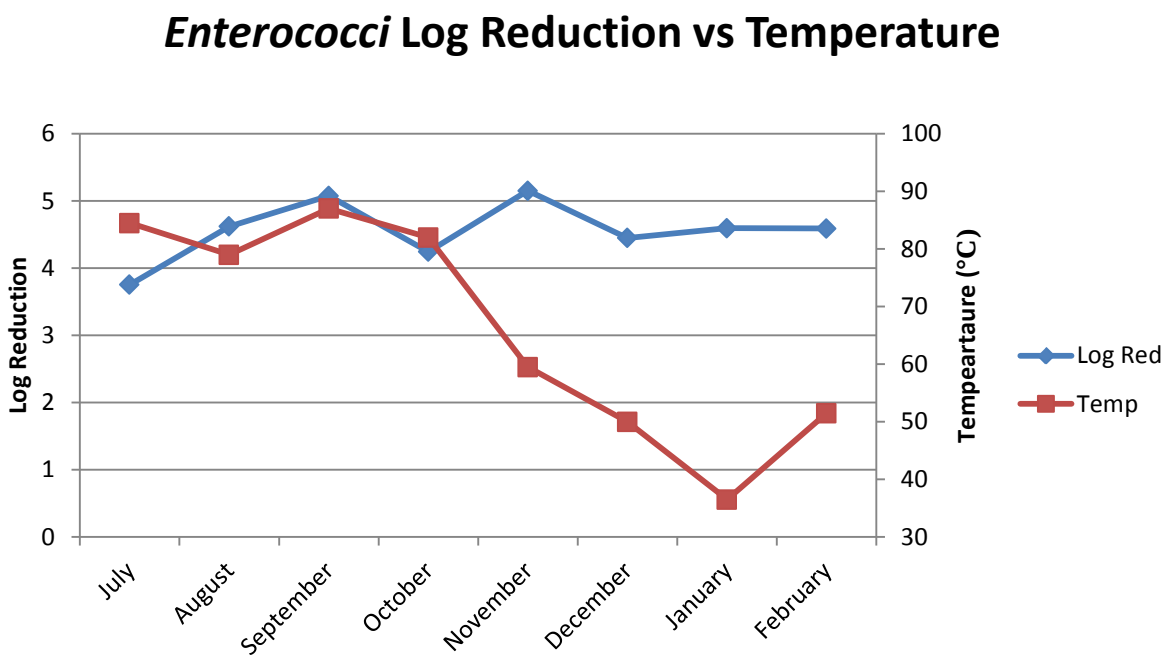
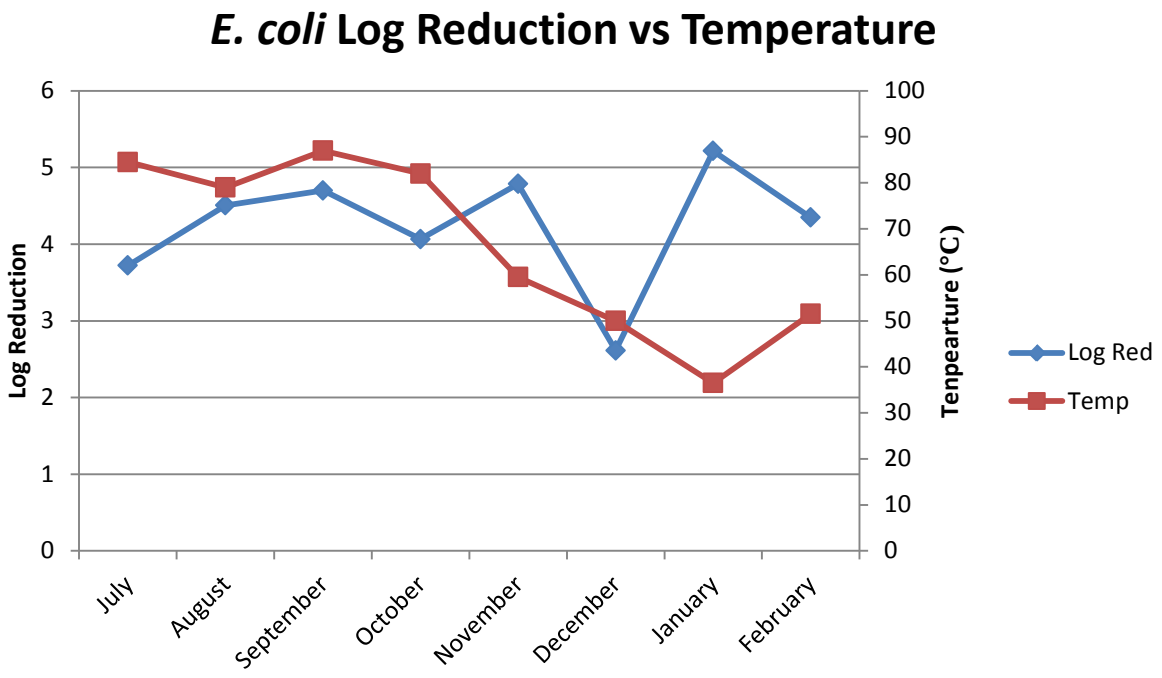


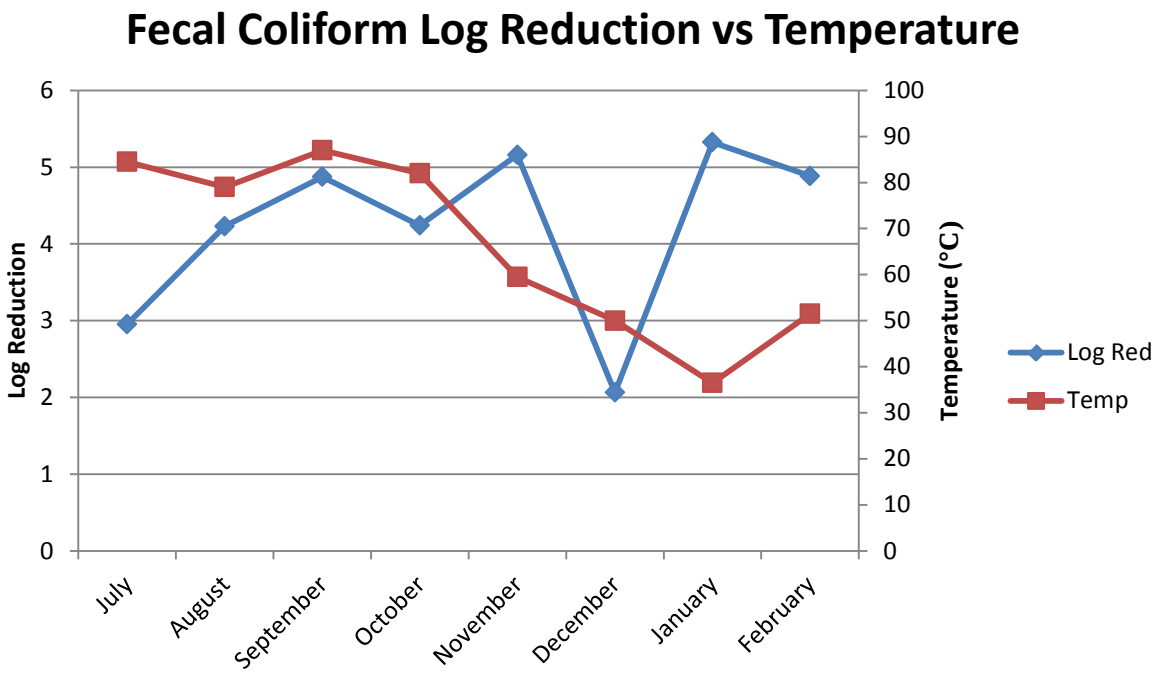
Figure 10 shows the filtration results for the detection and enumeration of *Escherichia coli*. Similar to the *Enterococci* results, the log reduction is hardly affected by the changing temperature. The only exception was in December, where there was a drastic decrease in the log reduction when compared to the other months. The only explanation for this outlier month is that there was overflow at the treatment facility, which caused the effluent wastewater to become contaminated, and that the sample we received contained effluent wastewater prior to a second cleaning.

Figure 10: Log reduction of *E. coli* compared to the changing temperature over time



The results for the third media, which detected and enumerated fecal coliforms, are shown in Figure 11. Similar to the *E. coli* results, the log reduction remained relatively consistent despite the changing temperature, with the only exception being December. The drastic drop in the log reduction in December is most likely due to an overflow and contamination problem at the plant. Overflow could have been caused by anything from an influx in influent wastewater, to a blockage in the equipment. The reduced log reduction could have been caused by the decreasing temperature if the log reduction had not increased by more than two log cycles from December to January.

Figure 11: Log reduction of fecal coliforms compared to the changing temperature over time



Figures 9, 10, and 11 show that overall when the temperature decreased, the bacterial log reduction remained relatively consistent. Based on these results, because there was no decrease in the log reduction of *Enterococci* in December, *Enterococci* might be more easily inactivated during the wastewater treatment process than *E. coli* and fecal coliforms. This however, is contrary to results from studies which showed that *Enterococci* were actually more resistant to inactivation through chlorination than *E. coli* (3, 6, 7).

4.2: Viral Enumeration and the Effect of Temperature Over Time

4.2.1: Coliphage reduction compared to bacterial reduction over time

While bacterial reduction was not greatly affected by the changing temperature, viral reduction appeared to decrease when the temperature decreased as seen in Figure 12.

Additionally, the overall log reduction of coliphage was usually at least one log cycle less than bacterial log reduction, as seen in Figure 13. These results are supported by a study performed by Tree et al. (2003), who showed that bacteriophages were more resistant to chlorination than the indicator bacteria (*E. coli*, *Enterococci*) (6).

Figure 12: Log reduction of coliphage compared to the changing temperature over time

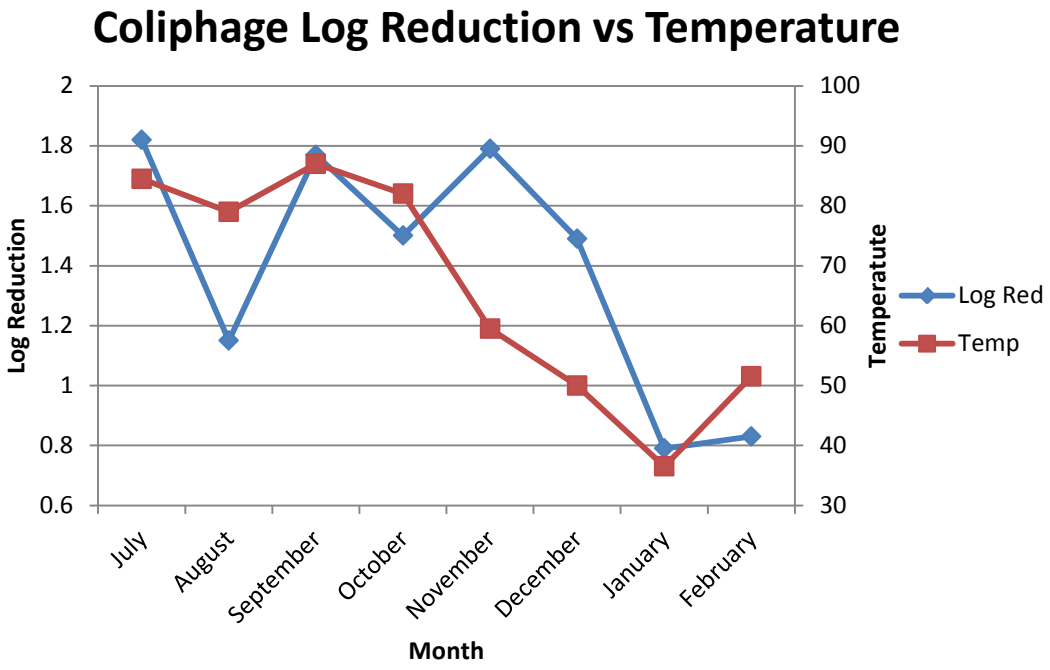
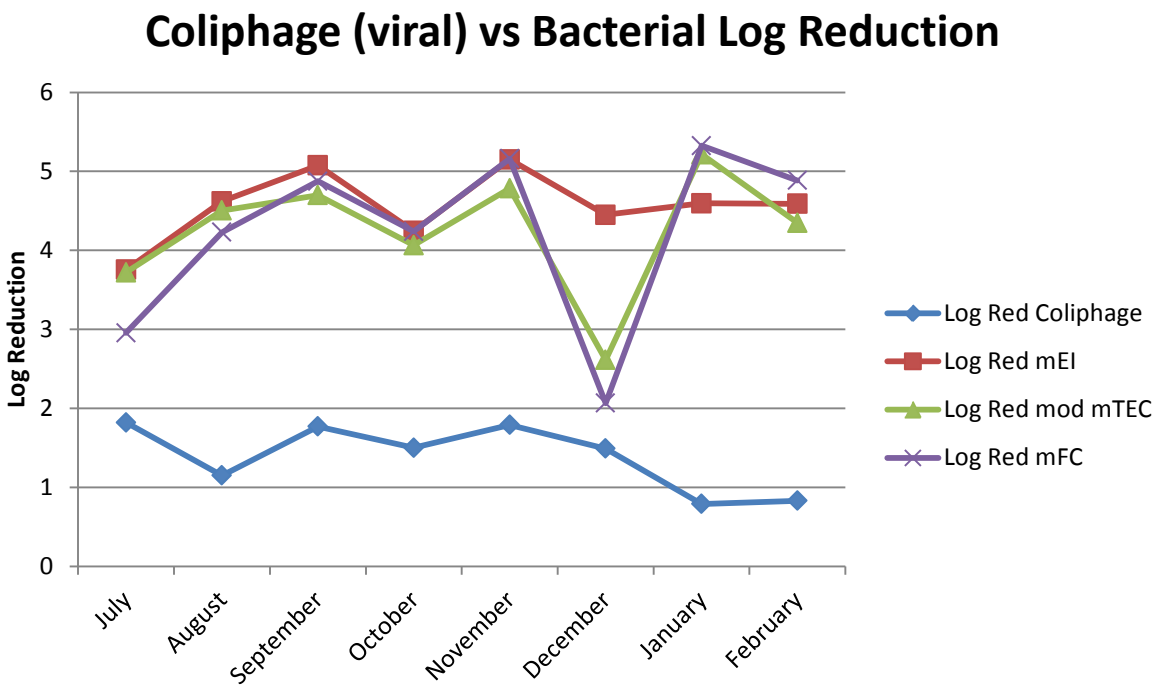


Figure 13: Log reduction of coliphage compared to the log reduction of *Enterococci*, fecal coliforms, and *E. coli* over time

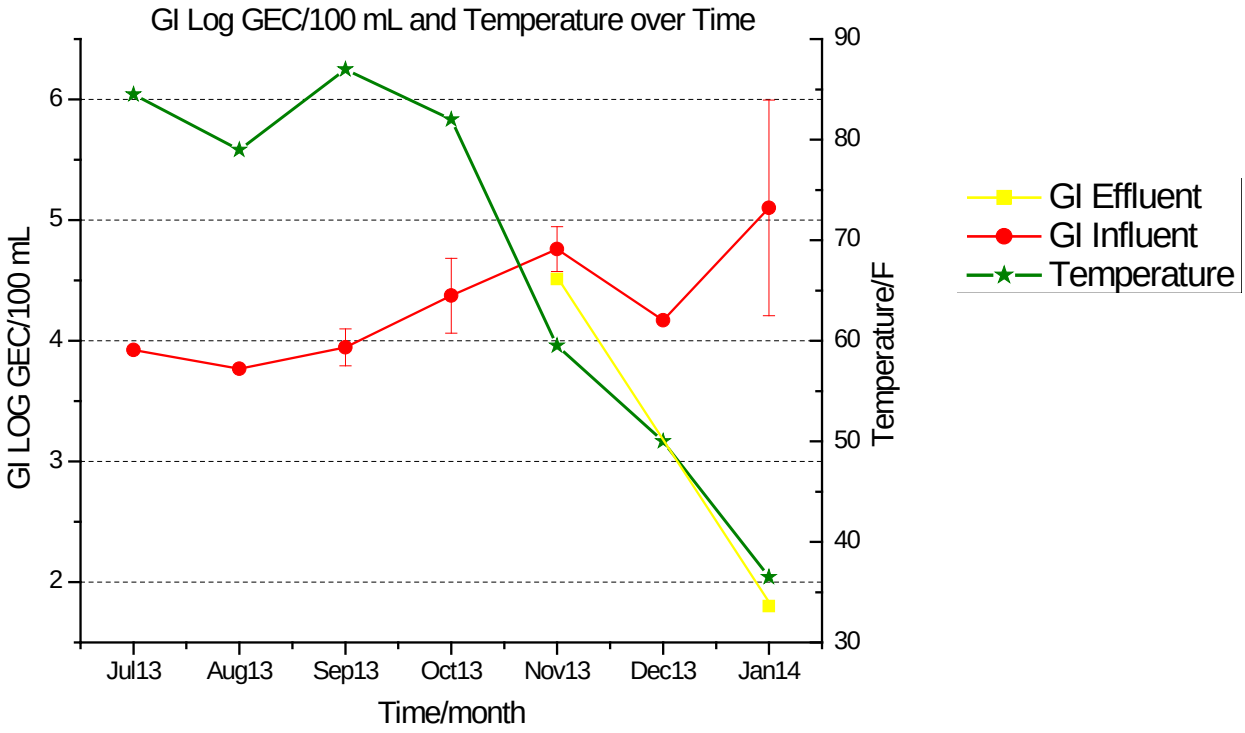


4.2.2: Norovirus GI and GII and enteric viruses compared to changing temperature over time

The human Norovirus cannot be cultured using media, the only way to detect and enumerate is through real-time reverse transcription-polymerase chain reaction (rt-qPCR). Rt-qPCR was run to detect and enumerate Norovirus GI, Norovirus GII, and enteric viruses that may be present in samples of influent and effluent wastewater. The rt-qPCR data was collected by Naim Montazeri, a PhD student in the Louisiana State University Nutrition and Food Sciences Department.

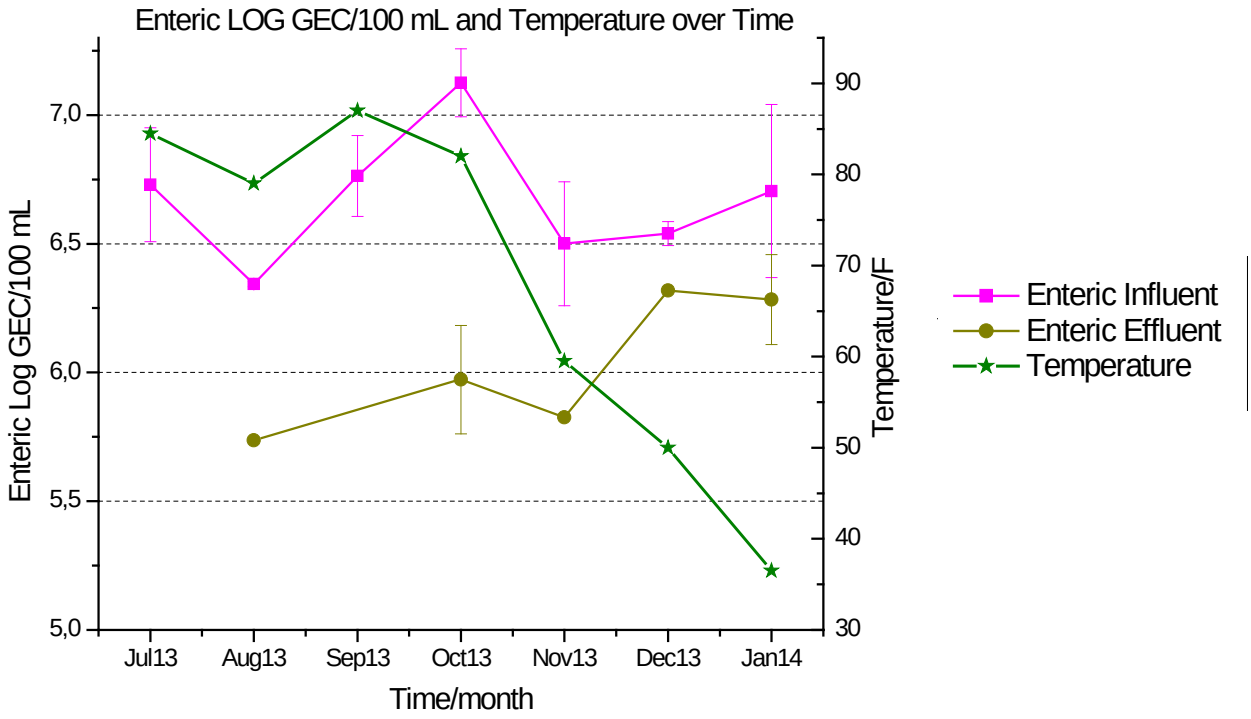
The rt-qPCR results can be seen in Figures 14, 15, and 16 below for Norovirus GI, enteric viruses, and Norovirus GII respectively.

Figure 14: GI log GEC/100 mL compared to the changing temperature over time



Results from Figure 14 show that the temperature affected the presence of Norovirus GI in the effluent wastewater. Overall, the concentration of Norovirus GI seemed to increase in the influent wastewater when the temperature decreased. Additionally, the only times Norovirus GI was detected in the effluent wastewater was in November and January, which were months with low temperatures. This is supported by a study performed by Bertrand et al. (2012), where it was concluded that at low temperatures, especially under 10°C (50°F), viral inactivation occurs more slowly (2).

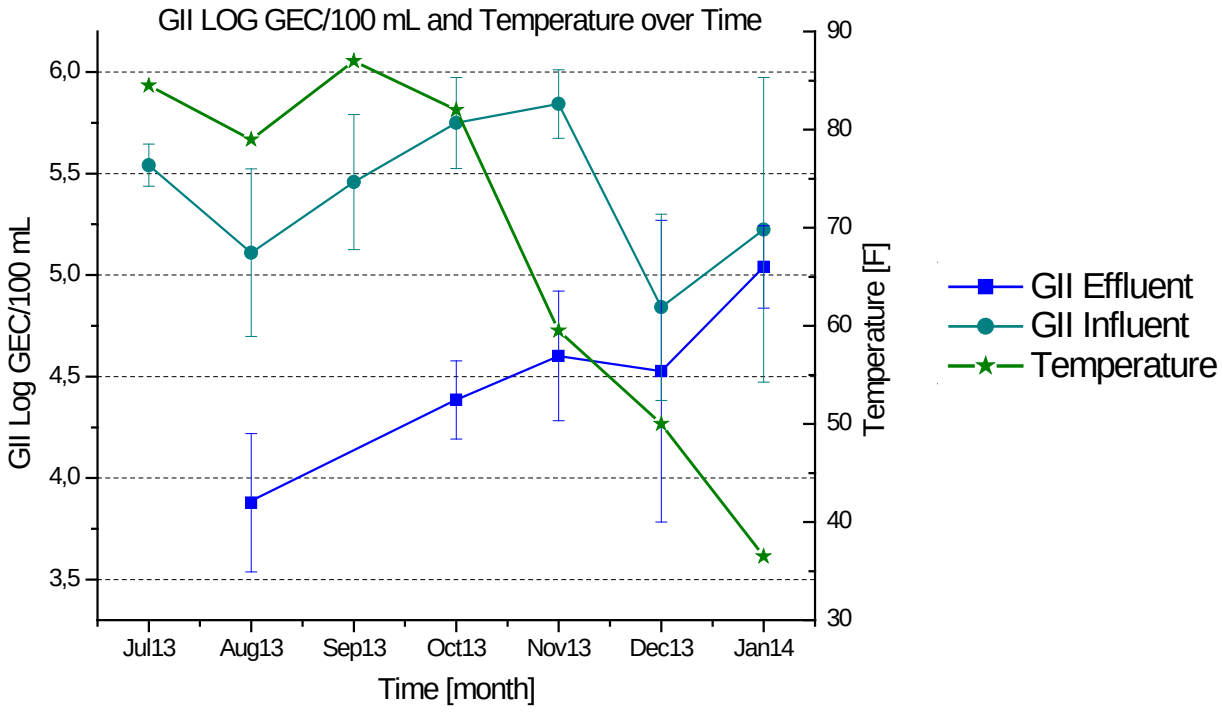
Figure 15: Enteric virus log GEC/100 mL compared to the changing temperature over time



Results from Figure 15 show that the temperature affected the enteric virus concentration. While the concentration of enteric viruses in the influent wastewater remained relatively even, the concentration in the effluent wastewater appears to increase when the temperature decreased.

Figure 16 shows that the temperature also affected the concentration of Norovirus GII. Overall, there was a general increase in the concentration of Norovirus GII in the effluent wastewater as the temperature decreased.

Figure 16: GII log GEC/100 mL compared to the changing temperature over time



4.3: References

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8. http://www.ncdc.noaa.gov/

CHAPTER 5: CONCLUSION

The analysis of contamination in wastewater from a New Orleans wastewater treatment facility has shown that chlorination is a moderately successful procedure in reducing both viral and bacterial contamination in effluent wastewater. For this research influent and effluent wastewater samples, the later chlorinated according to standard procedures, were taken, prepared, and analyzed. The amounts of viral and bacterial contamination were then compared to each other.

Different types of analyses were performed, one of which was the detection and enumeration of *Escherichia coli*, fecal coliforms, and *Enterococci* through cultures grown from membrane filtrates. Detection and enumeration of coliphage was also performed using Triptic Soy Agar. Lastly, for Norovirus GI, Norovirus GII, and other enteric viruses RNA was extracted and analyzed using RT-PCR.

Documenting the chlorination results over the time period from July 2013 until January 2014 has shown that ambient temperature slightly affects the efficiency of the chlorination treatment in the reduction of viruses while bacterial reductions remained relatively uniform, regardless of the ambient temperature.

Studies have shown that indigenous enteric viruses are generally resistant to disinfection by chlorination (4). It has been hypothesized that this could be due to them embedding themselves in suspended solids which could shield them from chlorination (4). Additionally, wastewater treatment works best at temperatures between 20-35°C (3). This all supports the results, which showed that with decreasing temperature, there was a slight increase in the virus concentration in the effluent wastewater.

As of today, there are no regulations for the concentration of Norovirus in wastewater. Studies have shown that the amount of chlorine used to treat water is not concentrated enough to

inactivate the Norovirus (2). Because the Norovirus is a major cause of epidemic gastroenteritis, and it can easily be spread in water (1), regulations should be made to help limit future outbreaks. This and further studies need to take into account factors such as chlorine concentration and temperature/weather conditions in order to ensure the best cleaning results and safe use of this important resource - water.

5.1: References

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